

Proton Magnetic Resonance Relaxation in *Pseudomonas aeruginosa* Cytochrome Oxidase Solutions*

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Yoav Blatt‡, Bojan Benko§¶, Israel Pecht‡, and S. Vuk-Pavlović§¶

From the §Macromolecular Biophysics Laboratory, Institute of Immunology, Rockefellerova 2, 41000 Zagreb, Yugoslavia and the ‡Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

We have measured the temperature and frequency dependence of solvent proton magnetic relaxation rates in solutions of *Pseudomonas aeruginosa* cytochrome oxidase (EC 1.9.3.2) in its native low spin oxidized, its reduced, and its carbonyl reduced derivative. In solutions of the native oxidized enzyme, a large paramagnetic enhancement of the proton NMR relaxation rates, propagated to the solvent by the fast exchange mechanism, is observed. The ratio $(T_1/T_2)_{\text{pmg}} = 1.25 \pm 0.10$ at 24 MHz demonstrates that dipole-dipole interaction of the neighboring paramagnets is the dominant relaxation mechanism. Measurements of proton NMR relaxation in solutions of cytochrome oxidase from which the hemes D have been extracted demonstrates that hemes C do not contribute to the observed paramagnetic effects. The electron spin relaxation time of the ferric hemes D of 3.2 ± 0.4 ns is calculated from the frequency dispersion data. This is the longest value reported for hemoprotein solutions so far. These features of a low spin ferric hemoprotein are similar to those found recently both for the microbial and for the microsomal cytochrome P-450. The calculated distances between the exchanging proton(s) and heme D iron ions demonstrate the high accessibility of the environment of heme D from the solvent side, also for molecules not penetrating the inner coordination sphere.

A considerable amount of evidence for the structural basis of reactivities of hemes has been derived by the solvent proton magnetic relaxation technique, especially in case of high spin ferric derivatives. This is due to the large magnetic moment of the high spin ferric iron which powerfully enhances the NMR rates of protons approaching it from the solvent. Experiments with low spin ferric hemoproteins have been of less interest, because the effects they exert on proton NMR relaxation rates of solvent are generally found to be small (Mildvan *et al.* 1971; Lanir and Aviram, 1975). Recently, however, it has been found that low spin derivatives of cytochrome P-450, both of microbial (Griffin and Peterson, 1975; Philson *et al.* 1979) and of microsomal origin (Ruckpaul *et al.* 1976; Rein *et al.* 1976; Grasdalen *et al.* 1978) exert paramagnetic effects comparable to those measured in solutions of high spin ferric hemoproteins.

In the present paper, we demonstrate that native oxidized cytochrome oxidase (ferrocytochrome c-551: oxygen oxidoreductase, EC 1.9.3.2) from *Pseudomonas aeruginosa*, a low

spin derivative, significantly enhances proton NMR relaxation rates. Of the two types of hemes (C and D), only hemes D are effective, because of their long electronic spin relaxation time of 3.2 ns, which is the longest reported for a hemoprotein in solution so far. The origins of the long electronic relaxation time are explained by the small g -tensor anisotropy of hemes D modulated by diffusion-like fluctuations of the protein interior.

MATERIALS AND METHODS

Cytochrome oxidase was isolated from *Pseudomonas aeruginosa* as described by Blatt and Pecht (1979). Protein samples for NMR measurements were prepared by dialysis against 0.1 M phosphate, 1 mM ethylenediaminetetraacetate, pH 7.0, and concentrated by ultrafiltration in ultrathimbles (Schleicher and Schull, Dassel, West Germany) up to the concentrations between 0.16 and 0.33 mM. The concentration of the oxidized protein was determined spectrophotometrically using the extinction coefficient $\epsilon_{410}^{\text{ox}} = 2.88 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ (Silvestrini *et al.* 1979).

Full reduction was attained by adding a few grains of sodium dithionite (Merck) into oxidase solution, while the carbonyl form was prepared by equilibration of the reduced sample by flushing carbon monoxide over the stirred solution. The cyanide derivative was obtained by adding solid potassium cyanide up to the concentration of 1.0 M to the oxidized enzyme and by equilibration during 24 h.

