Pseudomonas putida Cytochrome P-450
THE EFFECT OF COMPLEXES OF THE FERRIC HEMEPROTEIN ON THE RELAXATION OF
SOLVENT WATER PROTONS
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BRENDA WALKER GRIFFIN AND JULIAN A. PETERSON

From the Department of Biochemistry, The University of Texas Health Science Center, Southwestern Medical School, Dallas, Texas 75235

With pulsed nuclear magnetic resonance techniques, the effects of various complexes of ferric cytochrome P-450 on the relaxation rate of bulk solution water protons have been determined. For the camphor, metyrapone, and 4-phenylimidazole complexes, the experimental results are consistent with outer sphere relaxation effects. However, for the substrate-free enzyme, the magnitude and temperature dependence of the paramagnetic relaxation effects indicate the presence of exchangeable protons in the coordination sphere of the heme iron atom. The exchange rate (9.3 x 10^5 s^-1 at 25°C) and the thermodynamic activation parameters for the exchange process are very similar to those of acid metmyoglobin and acid methemoglobin, suggesting that a water molecule, and not an amino acid residue of the protein, coordinates to the ferric cation of the enzyme in the absence of added substrate or ligands. From the equations appropriate for coordination sphere protons, the distance between these protons and the ferric heme cation was evaluated as 2.1 Å, which further supports this interpretation. These experimental results demonstrate that the solvent accessibility of the ferric cation of substrate-free cytochrome P-450 is significantly reduced by the binding of substrate or nitrogenous ligands to the heme protein.

Cytochrome P-450, which was originally identified in liver and adrenal cortex microsomes (1-3), has since been shown to be widely distributed in mammals (4-6) and a number of lower organisms (7-9). Among the functions of this monooxygenase are steroid hydroxylation in adrenal mitochondria (10), drug metabolism in liver microsomes (11), and camphor hydroxylation by the bacterium Pseudomonas putida (12). A major problem in the study of cytochrome P-450 from mammalian sources has been the strong association of the enzyme with membrane and the resultant difficulty in obtaining purified active preparations. However, the cytochrome P-450 that can be induced in P. putida by growth on camphor is soluble and readily purified (12-14). Because this bacterial enzyme is similar in many respects to the mammalian enzymes (15-18), it is considered to be a valid and useful model system for understanding the reaction mechanism of the class of heme proteins termed cytochrome P-450.

The interaction of cytochrome P-450 with various ligands and substrates results in absorbance spectral changes that are characteristic of this class of heme proteins, for example, the 450 nm absorbance maximum of the carbon monoxide complex of ferrous cytochrome P-450 (3, 4). Substrate interaction with the ferric enzyme, which shifts the Soret absorbance maximum from 418 nm to about 390 nm (13, 19), has been extensively investigated. In the case of the purified bacterial cytochrome P-450, several techniques, including electron paramagnetic resonance (20), magnetic susceptibility (13), stopped flow spectrophotometry (21), and Mössbauer spectroscopy (22) have been utilized to probe the substrate-binding reaction. These studies have established a spin state conversion of ferric cytochrome P-450 associated with substrate binding (20), as well as the kinetics and thermodynamics of the reaction (21).

There is evidence that the low spin EPR signal of substrate-free ferric cytochrome P-450 may be due to the presence of a sulphydryl group as one of the axial ligands of the iron atom (23). Since substrate binding converts the enzyme to a high spin form, it has been inferred that substrate displaces this sulphydryl ligand from the coordination sphere of the cation (20). However, there is presently no direct experimental evidence concerning the identity of the sixth ligand of low spin ferric cytochrome P-450 or the axial ligand(s) of the high spin ferric enzyme.

In an attempt to answer these questions, studies were...
undertaken to determine the effect of various low and high spin forms of ferric bacterial cytochrome P-450 on the proton relaxation rate of bulk solution water molecules. Since water is one product of the enzymatic reaction, it was postulated that in the absence of added ligands or substrates a water molecule might serve as one of the axial ligands of the ferric cation. Experimental support for this hypothesis would be the demonstration of proton exchange between the first coordination shell of the cation and water molecules in the bulk solution (24). On the other hand, the exchange rate might be too small to be detected by this experimental technique, in which case no conclusions regarding the presence or absence of a coordinated water molecule could be drawn. In the latter case, however, it should be possible to evaluate the distance of closest approach of bulk solution water molecules to the paramagnetic ion and to compare these values for the several forms of ferric cytochrome P-450 studied.

