# Solvent Proton Magnetic Resonance Dispersion in Protocatechuate 3,4-Dioxygenase and Complexes with 3-Halo-4-hydroxybenzoate Inhibitors†

R. H. Felton,* Sidney L. Gordon, Anne L. Sowell, and Sheldon W. May

ABSTRACT: Solvent proton nuclear magnetic dispersion studies at 25, 100, and 300 MHz have been performed on protocatechuate 3,4-dioxygenase (PCD) and its complexes with 3-chloro-4-hydroxybenzoate and 3-fluoro-4-hydroxybenzoate. Longitudinal and transverse relaxation rates were measured for these compounds and for the apoenzyme. The paramagnetic enhancement of solvent $T_1$ is interpreted in terms of dominant dipole–dipole relaxation of fast-exchanging solvent protons with a negligible contribution from outer sphere relaxation and an electronic spin relaxation time of 0.5 ns for the high-spin ferric ion. A discrepancy between the observed $T_2$ at 300 MHz and that calculated by assuming the usual dipolar relaxation provides evidence for an additional Curie-spin dipolar or hyperfine interaction between the proton and iron. Quantitation of the additional relaxivity provides an estimated chemical exchange lifetime of 0.1–0.14 μs, which suggests proton exchange by a hydroxide ligand. Proton-to-iron distances are 2.7–3.1 Å in PCD and lengthen to 3.6–4.1 Å in the halohydroxybenzoate complexes.

Protocatechuate 3,4-dioxygenase (PCD) is a catechol-cleaving enzyme that catalyzes the oxygenation of protocatechuic acid to form β-carboxy-*cis*,*cis*-muconic acid (Que, 1980). The enzyme has an $M_r$ of $783 \times 10^3$ and is composed of eight identical subunits, each of which contains a high-spin iron ($S = {}^5/_2$) at the active site. Electron paramagnetic resonance (EPR) (Que et al., 1977), extended X-ray absorbance fine structure (EXAFS) (Felton et al., 1982), Mössbauer (Que et al., 1976), and resonance Raman (Felton et al., 1978; Que & Epstein, 1979) studies have shown that two histidines, two tyrosines, and at least one water are ligands of the iron. The presence of water was shown by a preliminary study (Que et al., 1977) of the 30-MHz relaxation rate of solvent, and that datum was interpreted as indicating one water located 2 Å from the iron. Subsequent $H_2{}^{17}O$ hyperfine broadening of the ferric EPR signal confirmed bound solvent (Lipscomb et al., 1982). New inhibitors and transition-state analogues have become available recently (May et al., 1979, 1982, 1983), and we have performed solvent nuclear magnetic dispersion (NMRD) on a number of these. In this paper, we present the results of a high-field NMRD study of native PCD and its complexes with two inhibitors, 3-fluoro-4-hydroxybenzoate (3-FHB) and 3-chloro-4-hydroxybenzoate (3-ClHB). The inhibitors were chosen on the basis of EXAFS evidence that suggests the PCD·3-ClHB complex exhibits pseudochelation with both the chloro and hydroxylic functionalities serving as ligands to iron. Consequently, the NMRD is expected to show solvent displacement from the inner sphere, since water is readily replaced by a strongly binding ligand such as the hydroxylic oxygen.

Our results provide evidence that bound water in native PCD is displaced upon binding of the halohydroxybenzoate inhibitors and further suggest that solvent is bound as the hydroxide. Additionally, a novel use of high-field transverse relaxation rates is advanced to obtain the proton exchange lifetime for solvent bound to the active site iron.

## Materials and Methods

PCD was isolated from *Pseudomonas aeruginosa* and purified as previously described (May et al., 1978). The apoenzyme was formed by dithionite reduction of PCD and removal of iron upon treatment with 1,10-phenanthroline under anaerobic conditions. The efficacy of the iron removal was monitored by NMR as described below and by atomic absorption measurements; the latter showed that the apoenzyme contained amounts varying from 10 to 15% as much iron as PCD, depending upon the preparation. 3-ClHB and 3-FHB were synthesized and purified by published procedures (May & Phillips, 1978, 1979).

