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NMRD studies on phthalate dioxygenase: evidence for displacement of water on binding substrate

Abstract Water proton T_1^{-1} measurements at magnetic fields between 0.01 and 50 MHz [nuclear magnetic relaxation dispersion (NMRD) measurements] have been performed on solutions of phthalate dioxygenase (PDO) reconstituted at the catalytic iron site with copper(II) or manganese(II). The data show evidence of a weakly coordinated water molecule in CuPDO; in the presence of the substrate, phthalate, this water appears to become even less tightly bound, and an additional tightly coordinated water can be detected. In PDO reconstituted with manganese, one tightly coordinated water is detected in the presence and in the absence of phthalate. An attempt is made to reconcile these data with low-temperature near-IR magnetic circular dichroism and X-ray absorption data, which show that PDO reconstituted with iron or cobalt is six-coordinate in the absence of substrate and five-coordinate in the presence of substrate.

Abbreviations *PDO* Phthalate dioxygenase; *NMRD* Nuclear magnetic relaxation dispersion, *apo-PDO* phthalate dioxygenase with the mononuclear divalent metal removed, *FePDO, ZnPDO, MnPDO, CuPDO*, phthalate dioxygenase that has been reconstituted with the indicated metal

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Introduction

The phthalate dioxygenase system is involved in the degradation of phthalate by the soil bacterium, *Pseudomonas cepacia* DB01. It catalyzes the first step in the biodegradation of phthalate by dihydroxylating the aromatic ring to produce the *cis*–4,5-dihydrodiol. This product is eventually converted to 3,4-dihydroxybenzoate, which is metabolized through the catecholic pathway [1]. The phthalate dioxygenase system is a member of a family that catalyzes the formation of *cis*–dihydrodiols from benzene, naphthalene, benzoate, biphenyl, and many other unactivated aromatic compounds (reviewed in [2, 3]). Each of the known dioxygenase systems uses an NADH-dependent FAD- or FMN-containing reductase and a terminal dioxygenase that contains a Rieske [2Fe-2S] center and requires ferrous iron for activity. The Rieske [2Fe-2S] center transmits electrons from the reductase to the ferrous mononuclear center. The phthalate dioxygenase system has two components: phthalate dioxygenase reductase, a 34-kDa protein, which has an FMN and a ferredoxin type of [2Fe-2S] center, and phthalate dioxygenase (PDO), an oligomeric protein of 200 kDa with one mononuclear ferrous center and one Rieske center per 50-kDa monomer [4].

The mononuclear center is thought to be the site where oxygenation occurs. The Fe^{2+} ion in the mononuclear site can be removed by dialysis against EDTA; it can then be replaced by a variety of divalent metals including Co^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+} [5]. None of these metals restores hydroxylation activity, but other properties are reestablished [6]. Although the three-dimensional structure of PDO is not known, X-ray absorption spectroscopy of PDO reconstituted with Co^{2+} in the absence of phthalate has shown that the average metal ligand distance is 2.08 Å, and that all coordination is through nitrogen and oxygen [7]. These studies also showed that when phthalate binds to the mononuclear site the average distance decreases from 2.08 Å to 2.02 Å, suggesting that the coordination number de-

creases from six to five. Similar results were obtained from PDO that had Fe^{2+} in the mononuclear site. Low-temperature near-infrared magnetic CD spectroscopic studies on the *d-d* transitions of the Fe^{2+} mononuclear site were consistent with the X-ray absorption studies, and implied strongly that upon binding phthalate, the coordination changed from six to five [8].

In the studies reported here we have used NMRD spectroscopy to investigate whether coordinated water can be detected and how it is affected by the binding of the hydrophobic phthalate ring near to the mononuclear iron.

Experimental

Materials and methods

Phthalate dioxygenase was prepared as previously described [4]. The standard buffer was 100 mM HEPES, pH 8. PDO was repeatedly washed with 0.1 mM dithiothreitol (DTT) in 100 mM HEPES buffer, pH 8, using a Centricon 10 K, to eliminate substrate and glycerol. The sample was then dialyzed against two changes of 2 dm^3 of 5 mM EDTA in the same buffer for 24 h to remove the mononuclear iron ion. EDTA was then removed by repeated dilutions with buffer and ultrafiltration with either an Amicon with a 100 K membrane or a Centricon 10 K. After this procedure the apoprotein (apo-PDO) was washed using the Centricon 10 K in the same buffer containing 0.1 mM DTT. Typical apo-PDO concentrations were approximately 1–1.5 mM (monomers) based on the [2Fe-2S] content. The protein solution was made anaerobic by purging with nitrogen and then was reconstituted to form holoprotein by adding stoichiometric amounts of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$ or $MnCl_2 \cdot 4H_2O$ buffer solutions to obtain FePDO, ZnPDO, or MnPDO, respectively. CuPDO was prepared by washing apo-PDO through a Centricon 10 K with buffer to eliminate DTT and then adding stoichiometric solutions of $CuCl_2 \cdot$ in buffer to the protein. The final concentrations were 1.2 mM for FePDO, 0.8 mM for CuPDO, 0.4 mM for MnPDO and 0.6 mM for ZnPDO. Substrate complexes were prepared by adding phthalate (up to 5 mM) to the metal-reconstituted solutions. The concentration of each sample was estimated by measuring the absorbance at 464 nm (= 7800 $M^{-1}cm^{-1}$) [4] with a Cary 17D spectrophotometer.