**THEORETICAL BACKGROUND**

The relaxation of proton spins in the coordination sphere of a paramagnetic ion in solution is described quantitatively by the Solomon-Bloembergen equations (27, 28) (Equations 1 and 2). In Equations 1 and 2, $T_{1m}$ and $T_{2m}$ are the longitudinal and transverse relaxation times, respectively; $\gamma$ is the proton gyromagnetic ratio; $\beta$, the Bohr magneton, and $S$ and $g$ are the total spin and $g$ value, respectively, of the paramagnetic ion. The distance between the ion and the proton is $r$. $A$ is the hyperfine coupling constant between the two spins, and $\hbar$ is Planck’s constant divided by $2 \pi$. The Larmor precession frequency of the proton is $\omega$, while that of the paramagnetic ion is $\omega_s$. The correlation time for the dipolar interaction, $\tau_{sr}$, is given by

$$ \frac{1}{\tau_s} = \frac{1}{\tau_{sr}} + \frac{1}{\tau_m} + \frac{1}{\tau_c} $$

and the correlation time for the hyperfine interaction, $\tau_{sr}$, is given by

$$ \frac{1}{\tau_{sr}} = \frac{1}{\tau_{sr}} + \frac{1}{\tau_m} $$

In Equations 3 and 4, $\tau_s$ is the rotational correlation time of the cation ligand complex; $\tau_m$, the residence time of the proton in the coordination sphere of the cation; and $\tau_{sr}$, the spin-lattice relaxation time of the paramagnetic species. If there is exchange between the protons coordinated to the paramagnetic ion and those of the bulk solution, the following equation is applicable (29):

$$ \frac{1}{\tau_{1p}} = \frac{n_m}{N_b} \frac{1}{T_{1m}} + \frac{1}{T_{1p}} $$

where $N$ is the molar concentration of the paramagnetic species; $T_{1p}$ designates the contribution of the paramagnetic ion to the measured relaxation of the bulk solution protons; $n_m$ is the number of protons coordinated to each cation; and $N_b$ is the molar concentration of the bulk solution protons. Similar expressions can be written for $T_2$ and $T_{2p}$.

**EPR EXPERIMENTAL RESULTS**

Quantitative analysis of experimental data by these equations requires that values be assigned to the various parameters. For the complexes of ferric cytochrome P-450 studied, values of $S$ were determined from EPR measurements at temperatures near that of liquid helium, where the substrate-free form is predominantly low spin ($S = 1/2$), while the camphor-bound form is predominantly high spin ($S = 5/2$). The EPR spectrum of ferric cytochrome P-450 in camporph-saturated buffer at 5.5 K is shown in Fig. 1. The low spin component of this sample was quantitated by double integra-
tion of EPR spectra recorded at 1 milli watt microwave power and comparison with either an $S = 1/2$ standard (copper-EDTA solution) or a sample of cytochrome P-450 that was essentially 100% low spin; the low spin signal accounts for approximately 5% of the total cytochrome P-450 concentration of the sample. In contrast to this result, Tsai et al. reported approximately 40% low spin content of the camphor-bound enzyme at 15 K (20).

Since the high spin portion of the EPR spectrum of camphor-bound ferric cytochrome P-450 has not been detected at temperatures above that of liquid nitrogen, the question of spin state of this species at room temperature must be considered. Magnetic susceptibility measurements of our preparation of cytochrome P-450 showed a temperature-invariant high spin content (80%) of the camphor-bound enzyme over the temperature range 100 to 250 K (19). This is less than the value of 95% determined from the liquid helium EPR data presented above because the present enzyme preparations have a higher degree of purity than the sample for which the magnetic susceptibility results were reported. From Moessbauer studies, Sharrock et al. evaluated the high spin content of camphor-bound cytochrome P-450 as 80% at 200 K (22). Thus, different experimental techniques employed by two different laboratories have established that near room temperature the camphor complex of ferric bacterial cytochrome P-450 is at least 80% high spin state. For the metyrapone complex, liquid nitrogen EPR spectra confirm that it is a low spin species, with $g$ values similar to those of camphor-free cytochrome P-450 (30). The EPR signal of the 4-phenylimidazole complex is very similar to that of the low spin N-phenylimidazole complex (20). Thus, for the metyrapone and 4-phenylimidazole complexes of ferric cytochrome P-450, $S$ was taken as $1/2$.