The apoprotein, containing only hemes C, was prepared by extraction of hemes D with cold acidified acetone (Hill and Wharton, 1977). The apoprotein was solubilized in 0.25 M phosphate, pH 9.5, or 0.75 M KI.

The temperature and frequency dependence of the proton NMR relaxation rates were measured with an apparatus described earlier (4 to 24 MHz; Maričić *et al.* 1977), Bruker Instruments SXP 90 (22.2 and 36.6 MHz), WH 90 (90 MHz), and WH 270 (270 MHz). The longitudinal magnetic relaxation times, T_1 , were determined by the inversion recovery technique, while for measurements of the transverse relaxation times, T_2 , the Carr-Purcell-Meiboom-Gill sequence was used. During the measurements, the temperature of the samples was kept constant within 0.5°C with a stream of thermostatted precooled nitrogen. All the temperature dependencies were measured at the frequency of 24 MHz and frequency dispersion determinations were done at 22°C.

RESULTS

A comprehensive treatment of the theory of nuclear magnetic resonance relaxation was given by Dwek (1973). Hence, in the forthcoming paragraphs, we use equations therefrom and define the symbols as in this book, but the reader should also consult original references cited therein.

Temperature and Frequency Dependence of the Proton NMR Relaxation Rates—The evaluation of the structural parameters, *i.e.* of coordination numbers and/or metal electron-to-proton interspin distances requires a knowledge of the relaxation times of nuclei bound or close to the paramagnetic center, $T_{1,2M}$, according to

$$R_{1,2,\text{pmg}} \equiv T_{1,2,\text{pmg}}^{-1} / N = \frac{n}{N_W} \cdot \frac{1}{T_{1,2M} + \tau_M} + R_{1,2,\text{out}} \quad (1)$$

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$R_{1,2,\text{pmg}}$ denotes the values of the paramagnetically enhanced relaxation rates normalized/concentration of paramagnetic species, N . N_W is the concentration of relaxing nuclei, n the number of these nuclei coordinated to the paramagnetic center, and $R_{1,2,\text{out}}$ stands for the proton NMR relaxation rates due to outer sphere effects. It is shown in Equation 1 that $R_{1,2,\text{pmg}}$ depends on the ratio of $T_{1,2M}$ and τ_M . If $\omega_1^2\tau_c^2 \ll 1$, a positive or zero slope in the Arrhenius-type plot of $\ln R_{1,2,\text{pmg}}$ versus reciprocal absolute temperature would then be representative of either outer sphere effect (*i.e.* $n = 0$ or $\tau_M \gg T_{1M}$), or of the so-called fast exchange mechanism, $\tau_M \ll T_{1M}$; here a negative slope of $\ln R_{1,2,\text{pmg}}$ versus $1/T$ is due to the thermally activated (slow proton exchange) mechanism. In case of $\omega_1^2\tau_c^2 > 1$, a negative slope may represent also the fast exchange mechanism. For $R_{2,\text{pmg}}$, the slope can be negative only in case of thermally activated proton NMR relaxation.

So, in order to determine the value of $T_{1,2M}$, one has to establish the nature of the actual mechanism of propagation of the paramagnetic relaxation enhancement into the solvent. Usually, this is done experimentally by measuring the temperature dependence of the proton NMR relaxation rates. The respective plots of the logarithms of the molar solvent proton longitudinal (R_1) and transverse (R_2) relaxation rates versus reciprocal absolute temperature in solutions of native, reduced, and carbonyl adduct of reduced *P. aeruginosa* cytochrome oxidase are presented in Fig. 1. These rates were obtained by subtracting the proton NMR rates measured in the protein free buffer from the corresponding values in protein solutions and dividing the difference by the concentration of protein. The values of R_1 and R_2 are composed of a diamagnetic ($R_{1,\text{dia}}$) and a paramagnetic term ($R_{2,\text{para}}$), the former due to the interaction of water with the protein matrix and the latter with the low spin ferric centers of the protein.

Assuming that the cross-relaxation effects between protons of the protein and of the solvent (Koenig *et al.* 1978) are equal both in the paramagnetic and in the diamagnetic derivative, the paramagnetic term $R_{1,2,\text{pmg}}$ is obtained as the difference of $R_{1,2}$ and $R_{1,2,\text{dia}}$. These differences are depicted in Fig. 1 as dashed lines.