NMR measurements were performed on 10-μL volumes of air-saturated samples, which contained 2.0 mM ferric ion (8 mol of iron/mol of enzyme) in 50 mM tris(hydroxymethyl)-aminomethane (Tris)–acetate buffer adjusted to pH 8.5. Sufficient inhibitor was present to ensure saturation of all binding sites. $T_1$ measurements at 25 °C were performed at 25 and 100 MHz (JEOL PFT-100) and 300 MHz (Bruker WM-300) by using an inversion–recovery technique. Sample temperatures were controlled at 300 MHz and were carried out at room temperature, 25 ± 1 °C, on the JEOL spectrometer. Variable-temperature experiments at 300 MHz showed that possible temperature variations introduced negligible error for all samples. $T_1$ for the buffer was 3.07 s. $T_2$ relaxation times were measured by a Carr–Purcell–Meiboom pulse train. At least three measurements were made at each frequency, and the reproducibility for all samples was better than 5% and usually was 2%. $T_1$ and $T_2$ were rechecked frequently to demonstrate that no change in sample properties occurred during the course of the experiments.

## Results

Paramagnetically enhanced longitudinal and transverse molar relaxivities were calculated at an intermediate state of analysis from (Koenig & Schillinger, 1969)

$$R^{(1,2)} = [(1 - V)/m](1/T_{1,2} - 1/T_{1,\text{buffer}}) \qquad (1)$$

where $m$ is the molarity of iron with the assumption that all eight iron-bindinding active sites are equivalent, and $V$ is the fraction of volume occupied by the protein. A density of 1.26 g/cm$^3$ (Satyshur et al., 1980) from preliminary X-ray crystallographic data was used to calculate $V$. Since atomic absorption analysis had indicated a remnant of PCD in the apo-PCD samples, relaxivities for the diamagnetic contribution of the apoenzyme had to be calculated. It was noted that relaxivities for the apoenzyme, computed according to eq 1, showed the same dispersive behavior at 100 and 300 MHz as

† From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332. *Received November 15, 1983; revised manuscript received March 14, 1984.* This work was supported in part by a grant from the National Institutes of Health (GM 23474).

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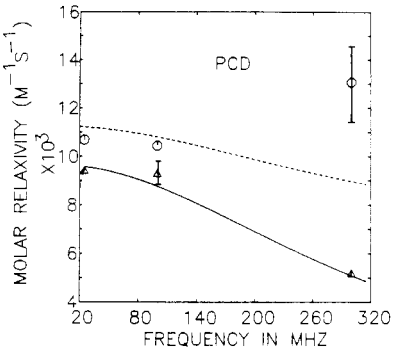



FIGURE 1: Paramagnetically enhanced solvent relaxation rates in PCD. Longitudinal rates, $R^{(1)}$, are data (▲) and theory (—) of Solomon (1955). Transverse rates, $R^{(2)}$, are data (○) and theory (--) of Solomon excluding contact contribution. Samples are 2.0 mM in Fe(III) at pH 8.5 and 25 °C.

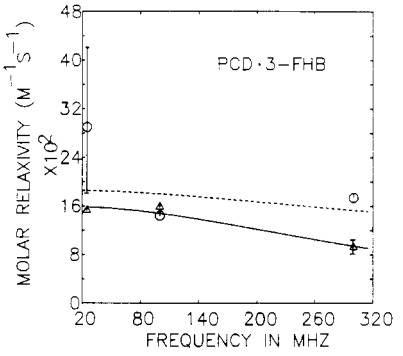



FIGURE 2: Paramagnetically enhanced solvent relaxation rates in PCD·3-FHB. Symbols are as in Figure 1.