In order to obtain an estimate of $\tau_s$ for ferric cytochrome P-450 complexes, the EPR signal linewidths of the camphor- and metyrapone-bound forms were measured as a function of temperature. In theory, the linewidth of each of these complexes should depend on the square of the temperature at temperatures greater than the Debye temperature (31). The experimental data plotted in this way are shown in Figs. 2 and 3. The linewidth extrapolated to room temperature determines $T_2*$, equated with $\tau_s$, to be $1.5 \times 10^{-11}$ s and $1.4 \times 10^{-11}$ s for the camphor and metyrapone complexes, respectively. This extrapolation procedure does not appear to be strictly valid for the linewidth data of the metyrapone complex (Fig. 3). An alternate empirical method is to plot the experimental linewidth as a function of reciprocal temperature. The linewidth extrapolated to room temperature was first corrected for the temperature-independent component measured near liquid helium temperatures and then used to calculate $\tau_s$. The $\tau_s$ values determined by this method are $3.0 \times 10^{-10}$ and $3.8 \times 10^{-10}$ s for the camphor- and metyrapone-bound forms, respectively.

Although neither of these extrapolation procedures is entirely satisfactory for determining a room temperature value of $\tau_s$, they do provide an estimate of the error in this parameter. It should be noted that $\tau_s$ values reported for other Fe$^{3+}$-containing proteins are of the order of magnitude of $10^{-10}$ s (32, 33).

With these estimates of $\tau_s$, Equations 1 and 2 can be simplified. Generally, $\tau_m^{-1}$ can be neglected in Equations 3 and 4. A reasonable estimate of $\tau_s$ is $10^{-8}$ s (34, 35); hence $\tau_s$ dominates both $\tau_c$ and $\tau_r$, i.e. $\tau_h \sim \tau_c \sim \tau_r$. Thus, $(\omega_c\tau_c)^2 \ll 1$ for both frequencies employed in this study. Typically, $a/h$, the hyperfine coupling constant, is less than 1 MHz (36); with this assignment, the hyperfine term of Equations 1 and 2 is negligible compared to the dipolar term, for $r$ values less than 20 A. Also, since $(\omega_c\tau_c)^2$ will be greater than one, the terms containing this factor in the denominator can be neglected. Incorporating all of these assumptions into Equations 1 and 2 yields

$$\frac{1}{T_{1m}} = \frac{b}{15} \left( \frac{S(S+1)}{I} \right) \frac{2^8 \gamma_r^2 \tau_s}{r^6}$$

(6)

and

$$\frac{1}{T_{2m}} = \frac{b}{15} \left( \frac{S(S+1)}{I} \right) \frac{2^8 \gamma_r^2 \tau_s}{r^6}$$

(7)

NUCLEAR MAGNETIC RESONANCE EXPERIMENTAL RESULTS

The dependence of $(NT_{1m})^{-1}$ on reciprocal temperature is shown in Fig. 4 for each of the ferric cytochrome P-450 complexes studied. The same experimental results were ob-
interaction between the ferric heme iron atom and protons outside the coordination sphere of the cation (24). Thus, if exchangeable protons reside in the coordination sphere of the cation, their rate of exchange with the bulk solution protons is too small to be detected at the temperatures of this study. For these complexes, one can calculate the distance $r$ between the cation and the nearest bulk solution protons. The appropriate equation is obtained by integrating Equation 1 (with the hyperfine term neglected) over the distribution of outer sphere protons, i.e. those not in the coordination sphere of the cation (37).

$$F(r, \omega, \tau_d) = \frac{2\pi \gamma S(S+1)\tau_s N_B}{90 r^3} 10^{-3} \quad (8)$$