Longitudinal water proton relaxation rates were measured with a Koenig-Brown field cycling relaxometer [9–11]. This instrument provides T_1 values with an error of less than ±1% in the 0.01–50 MHz proton Larmor frequency range. ^1H Nuclear magnetic relaxation dispersion (NMRD) profiles can thus be obtained by plotting proton relaxation rates as a function of applied magnetic field.

Theory

The measured values of water proton relaxation rates result from the sum of (1) all the diamagnetic contributions caused by the interaction with the protein, (2) any possible paramagnetic relaxation induced by the internal [2Fe-2S] clusters and (3) the paramagnetic enhancement induced by the metal ion bound at the catalytic mononuclear site [$R_{1(para)}$]. Therefore, proton relaxivities for the metal ion bound to PDO in solution with and without phthalate have been obtained by subtracting the relaxation rates [$R_{1(dia)}$] of the protein without the paramagnetic ion (we used ZnPDO for this; see results) from those of the protein with the paramagnetic ion (R_1), at the same protein concentration, frequency and temperature:

$$R_{1(para)} = R_1 - R_{1(dia)} \tag{1}$$

$R_{1(para)}$, which is normalized for 10^{-3} M of protein concentration, increases linearly with the metal ion concentration. The paramagnetic enhancement is given by the sum of two terms [10]

$$R_{1(para)} = f_m(T_{1M} + \tau_m)^{-1} \tag{2}$$

T_{1M} is the relaxation time of the protons bound to the paramagnetic center and τ_m is the residence time; both terms are multiplied by the molar fraction of protons, f_m, sensing the paramagnetic center. Any contribution from outer-sphere relaxation is neglected.

Nuclear relaxation theory for paramagnetic systems was originally developed by Solomon [12] under the assumption that the static Hamiltonian describing the electron spin system contains only an isotropic Zeeman term of the type

$$H = g_e \mu_B B_0 S_z$$

where g_e is the electron g-factor, μ_B is the Bohr magneton, B_0 is the external magnetic field, and S_z is the projection of the electron spin vector along the direction of the magnetic field. The effects due to g–tensor anisotropy and hyperfine coupling between the metal nucleus and the unpaired electron(s) have been considered through available programs (see later) [13, 14]; the hyperfine coupling proved to strongly influence the proton relaxivity if $A > \hbar\tau_c^{-1}$, in the range of frequency in which $A \geq g\mu_B B_0$, where A is the hyperfine constant with the metal ion and τ_c is the correlation time for nuclear relaxation [15]. In the same way, the presence of zero field splitting (ZFS) of the S manifold for $S > 1/2$ can strongly modify the shape of the NMRD profile when the splitting is larger than $\hbar\tau_c^{-1}$ [15–17]. The ZFS constants generally used, D and E, are defined in terms of the principal values of the traceless tensor D as:

$$D = D_{zz} - (D_{xx} + D_{yy})/2$$
$$E = (D_{xx} - D_{yy})/2$$

Contact relaxation [18] is known to provide no contribution to T_{1M} in copper systems, whereas it can be important in manganese systems [10].

A computer program recently made available [19] allows calculations of the nuclear relaxation enhancements arising from both contact and dipolar interactions for any I (metal ion spin quantum number) and S (electron spin quantum number) values, any g–tensor anisotropy, any ZFS and hyperfine coupling with any rhombicity, and any external magnetic field. It is calculated by evaluating the correlation functions in the equation

$$T_{1M}^{-1} = \left[\sum_{k=-1}^{1} \frac{(-1)^k k^2}{\hbar^2} \int_0^\infty < (\kappa F_k^{(1)}(t) - aS_k^{(1)}(t)) \right.$$
$$\left. (\kappa F_{-k}^{(1)}(0) - aS_{-k}^{(1)}(0)) > exp(ik\omega_I t)dt \right]_{sp.av.} \tag{3}$$