that exhibited by PCD. The ratio of apo-PCD to PCD relaxivities at the two frequencies was 0.057 and 0.054; therefore, the fraction of PCD was set to 0.056 in this sample. Next, the apo-PCD relaxivity at 25 MHz was corrected for 5.6% PCD present. This correction resulted in a diamagnetic apo-PCD relaxivity of 310 $M^{-1}$ $s^{-1}$ at 25 MHz and negligible relaxivity at 100 and 300 MHz. Similar correction of the apo-PCD transverse relaxivity was performed at the three frequencies. In this instance, $R^{(2)} \gg R^{(1)}$, for both apo-PCD and PCD, and a larger absolute error in the transverse relaxivity of PCD compared to that of $R^{(1)}$ represents the scatter in $R^{(2)}$ found by using not only the apo-PCD sample containing 5.6% PCD but also one that contained 10.4% PCD. Corrected, paramagnetically enhanced contributions $R^{(1)}$ and $R^{(2)}$ were found for PCD by this procedure and are shown in Figure 1 for PCD. PCD·3-FHB and PCD·3-ClHB paramagnetic relaxivities are found by subtracting the diamagnetic relaxivity contribution of the apoenzyme. The results are displayed in Figures 2 and 3. Corrected $R^{(2)}$ values for apo-PCD, used in the subtraction, are $10.7 \times 10^3$, $10.4 \times 10^3$, and $(13.0 \pm 1.5) \times 10^3$ $M^{-1}$ $s^{-1}$ at 25, 100, and 300 MHz, respectively.

If the value of $R^{(1)}_{apo}$ is normalized to the molarity of enzyme rather than that of iron sites, then its value is $2.5 \times 10^3$ $M^{-1}$ $s^{-1}$, which compares favorably to relaxivities exhibited by diamagnetic proteins of comparable molecular weight (Hallenga & Koenig, 1976). On the other hand, setting $R^{(1)}_{apo}$ to 0 at 100 and 300 MHz is equivalent to neglecting its high-field limiting value. However, few measurements have been made at high fields, and there is considerable error in the limiting value for proteins of large molecular weight.

*$T_1$ Measurements.* Relaxivities exhibit dependence upon several dynamic processes according to

$$R^{(1,2)} = [n/(2N_w)](T_{1,2M} + \tau_M)^{-1} + R^{(1,2)}_{out} \quad (2)$$

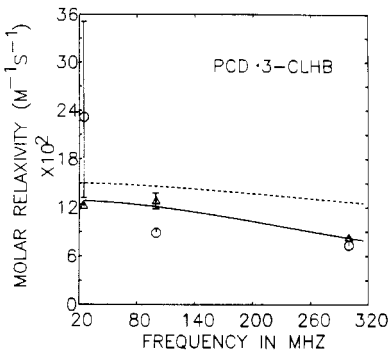



FIGURE 3: Paramagnetically enhanced solvent relaxation rates in PCD·3-ClHB. Symbols are as in Figure 1.

Here, $T_{1,2M}$ are the relaxation times of a solvent proton in the proximity of a paramagentic spin, $\tau_M$ is the chemical exchange lifetime of a proton, $N_w$ is the molarity of water, and $n$ is the number of solvent protons relaxed by the spin. $R^{(1,2)}_{out}$ values are outer sphere contributions arising from the diffusional modulation of the dipole–dipole interaction between the paramagnet and proton. A common feature of the data is the dispersion exhibited between 100 and 300 MHz by all samples, and this aspect of $R^{(1)}$ can be used to distinguish between outer sphere and paramagnetic or exchange contributions to the relaxation rates. Outer sphere relaxation contributions are dominated by a diffusional translation correlation time, $\tau_D$, when the proton-to-iron distance, $r$, is small and by the electronic spin relaxation time, $\tau_S$, if $r$ is large (Koenig & Schillinger, 1969). The theory (Pfeifer, 1961; Hausser & Noack, 1965) contains $\tau_S$, $r$, and the solvent diffusion constant $D$ as parameters. In addition, a factor $f$ is required for protein molecules when some directions of solvent access are excluded due to the protein matrix. This factor is $^1/_8$ for small $r$ and $^1/_6$ for large $r$, in a model that places the paramagentic ion a distance $r$ below a semiinfinite plane containing solvent (Koenig et al., 1981). We have utilized the equations of Pfeifer (1961) to calculate $R^{(1)}_{out}$ for a range of values of $r$. If $R^{(1)}_{out}$ is to disperse at frequencies above 25 MHz, then $\tau_D$ is on the order of $(1\text{–}3) \times 10^{-12}$ s, corresponding to iron–to–proton distances of 1–2 Å [$r = (3D\tau_D)^{1/2}$] with the diffusion constant of $H_2O$ taken as $2.5 \times 10^{-5}$ $cm^2$ $s^{-1}$. Since high-spin ferric ion relaxes slowly [$\tau_S \sim 10^{-9}$ –$10^{-10}$ s (Eaton & Phillips, 1965)], $\tau_S$ is much less than $\tau_D$, and the results are insensitive to the choice of $\tau_S > 10^{-10}$ s. An estimate for a physically acceptable distance of closest approach is 2.7–2.8 Å, corresponding to ligated water or hydroxide. Clearly, $r < 2$ Å is unacceptable. Additionally, the outer sphere contribution at 25 MHz is $4.1 \times 10^2$ $M^{-1}$ $s^{-1}$, using $f = ^1/_8$, and cannot account for the observed magnitude shown in Figure 1 for PCD. For a closest contact of $r = 2.7\text{–}3.0$ Å, $R^{(1)}_{out}$ is $(2.4\text{–}2.0) \times 10^2$ $M^{-1}$ $s^{-1}$ for a frequency of 25 MHz and is less than 3% of the paramagnetic relaxivity of PCD. Finally, $R^{(1)}_{out}$ under the cited conditions disperses more slowly than does $T_{1M}$, and most dispersion occurs between 25 and 100 MHz rather than between 100 and 300 MHz as is observed. In order to have outer sphere relaxations disperse properly, the electronic spin must now relax more rapidly than the diffusive correlation time, and $\tau_D > 10^{-9}$ s, corresponding to $r = 30$ Å. $R^{(1)}_{out}$ is 4 $M^{-1}$ $s^{-1}$ from employing $f = ^1/_6$ and $\tau_s = 0.5 \times 10^{-9}$ s (Table I); therefore, the outer-sphere contribution to $R^{(1)}_{PCD}$ can be neglected.