$B$ is Avogadro’s number and $\tau_d$ is a measure of the time in which a proton diffuses out of the range of the dipolar magnetic field of the paramagnetic ion. Haussler and Noack have computed $F$ for a wide range of $r$/$\tau_d$ and $\omega$/$\tau_d$ values (38) and have presented the results as a series of graphs, which will be employed in these calculations. If $\tau_d$ is assumed to be $1 \times 10^{-10}$ s (33), $F$ can be determined graphically from Reference 38 for the various extrapolated $\tau_d$ values. The $r$ values calculated from Equation 8 for each cytochrome P-450 species are given in Table I. Since $\tau_d$ cannot be specified precisely, we must consider the possible error in $r$ rising from error in the value of $\tau_d$. For example, as is also shown in Table I, the effect of increasing $\tau_d$ 4-fold is to increase the $r$ values. The experimental error in the $r$ value of the camphor complex may be a factor of 2; for the low spin complexes, it is less. However, the general trend of the $r$ values is clear: (a) of the three low spin complexes, the form in the absence of added substrates or ligands has the smallest $r$ value; and (b) the substrate-bound high spin complex of the enzyme appears to have the largest $r$ value of the complexes studied.

Since the value of $r$ for the camphor-free enzyme is smaller than that expected (2.8 A) for water molecules coordinated to the ferric cation (39, 40), it would appear that outer sphere relaxation effects alone cannot explain the observed $(NT_{1p})^{-1}$ values. In addition, the temperature dependence of $(NT_{1p})^{-1}$ for this species is characteristic of exchangeable protons in the coordination sphere of the paramagnetic ion. The equation to be used in the analysis (24) is analogous to Equation 6:

$$\frac{1}{NT_{1p}} - \frac{1}{(NT_{1p})_{\text{bulk}}} = \frac{n}{N_b} \frac{1}{T_{1m} + \tau_m} \quad (9)$$
Since, in this case, the outer sphere contribution \((NT_{\text{LP}})^{-1})_{\text{bulk}}\) is not negligible, it must be included in Equation 9. It was not possible to determine \((NT_{\text{LP}})^{-1})\) at sufficiently low temperatures to extrapolate a value of \((NT_{\text{LP}})^{-1})_{\text{bulk}}\) for the camphor-free enzyme. So \((NT_{\text{LP}})^{-1})\) for the metyrapone complex was used as an appropriate value of \((NT_{\text{LP}})^{-1})_{\text{bulk}}\), since both of these low spin cytochrome P-450 species have very similar EPR and optical spectral properties (13, 30). If the ligand with exchangeable protons is water, then only 1 water molecule can coordinate to camphor-free ferric cytochrome P-450. This restriction arises because this species of the enzyme is low spin and must have at least one strong field ligand. Since water is a weak field ligand, the possibility of 2 ligated water molecules is eliminated. Thus, for 1 ligated water molecule \(n_{H}/N_{S} = 1/(55.5 M)\).

The dependence of \((T_{\text{im}} + \tau_{r})^{-1}\), determined from Equation 9, on reciprocal temperature is shown in Fig. 5. One explanation for the shape of this curve is that \(\tau_{r} < T_{\text{im}}\), so that the temperature dependence of \(T_{\text{im}}\) dominates over the entire temperature range of the measurements. One must then explain the change in direction of the plot of Fig. 5. Both \(\tau_{r}\) and \(\tau_{s}\) decrease with increasing temperature, but \(\tau_{r}\) is not expected to become as small as \(\tau_{s}\) over this temperature range. However, if the temperature dependence of \(T_{\text{im}}\) were determined by \(\tau_{r}\), \((NT_{\text{ss}})^{-1})\) for camphor-free cytochrome P-450 should exhibit the same temperature dependence observed for the other low spin complexes, but clearly does not (Fig. 4).