obtained in the Kubo and Tomita description [20] of the relaxation phenomena, where *sp.av.* indicates the spatial average and a is the constant of contact interaction,

$$\kappa = -\frac{\mu_0}{4\pi} \frac{\sqrt{10}\,\hbar\,\gamma_I \mu B}{r^3}, \tag{4}$$
$$F_k^{(1)}(t) = \exp(\frac{i}{\hbar}H_0 t)F_k^{(1)}(0)\exp(-\frac{i}{\hbar}H_0 t),$$
$$S_k^{(1)}(t) = \exp(\frac{i}{\hbar}H_0 t)S_k^{(1)}(0)\exp(-\frac{i}{\hbar}H_0 t),$$
$$F^{(1)} = (G^{(1)} \otimes C^{(2)})^{(1)},$$
$$S_{\pm}^{(1)} = \mp 2^{-1/2}S\pm, \qquad S_0^{(1)} = Sz,$$

ω_I is the proton Larmor frequency expressed in rad s^{-1}, r is the distance between the paramagnetic center and the water proton, μ_0 is the permeability of a vacuum, γ_I is the proton magnetogyric ratio, G is the first-rank spherical tensor obtained as a product of the vector S and the g–tensor, C represents Racah's normalized spherical harmonics [21], and $\otimes$ is the symbol for the tensorial product. Additional internal magnetic fields due to the ZFS and hyperfine coupling are contained in the static Hamiltonian H_0 and modify the eigenvectors on which the electron spin operator acts.

The program can also take into account a possible field dependence of the electron relaxation time, τ_s, according to the Bloembergen-Morgan equation [22, 23]

$$\tau_s^{-1} = \frac{2\Delta^2(4S(S+1)-3)}{50}\left(\frac{\tau_\nu}{1+\omega_s^2\tau_\nu^2}+\frac{4\tau_\nu}{1+4\omega_s^2\tau_\nu^2}\right) \qquad (5)$$

where ω_s introduces the field dependence, Δ^2 is the average quadratic transient ZFS and τ_ν is the correlation time for electron relaxation. The quantity $(1/5)\Delta^2(4S(S+1)-3)\tau_\nu$ is also called τ_{S0}^{-1}, the low field limit for the electron relaxation rate. The range of validity and the limits of Eq. 5 are discussed in [24].

The correlation time that modulates proton relaxation, τ_c, is given by the shortest among three processes, rotation, electron relaxation and proton exchange,

$$\tau_c^{-1} = \tau_r^{-1} + \tau_s^{-1} + \tau_m^{-1} \qquad (6)$$

τ_r being the rotational correlation time.

In the present research the copper protein data were fit using the dipolar part of the electron proton coupling with a field-independent τ_s and the electron-metal nucleus hyperfine term, whereas the manganese protein data were fit with both dipolar and contact coupling and a field-dependent τ_s. The fitting of the data was satisfactory, and therefore there was no need to include the contribution of outer sphere relaxation [25], although the program would have permitted its inclusion [19].

Results

The evaluation of ^{1}H NMRD profiles is a powerful tool for obtaining reliable estimates of the parameters influencing nuclear relaxation. Since the contribution of dipolar relaxation to T_{1M} is proportional to r^6, the distance of the paramagnetic center from the protons of coordinated water can be calculated (Eqs. 3, 4). Therefore, information about water molecules coordinated to metal centers of proteins in aqueous solution can be derived. In the case of native PDO containing high spin iron(II), however, the effect of proton relaxation enhancement is small with respect to a blank which does not contain iron(II), and uninformative because magnetic field independent. As increasing concentrations of Fe^{2+} (from 0.5:1 to 1:1 iron:monomers) were added to the apo-PDO sample, an increase in R_1, proportional to the Fe^{2+} concentration, was detected over the whole range of measurement. The paramagnetic enhancement of the ^{1}H relaxation due to the presence of Fe^{2+} was found to be very small (about 0.6 s^{-1}mM^{-1}) over the whole range of frequencies investigated (Fig. 1) and was not significantly changed when phthalate was bound to PDO. The small relaxation enhancement, as well as its field independence, are due to the fast electron relaxation time of the iron(II) ion. Therefore, copper- and manganese-substituted samples have been prepared and studied.

