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Conference Paper

Solvent Proton Magnetic Relaxation in Solution of Rabbit Liver Cytochrome P450. - On the Correlation Time for the Electron Proton Dipole-Dipole Interaction.*

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Structural parameters can be derived from PMR measurements if the correlation time for the spin interactions is known. The relaxation rates induced by the ferric haem-iron of cyt P450 were found to be frequency independent from 10 to 37 MHz. The possible limits for the correlation time according to Solomon's theory are discussed with regard to the possible existence of a water molecule at the sixth coordination site of the haem-iron. No definite conclusion could be reached in this respect, but the results are definitely in favour of a haem environment which can accommodate several water molecules exchanging quickly with the bulk of solvent.

INTRODUCTION

We have been using the proton magnetic relaxation method to characterize the haem environment in cytochrome P450 from the liver, mainly in its oxidized, ferric state (m_L^0). It was possible to show¹ that the haem iron, in a solubilized, partially purified sample from the liver of phenobarbital pre-treated rabbits, is very accessible to solvent molecules. The results suggested a much more open haem »pocket«, if there is any at all, compared to the same structural domain in myoglobin. The more specific question as to whether a water molecule is here an axial ligand of the Fe^{3+} -ion could not be tackled because of lack of knowledge of the correlation time, τ_c , for the iron-proton spin interaction. Griffin and Peterson² considered the same problem for the

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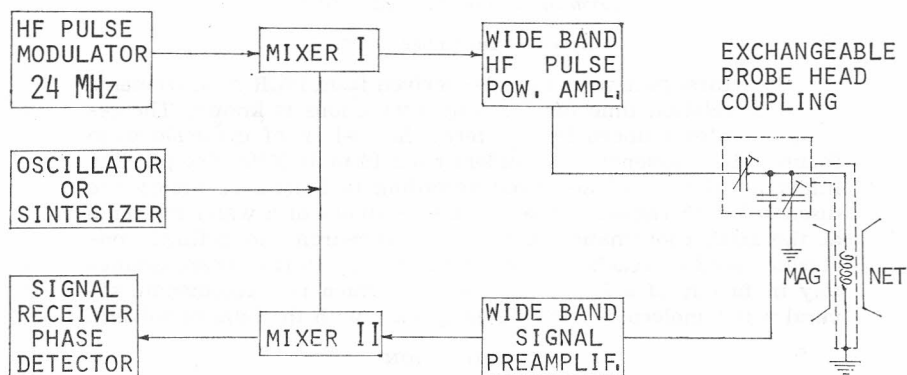
purified, soluble, *Pseudomonas putida* cyt P450 (m_L^0). They used a value of τ_c obtained by an extrapolation procedure from the low temperature electron spin resonance (ESR) spectra. It therefore seemed worthwhile to determine τ_c for a solution of m_L^0 by the only direct method — that of measuring the Fe^{3+} -induced relaxation rates of solvent protons in dependence on the magnetic field (*i. e.* on the Larmor frequency). The present report is such an attempt within a rather limited frequency range.

EXPERIMENTAL

The sample was prepared with slight modification of procedures from literature, as described in ref. 1. The solubilized m_L^0 was contained in 0.1 M phosphate, pH 7.4 aqueous 20% (v/v) glycerol with 1 mM EDTA, 0.4% (w/v) sodium cholate and 0.08% (w/v) nonionic detergent. The concentrations, determined as before¹, were: 306 μM cyt P450 (6.9 nmoles per mg), 13 μM cyt P420 (0.3 nmoles per mg), with a negligible amount of cyt b_5 . The total haem content corresponded to the total iron to within 5% as determined by atomic absorption. The ESR spectrum very closely resembled the low-spin one observed previously¹.

The sample was stored in ice/water except for very brief periods to prepare the portions of the sample for measurements, and the NMR-probe was always precooled to 5°C.

The apparatus used in our laboratory with two fixed channels (12 and 24 MHz) was adapted for variable-frequency measurements from 6 to 40 MHz, according to the block diagram:



The pulse modulator radio frequency output at 24 MHz is mixed with a C. W. signal of variable frequency $\nu_0 + 24$ MHz. The output of mixer I is amplified in a broad-band power amplifier. The probehead is provided with a fixed coupling capacitor and a set of exchangeable coils tunable with a small capacitor. The NMR signal after the r. f. pulse is amplified in a broad-band preamplifier and fed into the mixer II. The same C. W. signal is used as the local oscillator and 24 MHz r. f. signal fed into the basic spectrometer.