On the other hand, the temperature dependence of Fig. 5 can be explained if \(\tau_{s} \geq T_{\text{im}}\). Thus, the linear portion of this plot corresponds to the case where \(\tau_{s} > T_{\text{im}}\), that is, where the exchange of protons in the coordination sphere of the substrate-free enzyme with bulk solution protons dominates the relaxation process. The thermodynamic activation parameters calculated for this exchange process are compared in Table II with the corresponding values for acid metmyoglobin and acid methemoglobin. The results in Table II support this interpretation of the experimental data for substrate-free ferric bacterial cytochrome P-450 and the hypothesis that a water molecule coordinates to this form of the enzyme. Since it is known that \(\tau_{s}\) decreases with temperature while \(T_{\text{im}}\) increases (29), the deviation from linearity of the plot of Fig. 5 occurs in the higher temperature region where \(T_{\text{im}}\) becomes comparable to \(\tau_{s}\) (cf. Equation 9). Thus, the experimental data permit a determination of \(T_{\text{im}}\), from which \(r\), the distance between the cation and the exchangeable protons in its coordination sphere, can be calculated from Equation 6. From the extrapolated value of \(r_{m}\) (Table II) and the experimental value of \(T_{\text{im}} + T_{s}\) (Fig. 5), \(T_{\text{im}}\) is calculated as \(3.3 \times 10^{-6} s\) at 25°C. \(T_{\text{im}}\), we recall, is the actual relaxation time of protons in the coordination sphere of the ferric cation. These protons are relaxed by a magnetic dipole-dipole interaction that depends on the sixth power of the distance between the two dipoles, i.e., the proton and the paramagnetic ion. This distance is calculated from Equation 6 to have a value of 2.1 A. Thus, if the experimental data for substrate-free, low spin cytochrome P-450 is interpreted in terms of exchangeable protons in the coordination sphere of the cation, it can be determined that these protons are 2.1 A from the heme iron atom. Considering the approximations and extrapolations necessary for these calculations, this value is a reasonable one for coordinated water molecules, for which estimated and calculated values range from 2.5 to 2.8 A (39, 40).

**DISCUSSION**

For the camphor, metyrapone, and 4-phenylimidazole complexes of ferric cytochrome P-450, the temperature dependence of \((NT_{\text{LP}})^{-1})\), as well as the magnitude of the \(r\) values in Table I, indicate that the relaxation effects are due to outer sphere protons at a minimal distance of \(r\) from the cation. The molecular basis for the apparent difference in \(r\) values of the camphor and metyrapone complexes cannot be specified in detail, but the following qualitative explanation is proposed. First, what is known about the binding of various ligands and substrates to cytochrome P-450 will be considered. Metyrapone, which has two pyridyl rings, has been shown to coordinate to the ferric heme cation through the lone pair of electrons of one of the nitrogen atoms (41). Camphor, with a ketone function, probably does not coordinate directly to the iron. However, there are several lines of evidence that this substrate molecule binds in proximity to the heme group. First, camphor binding is competitive with metyrapone binding, which implies some overlap of the binding sites of these 2 molecules (30). Also, the heme group is considered to be the active site of the enzyme, since oxygen, the second substrate of this monooxygenase, binds at the iron atom of the ferrous hemeprotein (25). These experimental findings, together with the known stereo-specificity of the enzymic reaction (17), suggest that camphor is bound near the bound oxygen molecule and, by inference, near the heme iron atom. Because of the relatively large size of the compounds that bind near the heme group of cytochrome P-450 (compared to the usual ligands of metmyoglobin and methemoglobin), and because both substrate and oxygen are bound simultaneously near the heme iron atom, the protein “pocket” containing the heme group is considered to be both large and deformable.

The modifications of this protein pocket produced by camphor binding as contrasted with metyrapone binding probably relate to a specific molecular interaction between the substrate and the enzyme. Bacterial cytochrome P-450 exhibits a high degree of substrate specificity: only camphor and a few structurally related compounds are hydroxylated by this enzyme (17). In addition, only those compounds that are hydroxylated can convert the ferric hemeprotein to a high spin state by binding to it (42). These experimental findings indicate a very specific interaction between the protein and its substrate. On the other hand, the interaction of ferric cytochrome P-450 with a variety of isocyanides and nitrogenous bases (20, 30, 41–43), including metyrapone, appears to be a more general
cation-ligand coordination reaction. Thus, the molecular specificity of the interaction between camphor and ferric bacterial cytochrome P-450 is a general hypothesis that can explain why the substrate is the most effective compound of those studied in “closing” the heme pocket, thereby making the heme iron atom less accessible to solvent water protons. This experimental result is consistent with thermodynamic data for the camphor-binding reaction, which suggest that the driving force for substrate binding is the removal of the hydrocarbon from an aqueous environment to the more hydrophobic protein environment (21).