To evaluate the proton relaxation enhancement due to the presence of the paramagnetic metal ion, the diamagnetic contributions of the protein must be known (Eq. 1). Therefore, we investigated the ^{1}H NMRD profiles of apo-PDO and ZnPDO at 298 K. We noted that they exhibit some differences, especially in the low field region, the relaxation rate of the apo-PDO being smaller than that of ZnPDO. This difference could be due to
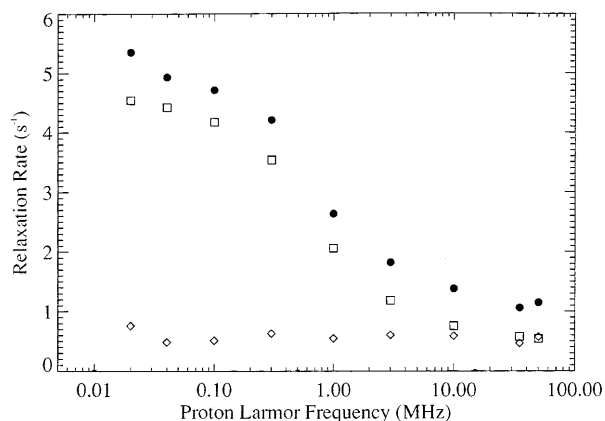

Fig. 1 298 K NMRD profiles of 1.0 mM (subunit concentration) FePDO (●), ZnPDO (□), and their difference (◊). The solutions were buffered with 100 mM HEPES buffer at pH 8 and contained 0.1 mM DTT

a different conformation of the protein in the absence of the metal ion, and perhaps, in consequence of this, to different contributions to relaxation from the [2Fe-2S] cluster. The relaxation rates of solutions containing ZnPDO are the same in the absence and in the presence of phthalate, in contrast to what occurs with apo-PDO. Therefore, we selected the relaxation rate of the Zn-substituted sample to use as $R_{1(dia)}$ in equation (1). The ^{1}H NMRD profiles for ZnPDO are reported in Fig. 2. The profiles have been fitted with an equation generally used for diamagnetic proteins [26] of the type

$$R_1 = R_{1w} + D + A \cdot Re\left[\frac{1}{1+(i\nu/\nu_c)^{\beta/2}}\right] \qquad (7)$$

where $R_{1w} \approx 0.35$ s^{-1} is the bulk relaxation rate of water; i stands for $\sqrt{-1}$, ν is the proton Larmor frequency, D, A, β and ν_c, are heuristic parameters, and Re stands for the real part of the bracketed expression that follows. In the field-dependent part of Eq. 7, the parameter A has been found to increase with decreasing the temperature, as expected [27]. The value β, which represents the steepness of the dispersion, was found to increase from 1.48 to 1.56 as the temperature decreases from 308 to 278 K. A value for β in the range 1.3–2 is typical for most proteins. The value of ν_c is related to the correlation time of the water-protein interaction, τ_c, by the equation $\tau_c = 1/(2\pi\nu_c\sqrt{3})$. This was found to be equal to 0.26, 0.22, 0.18, 0.14, 0.13 and 0.11 μs at 278, 283, 290.5, 298, 303 and 308 K, respectively. These values are consistent (even slightly longer) with the estimate of τ_r of a 200 000 MW protein from the Stokes-Einstein equation

$$\tau_r = \frac{4\pi r^3\eta}{3kT}, \qquad (8)$$

where T indicates the temperature, k is the Boltzmann constant, and η is the viscosity of the solvent, and sug-
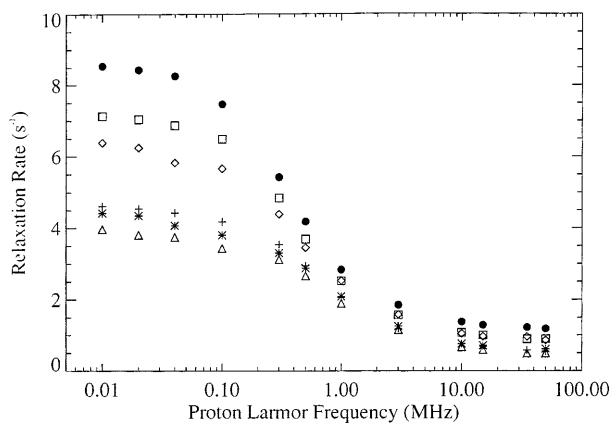
Fig. 2 NMRD profiles of solutions of ZnPDO (1.0 mM) at 278 (●), 283 (□), 290.5 (◊), 298 (+), 303 (*) and 308 (△) K. Other conditions are the same as in Fig. 1
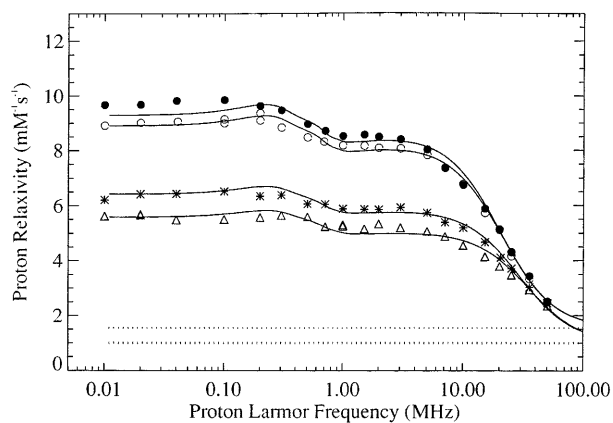