A $180^\circ - t - 90^\circ$ pulse sequence was used in a diode detection, generally with 20-30 different delay times, t , in each measurement of the longitudinal relaxation time, T_1 .

RESULTS

Figure 1 comprises all the measurements made at 10, 12, 24 and 37 MHz (abscissa). The ordinate is the longitudinal relaxation rate measured for the original sample, m_L^0 (O), and for the dithionite-reduced one with CO bound, $m_L^r \cdot CO$ (X).

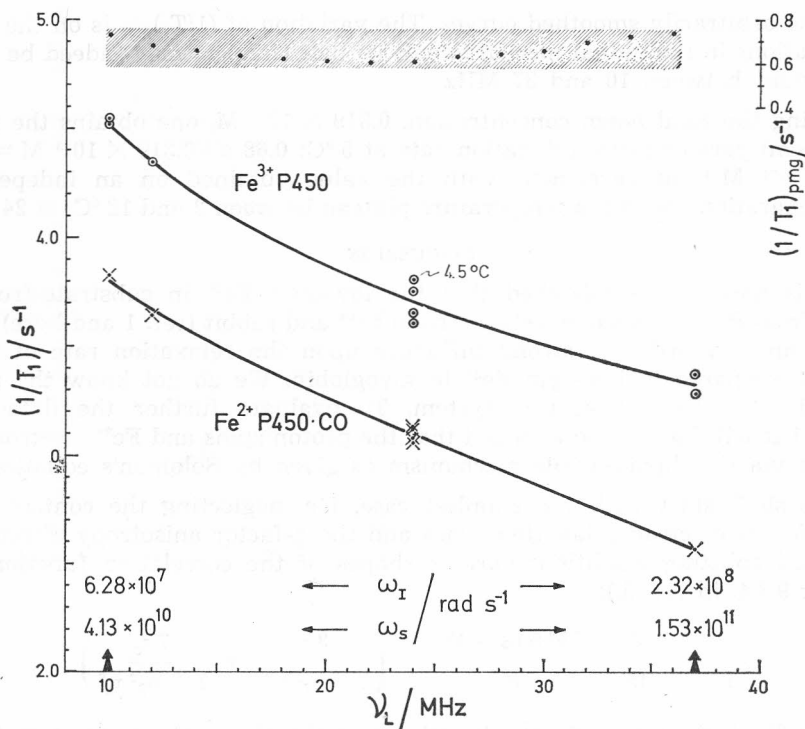


Figure 1. The longitudinal proton magnetic relaxation rates in dependence on the Larmor frequency for solutions of the native, ferric, cytochrome P450 ($m_L^O - O$) and its dithionite + CO reduced derivative ($m_L^r \cdot CO - X$) at $5^\circ C$. The shaped strip is the incremental relaxation rate, i. e. $(1/T_1) m_L^O - (1/T_1) m_L^r \cdot CO$, representing the paramagnetic contribution to the relaxation rates from the haem-iron electron spin.

The nuclear magnetization was first plotted on a semilog paper *versus* the delay-time, t . The plots clearly indicated two different relaxation times and were accordingly analyzed by a computer programme. Independent measurements with both samples (m_L^O and $m_L^r \cdot CO$) dialyzed against 2H_2O , showed that the faster relaxation was due to the aliphatic glycerol protons owing to the interaction of glycerol molecules (partial immobilization) and the protein. Hence, only the data due to the slower relaxing protons, that is, those from water molecules, are shown in Figure 1. They reflect the influence of the (diamagnetic) protein of $m_L^r \cdot CO$ (X), and that of the protein together with the paramagnetic centres in m_L^O (O), upon the relaxation rates of H_2O protons in solution. The data were collected within three weeks, but no systematic deviations were found. The temperature was between 5.0 and $5.6^\circ C$ (kept within $\pm 0.2^\circ C$ in each measurement), except for one case indicated in Figure 1.