Figure 1 shows the fit to $R^{(1)}$ for a process dominated by $T_{1M}$ and calculated according to the usual dipole–dipole interaction (Solomon, 1955) with no hyperfine contribution. Table I gives the correlation time and proton-to-iron distance.

Table I: Correlation Times and Proton-to-Iron Distances

| complex | correlation time (ns) | proton–iron distance (Å) $n = 1$ | $n = 2$ |
|---|---|---|---|
| PCD | 0.50 | 3.05 | 2.7 |
| PCD·3-FHB | 0.44 | 4.0 | 3.6 |
| PCD·3-ClHB | 0.40 | 4.1 | 3.7 |

In view of the strongly hydrolytic nature of iron, a value of $r$ ($n = 1$) corresponding to hydroxide is also included.

Figures 2 and 3 present a $T_{1M}$ least-squares fit to the two inhibitor–enzyme complexes. The derived distance has increased to 4.1 Å for a water molecule ($n = 2$), and at this distance, outer-sphere contributions are 120, 91, and 82 $M^{-1}$ $s^{-1}$ at 25, 100, and 300 MHz, respectively. The adduced values are <10% of the observed relaxivities.

*$T_2$ Measurements.* The contribution to $R^{(2)}$ arising solely from dipole–dipole relaxation is indicated in the figures. Parameters $\tau_C$ and $r$ are those listed in Table I. Within experimental error, calculated and experimental values of $R^{(2)}$ agree well for PCD at 25 and 100 MHz and for the inhibitor–enzyme complexes at all frequencies. However, there is a discrepancy, $\Delta R^{(2)}$, of $(4.0 \pm 1.5) \times 10^3$ $M^{-1}$ $s^{-1}$ for the native enzyme at 300 MHz.

## Discussion

The discrepancy, $\Delta R^{(2)}$, found at 300 MHz can be used to estimate the chemical exchange lifetime. In the preceeding analysis, $\tau_M \ll T_{1M,2M}$ has been assumed and remains to be verified. Usually, the temperature dependency of $R^{(1)}$ is measured in a nondispersive region, and the slope of an Arrhenius-type plot of ln $R^{(1)}$ vs. $1/T$ is employed to demonstrate if $\tau_M$ contributes to the longitudinal relaxivity. A distinct route to $\tau_M$ arises by considering various sources for the field dependency of the transverse relaxivity. One source could be a field-dependent electronic relaxation time, which is found, for example, in $Mn^{2+}$ (Koenig & Brown, 1981). Under conditions such that $\tau_S$ contributes strongly to the effective dipolar correlation time, the NMRD of the longitudinal relaxivity also exhibits $\tau_S$ variation. However, in our results $R^{(1)}$ dispersion is well described by the Solomon equation, and no evidence for $\tau_S$ variation is found. Consequently, we must exclude field dependency of the electronic relaxation time and examine causes that contribute strongly to $R^{(2)}$ but not $R^{(1)}$. The most plausible sources are dipolar or hyperfine interactions with the Curie magnetization of the electronic spin. For both interactions, it is the average spin component, $\langle S_z \rangle$, along the applied field that exhibits the field dependency. According to this treatment (Gueron, 1975; Vega & Fiat, 1976)