Fig. 3 Proton relaxivity profiles of CuPDO at 278 (●), 283 (○), 298 (*) and 308 (△) K. The *full lines* represent the best fit curves with the parameters reported in Table 1A. The *dotted lines* represent a field independent contribution

gests that the contribution to water relaxation from the [2Fe-2S] cluster (present in the protein) does not contribute significantly to R_1.

CuPDO samples

A computer program [19] was used to find the best-fit parameters of the experimental profiles. T_{1M} was derived as a function of the following parameters: (1) the distance r between metal ion and bound protons, (2) the electron relaxation time τ_s, (3) the proton exchange correlation time τ_m, (4) the electron-metal nucleus hyperfine coupling constants in the plane perpendicular to the direction of the z–axis of the molecular frame, $A_\perp$, and (5) the average angle ϑ between the water proton-metal ion direction and the molecular z–axis. The value of the hyperfine coupling constant along the direction of the z–axis, $A_\parallel$, was calculated from EPR measurements of the CuPDO sample ($A_\parallel = 0.0153$ cm^{-1} in absence of phthalate and $A_\parallel = 0.0168$ cm^{-1} in the presence of phthalate). The values of the rotational correlation times are fixed as given by Eq. 7.

Best-fit for CuPDO without phthalate

Figure 3 shows the relaxivity of CuPDO, i.e. the NMRD profiles normalized to 1 mM less all diamagnetic contributions estimated from the ZnPDO profiles. The profiles show a characteristic dispersion around 10–20 MHz attributable to the $\omega_I\tau_s = 1$ dispersion and a smaller dispersion at low field (0.1–1 MHz). The latter feature is often present in copper(II)-macromolecule complexes and results from a combination of the $\omega_s\tau_s = 1$ dispersion and contributions from the electron-metal nucleus hyperfine coupling [10]. The increase in $R_{1(para)}$ with decreasing temperature indicates that τ_m in Eq. 2 is negligible, i.e. protons are in fast exchange with those of bulk water.

A good fit to the proton relaxivity profiles was found by assuming that they derive from two protons that are 3.4 Å from the metal ion, relaxing in fast exchange, plus a small field-independent contribution. The best fits through these data are shown in Fig. 3, and the corresponding best fit parameters are given in Table 1A.

The electron relaxation times τ_s are in the range 4–8 ns. These values are in accordance with those for other type II Cu-proteins [28–32] and their trend with temperature is well described by the Arrhenius law

$$\tau_s^{-1} = m e^{-s/T} \tag{9}$$

with $m = 3.35 \times 10^{10}$ s^{-1} and $s = 1550$ K. The correlation time τ_c may be approximated as the electron relaxation time τ_s, since τ_r and τ_m are much longer than τ_s. This assumption will be valid in the presence or absence of phthalate. The fitting procedure provides the values for $A_\perp = 0.00274$ cm^{-1} and $\vartheta = 40°$.

The small field-independent contribution is only slightly greater than the experimental uncertainty of measurements, including those derived from the temperature control system. The temperature dependence of this small contribution is opposite to what could be expected in the presence of another water that is in slow exchange. We also showed that the introduction of outer-sphere relaxation [25] did not improve the quality of fittings.

In conclusion, these profiles indicate that in the absence of phthalate there is a fast exchanging water molecule about 2.5 Å from the copper nucleus. This distance is the same as that found for copper-superoxide dismutase [33].

Best-fit for CuPDO with phthalate

The proton relaxivity for CuPDO in the presence of phthalate is shown in Fig. 4. As in the case of the sub-

Table 1 Best fit parameters for proton relaxivity values of solutions of CuPDO in the absence (**A**) and in the presence (**B**) of sodium phthalate and MnPDO in the absence (**C**) and in the presence (**D**) of sodium phthalate

A CuPDO without phthalate

Parameter	Value					Units
Temperature		278	283	298	308	K
$\tau_s \times 10^9$		7.88	7.5	5.42	4.58	s
$\tau_r \times 10^7$		2.6	2.2	1.4	1.1	s
$A_\parallel$	0.0153					cm^{-1}
$A_\perp$	0.0027					cm^{-1}
ϑ	40					degree
r (1st water)	3.38					Å
2nd field-independent contribution[a]		1.56	1.53	1.02	0.97	s^{-1}