The two curves were drawn visually. The difference between them is the contribution to the total relaxation rate for m_L^O due to its paramagnetic iron, $(1/T_1)_{pmg}$. The shaded strip in the upper part of Figure 1. represents this relaxation rate difference. The dots within it indicate the actual values obtained

from the arbitrarily smoothed curves. The variation of $(1/T_1)_{\text{pmg}}$ is on the order of deviations in individual measurements so that $(1/T_1)_{\text{pmg}}$ may indeed be taken as constant between 10 and 37 MHz.

Using the total haem concentration, $0.319 \times 10^{-3} \text{ M}$, one obtains the molar (per haem) paramagnetic relaxation rate at 5°C : $0.68 \text{ s}^{-1}/0.319 \times 10^{-3} \text{ M} = 2132 (\pm 250) \text{ s}^{-1} \text{ M}^{-1}$, in agreement with the value obtained on an independent m_L^0 -preparation¹ within a temperature plateau between 2 and 12°C , at 24 MHz.

DISCUSSION

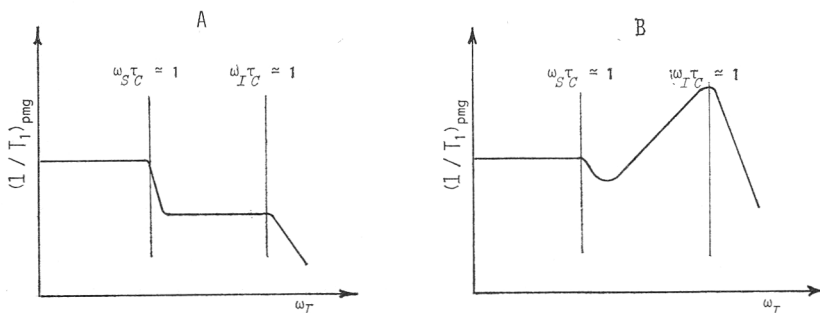
It is now well established that the low-spin Fe^{3+} in substrate-free m^0 , either from *Pseudomonas putida*² or from rat³ and rabbit (ref. 1 and here) liver, exhibits an extraordinary strong influence upon the relaxation rate of water protons compared to low-spin Fe^{3+} in myoglobin. We do not know the actual relaxation mechanism in this system. To evaluate further the data from Figure 1 it will have to be assumed that the proton spins and Fe^{3+} electron-spin interact via the dipole-dipole mechanism as given by Solomon's equation⁴ (1).

We shall start with the simplest case, *i.e.* neglecting the contact term, the different electron relaxation times and the g-factor anisotropy effects; the zero-field splitting condition; various shapes of the correlation function (see chapter 9.3.4. in ref. 5.):

$$\frac{1}{T_{1M}} = \frac{2}{15} \frac{\gamma_I^2 g^2 S(S+1) \beta^2}{r^6} \left(\frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2 \tau_c^2} \right) \quad (1)$$

where $1/T_{1M}$ is the longitudinal relaxation rate of water protons in fast exchange with the bulk solvent from defined site(s) near the paramagnetic centre (not necessarily from the first-coordination sphere of haem-iron⁶). We have already presented evidence¹ that the fast-exchange mechanism pertains in our solutions of m_L^0 , so that we may proceed to apply Eq. (1). The other symbols in this equation are: γ , the magnetogyric ratio; S , g and β are the electron spin value, its Lande splitting factor and the Bohr magneton, respectively; r is the distance between the interacting electron, S , and the nuclear, I , spin within a correlation time, τ_c ; ω_I and ω_S are the corresponding Larmor precession frequencies.

The correlation time may be frequency independent (A), or it may depend on the Larmor frequency (B). These two possibilities for $(1/T_1)_{\text{pmg}} = F(\omega_L)$ are depicted schematically below (after the Figure 9.10. in ref. 5):



The results in Figure 1. can be consistent with any of the three plateaux in graph A and B, based on this simple dipole-dipole relaxation mechanism.