From Fig. 1 it can be noted that the values of R_1 in solutions of the ferric cyanide complex as well as of the reduced and of the carbonyl complex of the reduced enzyme are indistinguishable within the experimental error, and that all these data give a measure of $R_{1,\text{dia}}$, although only the CO-reduced form is diamagnetic. The ferric cyanide complex is low spin ($S = 1/2$) and paramagnetic, the reduced hemes C are diamagnetic ($S = 0$), but ferrous hemes D are high spin ($S = 2$; Walsh *et al.* 1979) and thus paramagnetic. However, as in the case of other high spin ferrous hemoproteins (*e.g.* deoxyhemoglobin; Fabry and Reich, 1966), no electron spin resonance signals can be detected (Gudat *et al.* 1973) and we noted no proton NMR relaxation effects. These features of the ferrous high spin hemes are attributed, along with several other reasons, to a very short electron spin relaxation time (Johnson *et al.* 1977). Also, in the case of ferric cyanide complexes, the very short electron spin relaxation times have been mentioned as the cause for the lack of any effect on solvent protons (Wüthrich, 1970; Johnson *et al.* 1977). In this case, steric changes in the vicinity of the heme induced by the cyanide could certainly play a role as well. The observed molar longitudinal proton NMR relaxation rates (from $600 \text{ s}^{-1}\text{M}^{-1}$ at 40°C and $1200 \text{ s}^{-1}\text{M}^{-1}$ at 0°C measured at the Larmor frequency of 24 MHz) are typical for diamagnetic globular proteins of molecular weight of 120,000 (Hallenga and Koenig, 1976).

The dashed lines in Fig. 1, *a* and *b* represent the pure paramagnetically induced relaxation rates. The positive slope