B CuPDO with phthalate

Parameter	Value							Units
Temperature		278	283	290.5	298	303	308	K
$\tau_s \times 10^9$	$3.58 \times \exp(385/T)$	14.3	13.9	13.5	13.0	12.7	12.5	s
$\tau_r \times 10^7$		2.6	2.2	1.8	1.4	1.3	1.1	s
$A_\parallel$	0.0168							cm^{-1}
$A_\perp$	–							
ϑ	62							degree
r (1st water)	2.46							Å
$\tau_m \times 10^6$ (1st water)	$5.49 \times 10^{-4} \times \exp(2762/T)$	11.3	9.5	7.4	5.8	5.0	4.3	s
r (2nd water)	3.69							Å

C MnPDO without phthalate

Parameter	Value			Units
Temperature	283	290.5	308	K
Δ	0.016	0.018	0.020	cm^{-1}
$\tau_v \times 10^{12}$	8			s
$(\tau_{s0}{}^b \times 10^9$	2.1	1.7	1.3	s)
$\tau_r \times 10^7$	2.2	1.8	1.1	s
$\tau_m \times 10^6$	0.19	0.17	0.14	s
D	0.07			cm^{-1}
E	0.02	0.018	0.016	cm^{-1}
ϑ	90			degree
φ	90			degree
a/h	1.2			MHz
r	2.65			Å

D MnPDO with phtalate

Parameter	Value			Units
Temperature	283	290.5	308	K
Δ	0.031			cm^{-1}
$\tau_v \times 10^{12}$	2.2	2.1	1.9	s
$(\tau_{s0}{}^b \times 10^9$	2.1	2.2	2.4	s)
$\tau_r \times 10^7$	2.2	1.8	1.1	s
$\tau_m \times 10^9$	6.0	5.2	4.5	s
D	0.07			cm^{-1}
E	0.018	0.016	0.014	cm^{-1}
ϑ	90			degree
φ	90			degree
a/h	1.2			MHz
r	2.8			Å

[a] Probably due to slight differences in blank
[b] Not an independent parameter (see Eq. 5)

strate-free samples, a marked high field dispersion is present. In the 0.1–1 MHz range, a moderate increase in relaxivity is observed, as opposed to the decrease observed in the same range for the substrate-free sample. As noted above, the details of the profile in this region depend more on the electron-metal nucleus hyperfine coupling than on the $\omega_s \tau_s = 1$ dispersion, and an increase in relaxivity in this region is not uncommon [10]. There is a marked overall increase in ^{1}H relaxivity with increasing temperature, suggesting the presence of a contribution from additional water protons that are in quasi-slow exchange with the bulk solvent (see below).

This behavior is in contrast with the temperature dependence of the ^{1}H relaxivity of the sample without phthalate.

Attempts to fit the data with a single set of water protons as could be done for the phthalate-free sample were unsuccessful. Only the simultaneous presence of fast-exchanging and quasi-slow-exchanging protons yielded a reasonable fitting. The parameters giving the best fit to the data are reported in Table 1B. Two fast-exchanging protons are required at 3.7 Å from the paramagnetic center, relaxing with a correlation time equal to the electron relaxation time $\tau_s = 12$–14 ns.
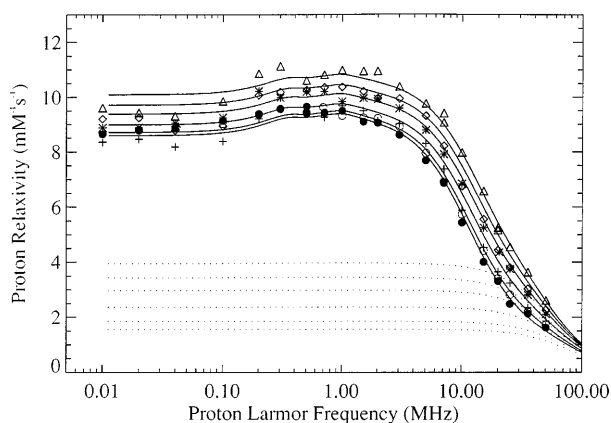
Fig. 4 Proton relaxivity profiles of CuPDO in the presence of 5 mM sodium phthalate at 278 (●), 283 (○), 290.5 (+), 298 (*), 303 (◊) and 308 (△) K. The *full lines* represent the best fit curves calculated with the parameters of Table 1B. The *dotted lines* represent the contribution of slowly exchanging protons that increases with increasing temperature

Fig. 5 Proton relaxivity profiles of MnPDO at 283 (●), 290.5 (+), and 308 (*) K. The *full, dotted* and *dashed lines* represent the best fit curves calculated with the parameters of Table 1C

Two other water protons in quasi-slow exchange ($\tau_m = 10^{-5}$–10^{-6} s) are present at a distance of 2.5 Å. The contribution of these protons to the total relaxation is indicated in Fig. 4 with dotted lines in the lower part of the frame. If the number (n) of protons that are effectively sensing the metal ion differs from 2, only slight changes in the distance between the protons and the paramagnetic center are required to keep n/r^6 the same.