The first dispersion steps (along increasing ω_L) correspond to $\omega_S \tau_c \approx 1$, while the second steps are obtained when $\omega_I \tau_c \approx 1$. The limiting values of ω_S and ω_I for the frequency range in these measurements are indicated in Figure 1 (at 10 and 37 MHz) for $\omega_S/\omega_L = 660$. The following correlation times must be considered:

$$\begin{array}{l} \text{if the dispersion step is at} \\ \nu_L < 10 \text{ MHz} \qquad \nu_L > 37 \text{ MHz} \\ \tau_c \text{ may be} \\ \text{from } 1/\omega_I \qquad \geq 1.6 \times 10^{-9} \text{ s} \qquad \leq 4.3 \times 10^{-9} \text{ s} \\ \text{from } 1/\omega_S \qquad \geq 2.4 \times 10^{-11} \text{ s} \qquad \leq 6.5 \times 10^{-12} \text{ s} \end{array}$$

The case of the frequency-dependent τ_c (graph B) does not pertain because a correlation time shorter than 6.5×10^{-12} would be required for the dispersion step to occur above $\nu_L = 37$ MHz and such a short τ_c is quite improbable.

For the remaining case, the experimentally observed plateau must be ascribed to one of the two in graph A. Too short a correlation time is again the argument against the first plateau. Therefore, the only possibility left is when the experimental data from Figure 1 are ascribed to the second plateau: the corresponding dispersion steps must be at $\nu_L < 10$ MHz (for ω_S) and $\nu_L > 37$ MHz (for ω_I), hence

$$2.4 \times 10^{-11} \text{ s} \leq \tau_c \leq 4.3 \times 10^{-9} \text{ s}.$$

The preceding reasoning has led to an expansion of the probability limits for τ_c from a factor of three, given by the limited frequency range of T_1 -measurements, to about two orders of magnitude. An inspection of the actual values of the correlation function (terms in τ_c and $\omega_{S,I}$ of Eq. (1) between 10 and 37 MHz, confirms that they coincide within this particular range of Larmor frequencies for values of τ_c between 5×10^{-11} s and 1×10^{-9} s. For such a span of τ_c -values the correlation function increases 20 times.

The ESR-extrapolation procedure used in ref. 2. yielded very similar values for τ_c in both *low* and *high* spin m_P^0 : $\sim 10^{-10}$ s. The present measurements for the *low* spin m_L^0 , while favouring the frequency-independent τ_c , do not suffice to determine τ_c more accurately, though, a value on the order of 10^{-10} s seems quite probable.

From Eq. (1) the interspin distance, $\text{Fe}^{3+} \rightarrow \text{proton(s)}$, can be evaluated:

$$r = [T_{1M} \cdot K_{IS} \cdot F(\tau_c)]^{1/6} \quad (2)$$

K_{IS} comprises all the physical constants, and with $S = 1/2$ it equals $0.185 \times 10^{-43} \text{ s}^{-2} \text{ m}^6$. $F(\tau_c)$ for $\tau_c \approx 1 \times 10^{-10}$ s equals $\sim 3 \times 10^{10}$ s. T_{1M} is obtained from

$$T_{1M} = \frac{n}{N_w} \cdot \frac{1}{(1/T_1)_{\text{pmg}}} \quad (3)$$

If we assume $n = 2$, that is, two protons from a fast-exchanging water molecule, equidistant from the haem-iron, then with the experimental value of

$(1/T_1)_{\text{pmg}} = 2132 \text{ s}^{-1} \text{ M}^{-1}$ and $N_w = 2 \times 55.5$ (the proton molarity neglecting the presence of 20% glycerol), $T_{1M} = 0.845 \times 10^{-5} \text{ s}$, so that

$$r = 1.90 \text{ \AA} \quad (\tau_c \approx 1 \times 10^{-10} \text{ s}).$$

With the upper limit of τ_c

$$r = 2.79 \text{ \AA} \quad (\tau_c \approx 1 \times 10^{-9} \text{ s}).$$

For one water molecule, as axial ligand at the m_L^0 -haem iron, the latter value of r is just what one would predict by analogy with metmyoglobin⁷ while any distance (r) shorter than 2.6 Å is improbable. Even for $r = 2.6 \text{ \AA}$ τ_c must be $\sim 7 \times 10^{-10} \text{ s}$. Therefore, it is desirable to have τ_c accurate to at least 30%.