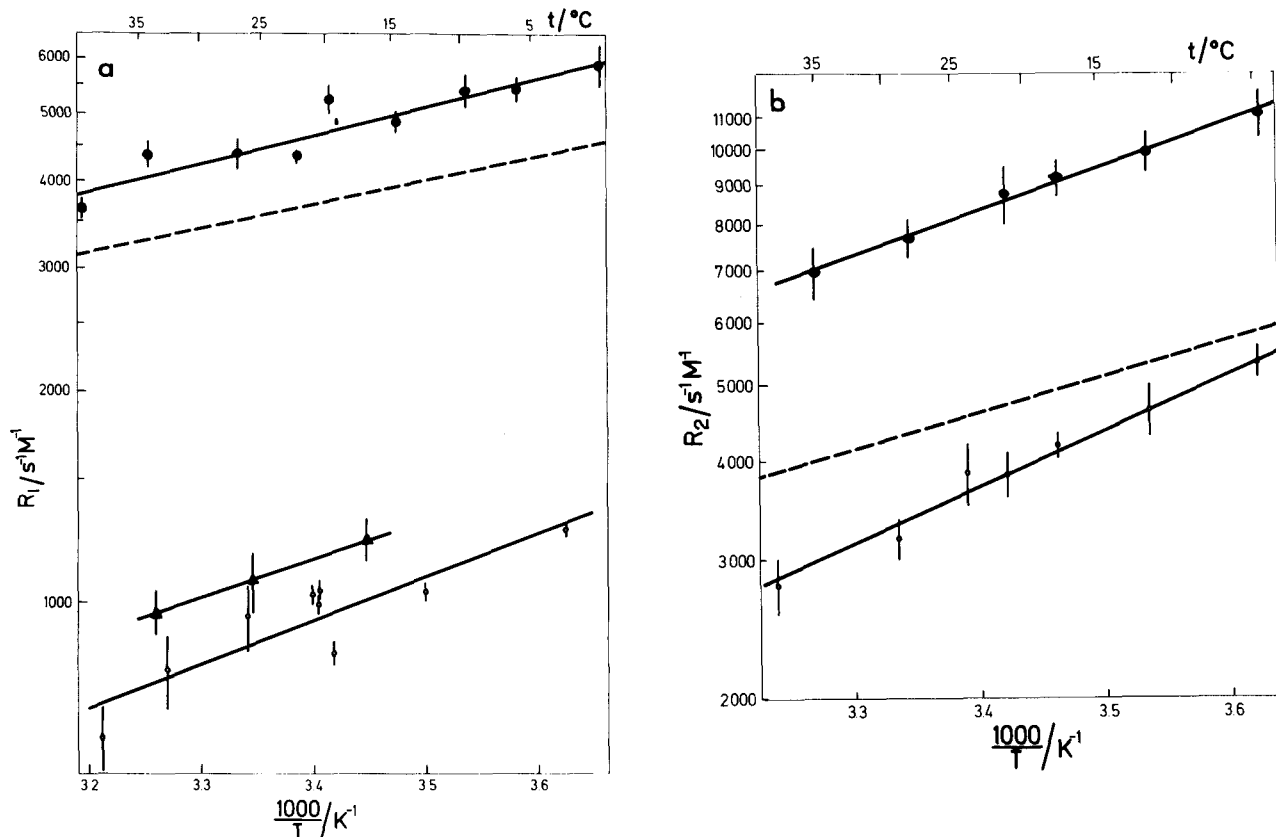


FIG. 1. The temperature dependence of the molar proton magnetic relaxation rates, R , in solutions of native oxidized *P. aeruginosa* cytochrome oxidase (●), its cyanide derivative (▲), and of its reduced carbonyl adduct (○). *a*, longitudinal relaxation rates, R_1 ; *b*, transverse relaxation rates, R_2 . The dashed lines represent the paramagnetic molar contribution to the magnetic relaxation rates. Buffer: 0.1 M phosphate, 1 mM ethylenediaminetetraacetate, pH 7.0. Protein concentration: 0.33 mM. Frequency: 24 MHz.

of $R_{1,2,\text{pmg}}$ in the Arrhenius plot is compatible both with the fast exchange and outer sphere mechanisms. The outer sphere relaxation can be dominated by τ_D , the water diffusion correlation time (as diffusion of the protein is negligible compared to that of water), or τ_S , the electron spin relaxation time (cf. Dwek, 1973). However, the respective correlation functions for the two processes differ considerably from each other. The fit of equations describing the outer sphere relaxation dominated by translational diffusion (cf. Dwek, 1973) to our data (Fig. 2) yields $\tau_D = 2 \times 10^{-8}$ s. As the translational diffusion of water is at least 2 orders of magnitude faster (cf. Page, 1972, and Dwek, 1973), this value appears to be unrealistically long. Moreover, using this calculated value of τ_D as the effective correlation time of the system, at 24 MHz we calculate the ratio $R_{2,\text{pmg}}/R_{1,\text{pmg}} = 2.2$ (cf. Equations 9.17 and 9.18 in Dwek, 1973). This ratio is significantly larger than the experimentally obtained $R_{2,\text{pmg}}/R_{1,\text{pmg}} = 1.25 \pm 0.10$.

The excellent fit of correlation functions for the τ_S dominated relaxation (Solomon, 1955) to our data (Fig. 2) yields the value of $\tau_S = (3.2 \pm 0.4) \times 10^{-9}$ s, and the calculated and observed ratios of $R_{2,\text{pmg}}/R_{1,\text{pmg}}$ agree well within the limits of experimental error. This also means that the relaxation rates in our system are due to the dipole-dipole interactions between the spins only, i.e. the contribution of the scalar interaction term, if any, can be neglected. As the inflection observed in Fig. 2 is due to the dispersion at ω_J , rather than ω_S (cf. Equation 9.13 in Dwek, 1973), the calculated value of τ_c is identical to $\tau_{S,1}$.