$$T_{2M}^{-1} = 4(\langle S_z \rangle g\beta\gamma_I/r^3)^2\tau_{eff}/5 \quad (3)$$

for dipolar relaxation, and

$$T_{2M}^{-1} = (A\langle S_z \rangle)^2\tau_M \quad (4)$$

for hyperfine relaxation of the transverse magnetization. Here

$$\langle S_z \rangle = g\beta HS(S + 1)/(3kT) \quad (5)$$

with $\gamma_I$ as the proton gyromagnetic ratio, $g$ as the electronic $g$ factor, $\beta$ as the Bohr magneton, and $H$ as the applied magnetic field. The correlation time of processes such as chemical exchange or internal rotation modulating the hyperfine coupling constant $A$ is $\tau_M$. For the dipolar term, the effective correlation time is $\tau_{eff}^{-1} = \tau_M^{-1} + \tau_R^{-1}$, with $\tau_R$ as the rotational correlation time for the macromolecule. The rates represented in eq 3 and 4 provide additional relaxation mechanisms for $T_{2M}$.

The two contributions to $\Delta R^{(2)}$ are estimated at 25 °C and a frequency of 300 MHz. We use $r = 2.7$ Å, $n = 1$, $g = 2$ (an isotropic $g$ factor appropriate for high-spin ferric ion), $H = 7.0$ T, and $A/h = 1.2 \times 10^7$ $s^{-1}$, which arises from the chemical shift of solvent protons observed in ferric nitrate solutions (Luz & Shulman, 1965). The values yield

$$\Delta R^{(2)}_{dipolar} = (3.7 \times 10^{10})\tau_{eff}$$

$$\Delta R^{(2)}_{contact} = (0.87 \times 10^{10})\tau_{eff}\ M^{-1}\ s^{-1}$$

with

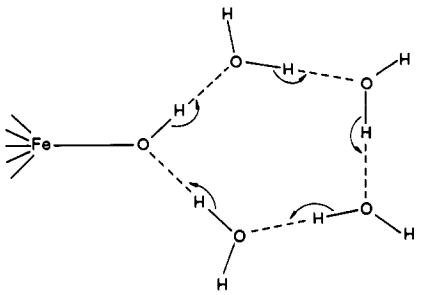
$$\Delta R^{(2)} = \Delta R^{(2)}_{dipolar} + \Delta R^{(2)}_{contact} \quad (6)$$

The rotational correlation time $\tau_R = 4.3 \times 10^{-7}$ s at 25 °C is calculalated from Stokes' law with a radius estimated from the 2.27-$Å^3$/dalton Matthews coefficient of crystalline PCD (Satyshur et al., 1980). A chemical exchange lifetime of $(1.1 \pm 0.5) \times 10^{-7}$ s is found by solving eq 6. It is likely that the contact contribution is overestimated, since it would provide an added 1500 $M^{-1}$ $s^{-1}$ to the usual dipolar $T_{2M}$ shown as a dotted line in the figures. Neglect of $\Delta R_{contact}$ alters $\tau_M$ to $(1.4 \pm 0.8) \times 10^{-7}$ s.

Thus, $\tau_M$ is smaller by 1 order of magnitude than the smallest value of $T_{1M} = 1.7 \times 10^6$ s or $T_{2M} = 1.5 \times 10^{-6}$ s and confirms the earlier assumption that chemical exchange is rapid in PCD for solvent protons contributing to NMRD. Since $\tau_{eff} \gg \tau_S$, the correlation times listed in Table I are the electron spin relaxation times, and furthermore, neither slow $\langle S_z \rangle$-modulated process contributes to $R^{(1)}$ in PCD.