Although the conclusion about the presence of an axial water molecule may be attractive, it should be viewed with great caution.

Firstly, we made an arbitrary assumption that there are only two exchanging protons. Our experience with various aquomethaemoproteins^{6,8,9} is that the axial water molecule is, in fact, stationary at its site on the NMR time scale, while a next-neighbour water molecule exchanges quickly with the bulk solvent. A water molecule of this kind with the protons $\sim 3.5 \text{ \AA}$ from the haem-iron, accounts for the observed solvent-proton relaxation rates in haemoglobins with a rather tight haem-pocket, but also in those with much more open ones⁹. Let us assume, that in m_L^0 there is also axial water as a rather static ligand, or that its site is blocked in some other way, so that the exchange of other water molecules accounts for the rather large relaxation rate of $2135 \text{ s}^{-1} \text{ M}^{-1}$. If these water molecules approached the haem-iron to some 3.5 Å, then, with a probable $\tau_c = 7 \times 10^{-10} \text{ s}$, there should be about 12 exchanging protons. This amounts to approximately 6 water molecules (or more, if $r > 3.5 \text{ \AA}$).

It would require of course, a very open protein environment around the haem in m_L^0 , which is consistent with our earlier qualitative conclusion obtained from measurements with the aliphatic protons of the glycerol molecule¹.

It is obvious that *no conclusion* can be reached as to whether a water molecule is an axial ligand or not. With two parameters (r and n) to fit one experimental value (even with a more stringent value of τ_c), several combinations of the two extrema described above can be envisaged. However, if it could be proved by a more accurate τ_c that the experimental value of $(1/T_1)_{\text{pmg}}$ is substantially larger than could be accounted for by the exchange of the axial water alone, its existence would become very probable, although not definitely proved by this method within the framework of Eq. (1).

Secondly, a comparison of the rates for the protein (m_L^0) tumbling, water exchange and electron spin relaxation shows, like in *high* spin methaemoglobins^{9,10,11}, that τ_c is, in fact, τ_s , the electron spin relaxation time. The puzzling feature of the result is the frequency-independent and rather long τ_s . In general, the mechanism of electron relaxation for paramagnetic metal ions bound to proteins is far from being understood (see Ch. 9. in ref. 5.), and no systematic study of τ_c for various haemoproteins is yet available. If the accepted notion, that τ_s is governed by the modulation of the zero-field splitting of the paramagnetic metal ion due to the impact by the ligand molecules, is true in this case, then one would have expected a far more efficient electron spin relaxation mechanism, because the haem-iron is very much exposed to the solvent molecules.

The initial neglect of the contact-term in Eq. (1) was justified because $\tau_c \equiv \tau_s$ in m_L^0 . Namely, such an additional term in Eq. (1) contains a correlation function similar to those in the dipolar term. As its characteristic time is also identified with τ_s , the contact mechanism, if operative at all, will not contribute to $(1/T_1)_{\text{PMG}}$ at the plateau after the ω_S -dispersion step in the present measurements.

Finally, the heterogeneity of our m_L^0 -preparations must be considered. The preceding evaluation of the proton magnetic relaxation measurements is only a rough estimate of some of the quantitative parameters involved in an attempt to define the immediate haem environment in more specific structural terms with regard to the solvent. Only after the relaxation mechanism has been elucidated, will it become necessary to check on the fine differences between various fractions¹² of m_L^0 .

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DISCUSSION

D. Bäckström:

Is it possible that the proton relaxation time that is seen can depend on a nitrogen ligated proton in the heme vicinity?

S. Maričić:

A probable candidate is the proton from the hydrogen bond between the sixth ligand water molecule and the nitrogen of the distal histidine, a view held by Gupta and Mildvan, for the case(s) of myoglobin or haemoglobin (*J. Biol. Chem.* **250** (1975) 246—253). We believe that the results we obtained using the methyl protons of methanol added to a deuterated haemoglobin solution exclude this possibility (*Biochim. Biophys. Acta* (Protein Structure), in press). The subject is still controversial and we cannot say anything definite in the case of cytochrome P450.

R. Austin:

Have you also measured relaxation rates for the substrate bound *P450*, and were there differences?