The question whether the paramagnetic effects in Fig. 1 are propagated to the solvent predominantly either by the outer sphere or by the fast exchange mechanism ($\tau_M \gg T_{1,2M}$ and $\tau_M \ll T_{1,2M}$; Luz and Meiboom, 1964) can be answered by the following consideration. From the data mentioned above, it follows that in our system the relation $\tau_D < \tau_{S,1}$ holds. In such a case, for the total electronic spin $S = 1/2$, the term $R_{1,\text{out}}$ is dominated by τ_D , rather than τ_S (Abragam, 1961). Assuming

that in a protein solution as dilute as ours the water diffusion coefficient, $D_{\text{H}_2\text{O}}$, is similar to that in pure water (at 22°C $D_{\text{H}_2\text{O}} = 2.2 \times 10^{-5}$ cm²s⁻¹; cf. Page, 1972) and with the smallest possible distance of closest approach of solvent protons to the iron $d = 0.45$ nm, we obtain the effective diffusion coefficient $D = \frac{1}{2}(D_{\text{H}_2\text{O}} + D_{\text{cyt.ox}}) \approx D_{\text{H}_2\text{O}}/2 = 1.1 \times 10^{-5}$ cm²s⁻¹. The diffusion correlation time $\tau_D (= d^2/D)$ is then 2×10^{-10} s. At the lowest experimental frequency of 4 MHz, the term $R_{1,\text{out}}$ contributes $140 \text{ s}^{-1}\text{M}^{-1}$ (cf. Equation 9.17 in Dwek, 1973). Using the same value of τ_D at 270 MHz, one calculates $R_{1,\text{out}} = 57 \text{ s}^{-1}\text{M}^{-1}$. This means that at high frequencies $R_{1,\text{out}}$ amounts even up to $\frac{1}{3}$ of the total $R_{1,\text{pmg}}$, while at low frequencies it can be neglected. If, because of steric reasons, $d > 0.45$ nm, a distance found in solutions of hydrated inorganic ions, τ_D would be longer, but $R_{1,\text{out}}$ would be even smaller (cf. Equation 9.17 in Dwek, 1973). As $R_{1,\text{out}}$ at low frequencies contributes less than 10% of $R_{1,\text{pmg}}$, we conclude that the fast exchange mechanism is operative. This means that τ_M vanishes and that $T_{1,2M}$ can be readily calculated (cf. Equation 1).

A piece of evidence supporting our conclusions about the dominance of τ_D in the outer sphere and of τ_S in the exchange-mediated proton relaxation comes from the analogous phenomena found in solutions of various ferrous nitrosyl proteins (Benko and Vuk-Pavlović, 1976; Benko and Maričić, 1978). As the electronic relaxation times in these $S = \frac{1}{2}$ complexes are $\tau_S \geq 3 \times 10^{-9}$ s (estimated from the electron spin resonance spectra by Shiga *et al.* 1969), here also the relation $\tau_D < \tau_{S,1}$ holds. In solutions of nitrosyl hemoprotein complexes where $R_{1,\text{out}}$ can be directly measured (Benko and Maričić, 1978) or its upper limit estimated (Benko and Vuk-Pavlović, 1976; Benko and Maričić, 1978), it was found to be $R_{1,\text{out}} \leq 150 \text{ s}^{-1}\text{M}^{-1}$. Assuming d as large as 1 nm, in case of τ_S -dominated $R_{1,\text{out}}$, it could not be smaller than $600 \text{ s}^{-1}\text{M}^{-1}$. This fact clearly demonstrates that in such cases diffusion-controlled relaxation mechanisms are operative in the outer sphere, even when the main contribution to $R_{1,\text{pmg}}$ comes from a process dominated by τ_S . Koenig and Brown (1973) also came to the same conclusion following a similar line of arguments based on their data obtained with copper proteins ($S = \frac{1}{2}$).

The pH Dependence of $R_{1,\text{pmg}}$ —In an attempt to determine the pK of the exchanging species, a pH titration of the native oxidized enzyme was monitored by proton NMR relaxation. Surprisingly enough, the $R_{1,\text{pmg}}$ is pH independent within the range between pH 5.20 and 10.65 and amounts $3450 \pm 180 \text{ s}^{-1}\text{M}^{-1}$ at 25°C and 24 MHz. This means either that the pK of the group from which the proton(s) exchange with the solvent lies outside this region, or that the exchangeable species are the entire water molecules (Fabry and Eisenstadt, 1974). However, from this result we conclude that the conformation of the intimate environment of the paramagnetic center is not affected by the pH change within this range to any significant degree.