The electronic spin–lattice relaxation time for PCD is $0.5 \times 10^{-9}$ s and is in the range found for other high-spin ferric ions in transferrin ($\tau_S = 0.3 \times 10^{-9}$ s; Koenig & Schillinger, 1969) or horseradish peroxidase [$\tau_S = (0.1–0.5) \times 10^{-9}$ s; Vuk-Pavlovic & Benko, 1975). In the PCD complexes, $\tau_S$ decreases slightly.

*Chemical Exchange Rate.* A proton chemical exchange rate of $10^7$ $s^{-1}$ exceeds the diffusion limit if the catalytic process involves exchange of $H^+$ or $OH^-$. In a pH 8.5 buffer, the rate for that mechanism cannot be faster than $10^{10} \times 3 \times 10^{-6} = 3 \times 10^4$ $s^{-1}$. Similar fast rates for proton exchange have been observed in solutions of $Fe(NO_3)_3$ (Luz & Shulman, 1965), $Ru(NH_3)_6^{3+}$ (Waysbort & Navon, 1973), $Cr(H_2O)_5(OH^-)^{2+}$ (Melton & Pollak, 1969), and Co(II)-substituted carbonic anhydrase (Bertini & Luchinat, 1983). For the Ru and Cr complexes, a Grotthus-type mechanism is invoked, in which a hydroxide or amide ligand, a neutral aquo or amine ligand, and water molecules participate in a cyclic intermediate (Melton & Pollack, 1969; Grunwald & Fong, 1972). This mechanism requires two cis ligand sites and exchange of all protons of the basic and neutral ligands. The mechanism has been modified to the situation in which only one hydroxide ligand is involved (Bertini & Luchinat, 1983), viz. Here,




proton exchange is fast, it involves no ionic intermediates, and

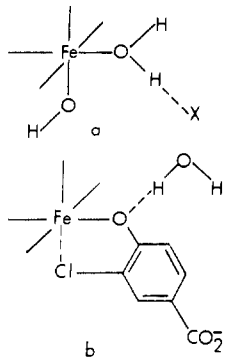



FIGURE 4: Suggested structures containing solvent molecules and consistent with NMRD data. (a) Native enzyme with hydroxo and aquo ligands, the latter exchanging slowly perhaps due to hydrogen bonding. Alternatively, the site occupied by water could be vacant or occupied by an aprotic amino acid residue. (b) PCD·3-ClHB or PCD·3-FHB containing a water molecule held by hydrogen bonding at the active site. Bonding to phenolic oxygen is shown as one possibility.

oxygen is retained. In the absence of data on $^{17}O$ exchange rates in PCD, the mechanism is compatible with our measured proton exchange rate. In particular, the volume needed to accomodate the structure shown is about that of the protocatechuate substrate; thus, sufficient space should be available at the active site of PCD.

All mechanisms for fast proton exchange from a coordinated group involve the basic form of the ligand. Whether this is the predominant form in PCD at pH 8.5 is not established, but in this context, we note that $pK_1$, $pK_2$, and $pK_3$ for successive hydrolysis of hexaaquoferric ion are 2.46, 4.7, and 6.4 (estimated) (Lamb & Jacques, 1938), and proton release studies on transferrin, which contains the same two anionic tyrosyl ligands as PCD, are interpreted in favor of a bound hydroxo ligand (Pecoraro et al., 1981), stable over the pH range 6.5–9.4. In the ferric–ethylenediametetraacetate complex, ferrihemoglobin, and protoheme, the $pK_a$'s for water ionization are 7.8 (Manley, 1971), 7.5–9 (Brunori et al., 1971), and 7.5 (Shack & Clark, 1947), respectively. The adduced values favor coordinated hydroxo at pH 8.5.

The other mechanism referred to previously and advanced for rapid proton exchange requires an additional water ligand. In the ferric(aquo)(hydroxo) complex, if at least one proton in the aquo species and the hydroxo proton exchange at comparable rates, then an unreasonably large value of $r$ (vide infra) is calculated. On the other hand, the presence at the sixth coordination site of a species (Figure 4a) that does not particpate in fast proton exchange is consistent with our data, as is a vacant site.