MnPDO samples

Since EPR measurements have shown that MnPDO samples contain free Mn ions (data not shown), the paramagnetic enhancement due to the Mn ion bound to the protein has been obtained by subtracting from the observed data the contribution to the relaxation provided by the free metal ion according to its concentration. The concentration of free Mn has been estimated to be about 15% of the total concentration of metal ions for the sample without phthalate and about 5% for the sample with phthalate.

T_{1M} has been derived as a function of the following parameters: (1) the distance r between metal ion and bound protons, (2) the electron relaxation time τ_s, (3) the dependence of τ_s on the magnetic field, which is in turn a function of the transient ZFS, Δ, and the correlation time for electron relaxation τ_v, (4) the proton exchange correlation time τ_m, (5) the static ZFS constants, D and E, (6) the average angles ϑ and φ that define the position of the water proton in the molecular coordinate frame, and (7) the constant of contact interaction. As in the previous cases, the values of the rotational correlation times have been fixed as given by Eq. 7.
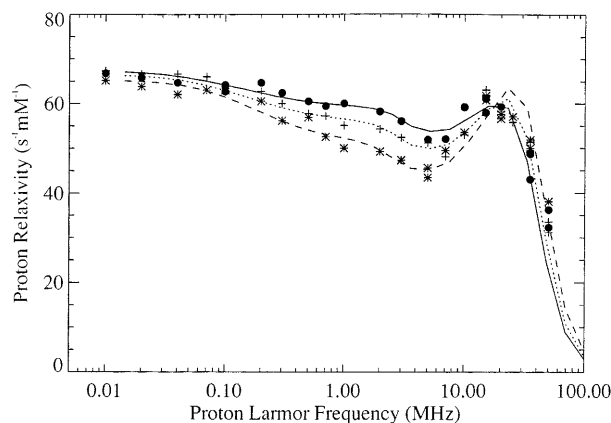
Best fit for MnPDO without phthalate

The proton relaxivities for MnPDO in the absence of phthalate at different temperatures are shown in Fig. 5. The profiles look very similar to those of diiron transferrin (Fe₂TRN) [24]. Thus by inference we assign the first inflection (centered at 0.2 MHz) to the ω_s dispersion, the second inflection (centered at 2 MHz) to the ZFS, and the following hump (centered at about 20 MHz) to a field-dependent electron relaxation. This last feature indicates that the electron relaxation time must be the correlation time for nuclear relaxation. Finally, the last inflection will be related to the ω_I dispersion.

These profiles can be fitted equally well by using different values of several parameters. A satisfactory set of parameters is shown in Table 1C. Extensive calculations have shown that the values of Δ, τ_v, τ_m, D, E and a/h can vary at most within a factor two without affecting the fit appreciably. The angular parameters ϑ and φ are strongly dependent on the D and E values and are therefore ill-determined when D and E are readjusted with the factor two. In any case, the r values are always indicative of the presence of metal-coordinated water, whose protons are in the range of 2.6–2.8 Å from the metal ion.

Best fit for MnPDO with phthalate

Proton relaxivities and best fit parameters for MnPDO in the presence of phthalate at different temperatures are reported in Fig. 6 and Table 1D, respectively. Three inflections are again observed; the second one indicates the presence of ZFS, with a value of D equal to 0.08 cm⁻¹. The hump around 20 MHz is much less evident than for the MnPDO sample without phthalate, and a smaller value for τ_v is thus found. Good fits are obtained by assuming the presence of one water mole-
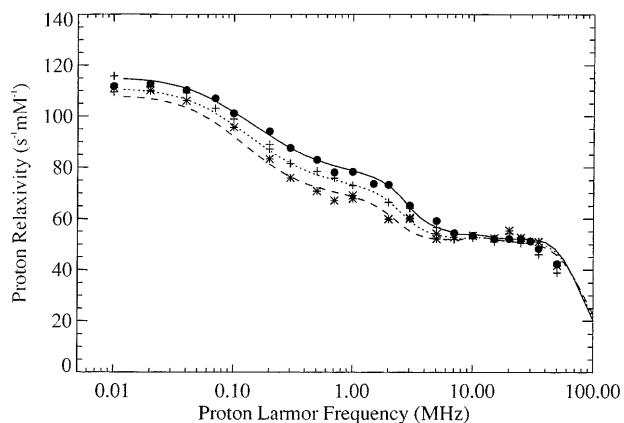
Fig. 6 Proton relaxivity profiles of MnPDO in the presence of 5 mM sodium phathalate at 283 (●), 290.5 (+), and 308 (*) K. The *full*, *dotted* and *dashed lines* represent the best fit curves calculated with the parameters of Table 1D

cule at 2.8 ± 0.1 Å from the paramagnetic center. The low field dispersion contains a contact relaxation contribution that yields a constant of contact interaction of approximately 1.2 MHz.