S. Maričić:

Yes, we tried metphenetamine and aniline (see *Biochim. Biophys. Acta (Reports)* **446** (1976) 325—330) and there was practically no difference with the first one, while aniline, surprisingly, increased the paramagnetically induced relaxation rates. In view of the multiple forms of *P450* in our preparation, and the possible spin-state change, these data are very difficult to interpret in structural terms.

L. K. Hanson:

Would you care to comment on whether or not a water is bound to the heme in the high spin ferric forms?

S. Maričić:

I take this question to mean whether the PMR approach can tell us anything about it. As I tried to explain we ended up in a draw even for our low-spin *P450*, and I really cannot say anything about the high spin form for which we have no measurements.

S. Vuk-Pavlović:

The rates of electron-spin relaxation of the low-spin ferric haem-iron are very important for the understanding of the mechanism of solvent-proton magnetic relaxation measurements for *P450*. Namely, Wüthrich (*Structure and Bonding* **8** (1970) 53) estimated the electron-spin relaxation time for the (low spin) cyanomethaemoglobin solutions to be of the order of 10^{-12} s, based on the line-widths of the hyperfine shifted resonances of the porphyrin-protons in the high resolution NMR spectra. However, similarly as shown here for the low spin *P450*, I have recently measured large solvent-proton magnetic relaxation rates in the solutions of cyanide derivative of bovine liver catalase, another low spin example of haem iron. On the basis of these preliminary results it appears that the iron spin state is not determined solely by the axial ligand, but that other sterical factors may be of equal importance.

S. Maričić:

We really badly need a theoretical reinvestigation of the electron spin relaxation mechanism for the two spin states of the haem-iron in two different protein environments: one a tight protein crevice, and the other more open to the solvent.

H. Schleyer:

I'd like to follow this up and add a comment on the use and interpretation of data from the literature, especially of results which were obtained under very different experimental conditions. Metmyoglobin in solution at a neutral pH exists almost fully in a $S = 5/2$ spin state as is well documented by optical and EPR spectroscopy and in direct measurements of the magnetic susceptibility. Yet when Winslow Caughey and his colleagues undertook a careful study of metmyoglobin by Mössbauer spectroscopy some 8—9 years ago they found that — whatever interpretation of these spectra they were attempting to use — their material could only be of a low spin, $S = 1/2$ -character. For quite unrelated reasons we had looked at about the same time into effects which certain »physical« treatments of heme proteins in solution or as single crystals caused on certain parameters of EPR spectra. Our series of »physical« treatments included then partial drying of crystals, lyophilization of samples, repeated freezing and thawing cycles etc. In the course of our studies we found empirically that lyophilization or any similar process of partial or total drying of sperm whale myoglobin at neutral pH led to the formation of low spin forms of the heme protein. The simple explanation of Caughey's unexpected results — which he correctly interpreted — was that he had used a lyophilization process in preparation for his Mössbauer measurements! One could cite quite a few more unexpected results of this kind from recent years. Quite clearly then one must be extremely cautious with the interpretation of experimental data on heme proteins which were obtained under different conditions, and must worry about the limitations of extrapolation from one set of experimental conditions to another.

SAŽETAK

**Magnetska relaksacija protona otapala u otopini citokroma P450 iz jetre zeca. —
O korelacijskom vremenu za dipolnu interakciju elektrona i protona**

*S. Maričić, S. Vuk-Pavlović, B. Benko, *J. Porok, H. Rein, G.-R. Jänig i K. Ruckpaul*

Strukturni se parametri mogu izvesti iz mjerenja PMR, znade li se korelacijsko vrijeme za interakciju spinova. Brzine relaksacija inducirane paramagnetskim željeznim ionom hema u citokromu P450 bile su nezavisne o frekvenciji između 10 i 37 MHz. Moguće granice za korelacijsko vrijeme prema Solomonovoj teoriji razmotrene su s obzirom na mogućnost postojanja jedne molekule vode kao šestog liganda željeza hema. O tome se nije moglo ništa definitivno zaključiti, ali rezultati idu svakako u prilog okolici hema u kojoj se može smjestiti nekoliko molekula vode koje se brzo izmjenjuju s masom otapala.

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