R_1 in the Apoprotein Solution—The question of paramount importance for the interpretation of our data is whether both types of hemes (C and D) contribute to the observed proton NMR relaxation enhancement. In order to answer this question, we used a solution of cytochrome oxidase from which the hemes D were extracted by the cold acidified acetone treatment (Hill and Wharton, 1977). The apoprotein, insoluble at neutral pH, was partly soluble in 0.25 M phosphate, pH 9.5 (Hill and Wharton, 1977) and in 0.75 M KI, pH 7.0. In these solutions, the value of $R_1 = 920 \pm 200 \text{ s}^{-1}\text{M}^{-1}$ at 24 MHz was determined. Since we have found that $R_{1,\text{pmg}}$ in the holoenzyme solution is pH-independent, we believe that this value (which is equal to the corresponding value in the diamagnetic

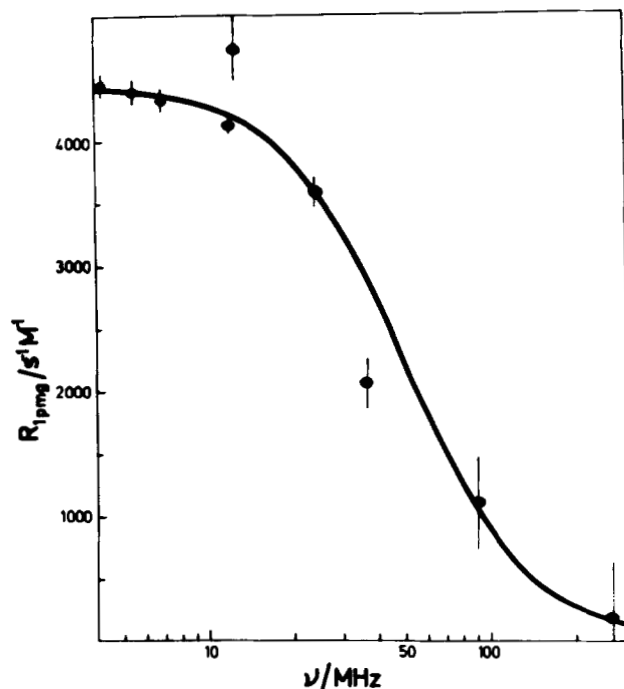


FIG. 2. The frequency dependence of the molar longitudinal paramagnetically induced relaxation rates in solutions of oxidized native *P. aeruginosa* cytochrome oxidase. Data obtained as the difference of the relaxation rates of the native oxidized and reduced carbonyl adduct. Temperature: 22°C, other conditions as in Fig. 1.

enzyme solution; Fig. 1) demonstrates the proton NMR relaxation inertness of hemes C in the holoprotein at neutral pH.

The Iron-to-Proton Interspin Distance—Based on the above finding that only hemes D exert measurable paramagnetic effects and using the experimentally determined effective correlation time $\tau_c = 3.2 \times 10^{-9}$ s and assuming one proton exchanging from the sphere of closest approach to each of the heme D irons, Equation 1 and the well known Solomon's equation (1955) yield the heme D iron-to-proton interspin distance $r = 0.29 \pm 0.02$ nm. As the ratio of the electron g -values of hemes D is close to 1, the error due to the neglect of g -tensor anisotropy can be ignored (Dwek, 1973).

DISCUSSION

The most striking feature of all the results presented is the magnitude of the molar paramagnetically enhanced longitudinal and transverse proton NMR relaxation rates measured in a solution of a low spin ferric haemoprotein. These rates are comparable to those typically measured in solutions of high spin ferric hemoproteins (cf. Table I in Vuk-Pavlović, 1976), although the constant term in the Solomon's equation is in the latter case 12 times larger. However, recently, the low spin ferric cytochrome P-450 from *P. putida* (Griffin and Peterson, 1975; Philson *et al.* 1979), as well as the detergent-solubilized cytochrome P-450 of mammalian origin (Ruckpaul *et al.* 1976; Rein *et al.* 1976; Maričić *et al.* 1977, 1979; Grasdalen *et al.* 1978), has also been found to exert a surprisingly large enhancement of the proton NMR relaxation rates.