For the enzyme–inhibitor complexes, the $T_1$ relaxation is not lifetime limited and $T_2$ is not field-dependent, and these observations lead to $\tau_M \leq 0.2$ μs. Thus, the proton exchange rate is too fast to be an acid–base–catalyzed exchange of an amino acid residue. The present case may be analogous to that found in fluromethemoglobin (Koenig et al., 1981), in which one proton of a water molecule is hydrogen bonded to the fluoride ion ($r$ = 3.1–3.7 Å) and undergoes rapid exchange with the solvent ($\tau_M \leq 0.05$ μs). In PCD with bound 3-FHB or 3-ClHB, the phenolic oxygens of the inhibitor or of the tyrosine ligands could participate in hydrogen bonding to water (Figure 4b). In this configuration, relaxivity is dominated by the hydrogen-bonded proton. Indeed, in the crystal structure of a copper complex containing imidazole and 2,4,6-trichlorophenol as ligands (Wong et al., 1976), one water proton is located at a distance of 3.1 Å from the metal due to hydrogen bonding. The simplest mechanism one can envisage is diffusion by the solvent into the active site and retention of the molecule via hydrogen bonding for a time $\tau_M$ less than $T_{1M}$ but greater than $\tau_S$, followed by diffusion back to bulk solvent. This mechanism involves whole water exchange and, in principle, is testable by measuring $^{17}O$ relaxation.

*Proton-to-Iron Distance.* Values of $r$ are 2.7, 3.1, and 3.3 Å depending upon one, two, or three protons exchanging with bulk solvent molecules. The accuracy of the distance requires that Solomon's equation for dipole–dipole relaxation is valid. Either $g$-factor anisotropy or zero-field splitting (Koenig & Brown, 1980) can alter derived distances. In high-spin ferric ion, the state is derived from a $^6S$ atomic term and, consequently, the $g$-factor anisotropy will be quite small. Zero-field splitting (ZFS) influences the relaxation rates by either changes in transition probabilities of the electronic spin or altering the spacing between energy levels of the coupled electron and nuclear spins. At frequencies encountered in this study, dispersion arises from nuclear spin flips without a simultaneous electron spin flip. Thus, even though the Zeeman and ZFS energies are comparable in PCD and PCD·3-FHB (Que et al., 1977), we can demonstrate by direct calculation of the energies and eigenfunctions of the spin Hamiltonian that the total transition probability changes by 10% at most and the value of $r$ would change by 2%. This calculation also demonstrates that eq 5 remains valid for ZFS in the electronic spin manifold.

Our model to this point postulates one bound hydroxo and possibly an additional aquo ligand from which proton exchange is slow (Figure 4a). If the hydroxo proton is being detected by NMRD, then this implies $r$ = 2.7 Å, which is consistent with the observation of $^{17}O$ broadening of the EPR signal in PCD (Lipscomb et al., 1982). The requirement that proton exchange is slow in the aquo ligand arises from the fact that two or three fast-exchanging protons lead to $r$ = 3.1 and 3.3 Å and is incompatible with $r$ = 2.7 Å expected in a ferric(aquo)(hydroxo) species. A more complex model postulates that NMRD detects a nonligated water molecule at a distance of 3.1 Å but does not sense a bound and slowly exchanging aquo ligand. Since the bound aquo ligand is presumably present as $OH^-$ at pH 8.5 and we cannot envisage a reason why exchange of this proton is slow, the model is excluded. Therefore, we favor the view that both spectroscopic techniques are sensing ligated solvent species. These may be the same molecule, i.e., hydroxo, or the species may be hydroxo and aquo ligands that exchange at different rates (Figure 4a). The notion of two waters with different exchange rates and both bound at the same metal has been discussed in the NMRD study of the Mn binding site of concanavalin A (Burton et al., 1979; Koenig & Brown, 1980). The larger value of $r$ in inhibitor–enzyme complexes implies the NMRD detectable water is nonbonding (Figure 4b). A similar observation has been made by Whittaker & Lipscomb (1983), who observe no $^{17}O$ broadening in PCD·3-ClHB and suggest that two solvent molecules or one solvent and one amino acid ligand are displaced upon binding of 3-ClHB.

**Registry No.** PCD, 9029-47-4.

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