Discussion

Hydration of mononuclear metal in PDO

The present investigation provides evidence for the presence of exchangeable protons coupled with the paramagnetic metal ion in both CuPDO and MnPDO, both with and without the natural substrate phthalate. In the case of CuPDO, an analysis of the data with the available theory has allowed a very loosely bound water molecule to be revealed. This could be consistent with semicoordination (i.e. binding at a distance longer than usual), which is often displayed by copper systems, especially in tetragonal symmetry [34]. The technique does not provide any information on the other ligands or on any other coordinated water molecule(s) with exchange time(s) longer than $\sim 10^{-5}$ s. Addition of phthalate causes a slight further loosening of the interaction of the semicoordinated water with the mononuclear metal. However, a close analysis of the data reveals the existence of another water molecule with an exchange time of the order of T_{1M}, whose protons can be set at an upper distance of about 2.7 Å from the metal ion. This value, which would provide a Cu-O distance of 2.0 Å or less, thus reveals another, regularly coordinated, water molecule.

Comparison of the data with and without phthalate would at first glance suggest that the binding of substrate causes the binding of a further water molecule. This is very unlikely in the light of MCD data on FePDO and CoPDO that indicate that upon binding of substrate the coordination sphere of the metal ion passes from 6 to 5 (see below) [8]. A more plausible

model would be one in which one water molecule is always present in the coordination sphere and one is semicoordinated. The molecule that is always present, which is not detected in CuPDO because it is in very slow exchange, would be mobilized by the substrate. The semicoordinated molecule would be further removed from coordination by the substrate.

The interpretation of the data on MnPDO represents a considerable challenge, owing to the many features present in the profiles. The peak in relaxivity at about 20 MHz indicates a frequency-dependent correlation time. In the presence of phthalate the values of τ_v are much smaller than those obtained in the absence of the substrate. This determines longer values for τ_s at low fields. Despite the complexity of the analysis, the data show clear evidence of at least one regularly coordinated water both in the absence and in the presence of phthalate. The slightly shorter r value (2.65 Å vs. 2.8 Å) observed in the absence of substrate actually suggests that the substrate-free form may contain two water molecules, more or less regularly bound. No evidence is found for slow-exchanging water in either case.

Implications for the function of PDO

The present investigation is limited to the monitoring of protons coupled with the unpaired electrons of the metal ion. Although the interpretation of these data is not straightforward, the investigation has provided clear evidence of coordinated water to the mononuclear metal both in the absence and presence of substrate. This result taken by itself, is not conclusive in developing an understanding of the structure and function of the protein, but if viewed alongside the general knowledge of the enzyme, adds some meaningful information.

Studies on the analogous Fe and Co derivatives have shown that the metal is five-coordinate in the presence of phthalate [8]. We have evidence that in CuPDO and MnPDO in the presence of substrate there is still a water molecule regularly coordinated. We may therefore propose that there are at most four protein ligands and that one or two water molecules are also coordinated, depending on whether phthalate is bound in the cavity. This model can easily be reconciled with the present findings. Dioxygen would only bind effectively in the presence of substrate, which makes available the sixth coordination site.

A class of proteins related to the iron(II)-hydroxylating dioxygenases are the extradiol ring-opening catechol dioxygenases. They have in common a relatively low affinity for iron [35], the presence of histidine ligands [36], and the apparent capability of binding and activating dioxygen. Recent X-ray data on the biphenyl-cleaving extradiol dioxygenase from a PCB-degrading strain of *Pseudomonas cepacia* have shown the metal to be five-coordinate with two histidines, one aspar-

tate and two water molecules [37]. The observed hydration of the mononuclear metal active sites both in hydroxylating dioxygenases like PDO and in the catechol dioxygenases may simply reflect the solvent accessibility of the active site cavity, which must be wide enough to accommodate both the aromatic ring and the dioxygen substrate. The relatively low affinity of iron for the site in both classes of proteins could originate from both the oxidation state 2+ (as opposed to the 3+ state that usually gives rise to more stable protein adducts [38]) and from the limited number of protein ligands required to leave a wide active site cavity.

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