The actual NMR relaxation rate of proton(s) at the sites of closest approach to the paramagnetic center (all other parameters being constant), depends on the metal-to-proton interspin distance, r , and on the effective correlation time of the modulation of the local magnetic fields at the relaxing nucleus, τ_c . From the frequency dependence of the proton NMR relaxation rates (Fig. 2), we have calculated $\tau_c = 3.2 \times 10^{-9}$ s. This value is much shorter than the expected rotational relaxation time, $\tau_R = 2 \times 10^{-7}$ s, for a protein of comparable molecular weight (Hallenga and Koenig, 1976). The effective correlation time is given by $\tau_c^{-1} = \tau_s^{-1} + \tau_R^{-1} + \tau_M^{-1}$ (where τ_s is the iron electron relaxation time and τ_M is the proton residence time close at the iron), and hence $\tau_c \approx \tau_s$ (cf. Lahajnar *et al.* 1974). Our value of τ_c is comparable to the corresponding typical values determined for various high spin hemoproteins (Lahajnar *et al.* 1974, 1976; Gupta and Mildvan, 1975; Vuk-Pavlović *et al.* 1976; Vuk-Pavlović and Williams-Smith, 1977) and much longer than those determined for their low spin

derivatives (Johnson *et al.* 1977). However, our measured value of τ_c is similar, yet longer, to τ_c found for cytochrome P-450 from *P. putida* (Griffin and Peterson, 1975; Philson, 1977; Philson *et al.* 1979) and rabbit liver (Maričić *et al.* 1977, 1979). Hence, the answer to the question for the cause of the large proton NMR relaxation effects in bacterial native oxidized *Pseudomonas* cytochrome oxidase solutions depends on the explanation of the reasons for the comparatively inefficient iron-electron relaxation (long τ_s). We believe that the value of τ_s is not directly related to the chemical nature of the heme side groups and to the extent of saturation of the constituent pyrrole rings. The large variance of the τ_c values in various protoheme IX-containing proteins on the one hand, and the similarity of the τ_s values between cytochrome P-450 (with protoheme IX) and cytochrome oxidase (with heme D efficient in proton NMR relaxation) support this notion.

In the absence of a comprehensive theory of electron spin relaxation in paramagnetic transition metal complexes with $S = 1/2$, where also the Redfield approximation ($\tau_s \gg \tau_R$) does not hold, we relate our findings to the approach by Doddrell and co-workers (Pegg and Doddrell, 1976a, 1976b; Doddrell *et al.* 1977). In order to explain the relaxation times of methyl protons from the heme rings in metmyoglobin cyanide (Doddrell *et al.* 1977), these authors modify the theory originating from the solid state physics (Breen *et al.* 1969; Williams and Krupka, 1969) and propose that dynamic Jahn-Teller forces provide the mechanism of electronic relaxation (Doddrell *et al.*, 1977). According to this treatment,

$$\tau_S = \frac{9}{2} \frac{g_{av}^2}{\Delta g^2} \tau \quad (2)$$

where g_{av} is the value of g ($= (g_x + g_y + g_z)/3$), Δg is the g -tensor anisotropy ($(g_{av} - g_x)^2 + (g_{av} - g_y)^2 + (g_{av} - g_z)^2/g_{av}^2$). The symbol τ denotes the characteristic time of the phonon-induced lattice pseudorotation in the solid state. In solutions of inorganic complexes, it can probably be approximated by the interval between collisions with the molecules of the solvent.

Table I displays the calculated values of τ for four different hemoproteins, where both τ_S and g -tensor anisotropy are known. It is evident that the values of τ are of the order of picoseconds. As translational diffusion of water is at least 2 orders of magnitude slower, the motions influencing the electronic relaxation (and characterized by τ) cannot originate from the solvent. It is relevant to note that the identity of the τ_c values for cytochrome P-450 (from *P. putida*) as obtained

TABLE I
The values of g , τ_S and τ for some low spin ferric hemoprotein derivatives

Hemoprotein	g_1	g_2	g_3	τ_S/ps	τ/ps
Hemoglobin cyanide	3.4	1.89	0.74 ^a	25 ^b	5
Cytochrome c	3.06	2.24	1.24 ^c	~7 (2) ^d	~0.5 (0.2)
Cytochrome P-450 (<i>P. putida</i>)	2.45	2.26	1.91 ^e	400–500 ^{e,f}	~3
Cytochrome P-450 LM ₂ rabbit	2.43	2.25	1.93 ^e	100–1000 ^e	0.8–8
Cytochrome oxidase (<i>P. aeruginosa</i>)					
Hemes C	2.93	2.31	1.4 ^h	3200 ⁱ	50
Hemes D	~2.46	~2.46	1.71 ^h		

^a Blumberg and Peisach, 1970, cited in Sheard *et al.* 1970.