Although there are two possible interpretations of the proton relaxation data for camphor-free cytochrome P-450, both yield the result that bulk solution water protons can approach the cation to within a distance of approximately 2 Å. The interpretation that a water molecule occupies the sixth ligand position of this species provides a satisfactory explanation of the observed temperature dependence of \(\left(NT_m\right)^{-1}\). Also, the rate and thermodynamic activation parameters of the exchange of coordination sphere protons with solvent protons are in good agreement with the corresponding parameters of acid methemoglobin and acid metmyoglobin (44), both of which have a coordinated water molecule (45, 46). Since the parameters in Table II are not necessarily unique to coordinated water molecules, they do not prove that water is a ligand of substrate-free ferric cytochrome P-450. However, under the conditions of these experiments in which neither substrate nor ligands were added, the only possible ligands of this enzyme species are water or an amino acid residue of the protein. The latter case is highly unlikely, as shown by the following arguments.

In order to resolve the question about other possible ligands of camphor-free ferric cytochrome P-450 one must first consider the possibility of observing exchange effects with an added ligand that has exchangeable protons; for this reason, the experiments with 4-phenylimidazole were undertaken. Both metyrapone and 4-phenylimidazole coordinate to the ferric cation through the lone pair of electrons of a ring nitrogen atom (41). At the pH of these studies, metyrapone has no exchangeable protons, but 4-phenylimidazole has a second ring nitrogen atom less accessible to solvent water protons. This experimental result is consistent with thermodynamic data for the 4-phenylimidazole complex of ferric cytochrome P-450, it was not detected experimentally, as would be predicted by these arguments. Indeed, the experimental data for the 4-phenylimidazole and metyrapone complexes are essentially identical and can be attributed to paramagnetic relaxation effects on outer sphere protons, those not coordinated to the heme iron atom.

If, on the other hand, the ligand of the camphor-free enzyme is endogenous, i.e. an amino acid residue of the protein, the conditions for observing exchange are even more restrictive: direct proton exchange between bound ligand and solution water molecules must occur, and the concentration of the ligand can be no greater than the protein concentration. Thus, it is very unlikely that an endogenous ligand with exchangeable protons could give rise to the effect produced by substrate-free ferric cytochrome P-450 on the solvent proton relaxation rate. From these considerations, it is concluded that the exchange effect observed for this enzyme species is most likely due to a coordinated, rapidly exchanging water molecule.

In summary, the results of this study indicate that the accessibility of the heme iron atom of low spin, ferric bacterial cytochrome P-450 to solvent water molecules is significantly reduced by the binding of nitrogenous ligands or camphor, the substrate hydroxylated by this monooxygenase. The significance of this finding for the mechanism of the enzyme reaction can only be speculated at present, but is being actively investigated.

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**Table II**

Exchange parameters for protons in coordination sphere of ferric heme proteins at 25°C

<table>
<thead>
<tr>
<th>Hemeprotein</th>
<th>(\varepsilon_m) kcal/mol</th>
<th>(\Delta H^\circ) kcal/mol</th>
<th>(\Delta S^\circ) cal/(mol - K)</th>
<th>(1/\tau_m) s^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-450</td>
<td>6.6</td>
<td>6.0</td>
<td>-15.5</td>
<td>9.3 x 10^4</td>
</tr>
<tr>
<td>Met-Hb (44)</td>
<td>6.6</td>
<td>6.6</td>
<td>-21.3</td>
<td>5.6 x 10^3</td>
</tr>
<tr>
<td>Met-My (44)</td>
<td>10.0</td>
<td>9.4</td>
<td>-7.8</td>
<td>1.4 x 10^4</td>
</tr>
</tbody>
</table>

*a* Camphor-free.

*b* Determined as control in this study.
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