^b Johnson *et al.* 1977.

^c Salmeen and Palmer, 1968.

^d Wüthrich, 1970; From the linewidth of the Met-80 methyl resonance ($\Delta\nu_{100}$ MHz \approx 100 Hz, $\Delta\nu_{220}$ MHz \approx 130 Hz, Wüthrich, 1970) and methyl proton to iron distance $\langle r_{\text{Me-Fe}} \rangle = 0.36$ nm, we calculate $\tau_c \sim 7 \times 10^{-12}$ s. The line broadening due to the Curie spin (Guéron, 1975) is smaller than 7% at 220 MHz.

^e Griffin and Peterson, 1975.

^f Philson *et al.* 1979.

^g Maričić *et al.* 1979.

^h Gudat *et al.* 1973.

ⁱ This work.

from the extrapolation of the low temperature ESR data to room temperature (Griffin and Peterson, 1975), and as determined by proton NMR relaxation (Philson *et al.* 1979) is compatible with the idea that the electronic relaxation is probably caused by the same processes both in the liquid and in the frozen state, where in the latter case solvent dynamics is negligible. Therefore, we conclude that motions on the picosecond time scale must be those diffusion-like fluctuations of the protein interior predicted by the theory (McCammon *et al.* 1977; Northrup *et al.* 1980).

We noted that in $S = \frac{1}{2}$ hemoproteins, the low g -tensor anisotropy is accompanied by the comparatively long electron spin relaxation time (Table I), in line with the theory by Doddrell *et al.* (1977). Significant solvent-proton magnetic relaxation enhancements have been observed only in solutions of such proteins. The results of direct measurements of τ_s , when available, will be the final test of the applicability of Doddrell's theory to explain the unusually long (inefficient) electronic relaxation in low spin ferric hemoproteins with small g -tensor anisotropies.

Irrespective of the mechanism of electronic relaxation, the value of τ_e calculated from the data in Fig. 2 enables a straightforward calculation of the iron-to-proton interspin distance r using Solomon's equation. The calculated value of r_{Fe-H} for hemes D lies between 0.27 and 0.31 nm (see "Results"). This means that situated at this distance from the iron there is a site from which protons exchange with the bulk solvent. This free exchange of proton(s) from the close proximity of the ferric ion demonstrates the high accessibility of hemes D from the solvent side. The existence of exchangeable proton(s) at this distance lends evidence to the idea that in the oxidized state hemes D are hexacoordinated as proposed by Walsh *et al.* (1979). The axial ligand(s) can be displaced by cyanide which abolishes every paramagnetic effect of hemes D, both because of ligand replacement and of shortening of τ_e . Although proton NMR relaxation data do not reveal identity of axial ligands, the independence of the relaxation rates on pH between 5.20 and 10.65 shows that the pK value of the exchangeable proton(s) lies outside this region or that the exchangeable species is a water molecule. It is not likely, however, that this water molecule is at the sixth ligand position, because the pure low spin character of ferric hemes D (Walsh *et al.* 1979) suggests that both axial positions are occupied by strong field ligands. This conclusion is further corroborated by the unusually low binding rate of cyanide (Barber *et al.* 1976), a fact similar to cytochrome P-450 (Jefcoate *et al.* 1969; Ruckpaul *et al.* 1976) meaning probably that this anion competes with another strong ligand for the axial site on the iron. On the basis of all the mentioned similarities between the properties of hemes in cytochrome P-450 and cytochrome oxidase, it is not unreasonable to argue that similarities in the nature of axial ligands to the hemes of the two proteins may exist. Since it has been suggested that hemes D are pentacoordinated in the reduced state (Walsh *et al.* 1979), it is plausible to assume that the association/dissociation of an axial ligand to hemes D could play a role in the oxidation-reduction mechanism of cytochrome oxidase.

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