Evidence for a Magic Magnetic Configuration between FMN and the [2Fe−2S]$^+$ Center of Phthalate Dioxygenase Reductase of *Pseudomonas cepacia*

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Abstract: The enzyme phthalate dioxygenase reductase of *Pseudomonas cepacia* contains two prosthetic groups, a flavin mononucleotide, and a [2Fe−2S] center. Although these centers are separated by only 12 Å according to the X-ray crystal structure, no magnetic coupling effects are detected on their EPR spectrum in the NADH-reduced state of the enzyme in which both centers are paramagnetic. In this paper, we demonstrate that the peculiar arrangement of the prosthetic groups together with the location of the reducible iron site gives rise to a "magic magnetic configuration" for which the dipolar interactions cancel.

Introduction

Phthalate dioxygenase reductase (PDR) mediates electron transfer from reduced nicotinamide adenine dinucleotide (NADH) to phthalate dioxygenase in the bacterium *Pseudomonas cepacia*. It belongs to class I of the aromatic dioxygenases, which contain a flavin group and a [2Fe−2S] center in the same molecule.[1] Reduction of the enzyme with 1 equiv of NADH results in the appearance of a low-temperature EPR spectrum that is apparently the simple superposition of signals given by a [2Fe−2S]$^+$ center belonging to the $g_{av} \simeq 1.96$ class[2] and a neutral semiquinone radical.[1] The absence of magnetic interaction between the two prosthetic groups, which seems to be general in this class of dioxygenases,[3,4] is puzzling since their center-to-center distance is only 12 Å according to the crystal structure recently determined at 2.0 Å resolution.[5] Other iron−sulfur proteins in which paramagnetic centers are separated by the same distance give a complex EPR spectrum resulting from intercenter magnetic interactions.[6,7] In this paper, we demonstrate that the EPR spectrum given by PDR is in fact perfectly consistent with the X-ray crystal structure. The peculiar arrangement of the prosthetic groups together with the position of the Fe(II) ion in the dinuclear cluster gives rise to a "magic magnetic configuration" for which the dipolar interactions cancel.

Materials and Methods

EPR spectra were numerically computed by using the program DIPLOC, which calculates the spectrum resulting from the magnetic dipolar and exchange interactions between a dinuclear cluster characterized by $S_A = {}^1/_2$ comprising two metal sites A_1 and A_2 and a mononuclear complex or a radical species characterized by $S_B = {}^1/_2$. This program is based on a local spin description in which the interactions between all the metal sites A_1, A_2, and B are explicitly considered.[8] The values of the ferric and ferrous **g** tensor components that are needed in the local spin treatment were determined by resorting to the ligand field model applying to the [2Fe−2S]$^+$ clusters with $g_{av} \simeq 1.96$ as previously described.[8] In all the calculations, the broadening of the EPR lines was considered as arising from pure *g*-strain effects. The distribution of the **g** tensor around a mean tensor $\tilde{\mathbf{g}}_0$ was described by a three-dimensional tensor $\tilde{\mathbf{p}}$ whose principal elements are random variables p_i characterized by their standard deviation σ_i.[8] Good simulations were achieved by assuming that $\tilde{\mathbf{g}}_0$ and $\tilde{\mathbf{p}}$ are collinear and by taking the random variables to be fully positively correlated for both the [2Fe−2S]$^+$ cluster and the semiquinone radical. In the first step, DIPLOC was used to determine the magnetic parameters (**g** tensor components and line-width parameters σ_x, σ_y, and σ_z) of the [2Fe−2S]$^+$ cluster and of the flavin semiquinone radical species by simulating the spectrum of PDR obtained by digitization of the spectrum represented in Figure 4 of ref 1. These parameters were subsequently incorporated into the calculation of the EPR spectrum resulting from the arrangement deduced from the X-ray crystal study (Figure 1).

Results

According to the X−ray crystal structure, the prosthetic groups of PDR are located in two different domains and are oriented so that the [2Fe−2S] core and the flavin ring are nearly perpendicular.[5] This arrangement is schematically depicted in Figure 1. In this figure, we have also represented the spin density distribution expected for a neutral semiquinone radical[9] and its barycenter G. This barycenter is close to nitrogen N_5, which carries about 50% of the total spin density. By using the spin distribution reported in ref 9 and the crystal coordinates of PDR (Protein Data Bank, entry 2PIA), the polar coordinates of G in the reference frame (*x*, *y*, *z*) represented in Figure 1 could be deduced: $r = 12.3$ Å, $\theta = 131°$, and $\varphi = 265°$.

In a recent study, we have shown that the EPR spectrum given by a system containing polynuclear clusters coupled by magnetic interactions is very sensitive to the delocalization of the magnetic moments within the clusters. These effects can be taken into

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(1) Batie, C. J.; Ballou, D. P.; Correl, C. C. In *Chemistry and Biochemistry of Flavoenzymes*; Muller, F., Ed.; CRC Press: Boca Raton, FL, 1991; Vol. 3, pp 543−556.

(2) Bertrand, P.; Guigliarelli, B.; More, C. *New J. Chem.* **1991**, *15*, 445−454.

(3) Twilfer, H.; Bernhard, F. H.; Gersonde, K. *Eur. J. Biochem.* **1981**, *119*, 595−602.

(4) Schweizer, D.; Markus, A.; Seez, M.; Ruf, H. H.; Lingens, F. *J. Biol. Chem.* **1987**, *262*, 9340−9346.

(5) Correl, C.; Batie, C. J.; Ballou, D. P.; Ludwig, M. L. *Science* **1992**, *258*, 1604−1610.

(6) Mathews, R.; Charlton, S.; Sands, R. H.; Palmer, G. *J. Biol. Chem.* **1974**, *249*, 4326−4328.

(7) Stevenson, R. C.; Dunham, W. R.; Sands, R. H.; Singer, T.; Beinert, H. *Biochim. Biophys. Acta* **1986**, *869*, 81−88.

(8) Bertrand, P.; More, A.; Guigliarelli, B.; Fournel, A.: Bennett, B.; Howes, B. *J. Am. Chem. Soc.* **1994**, *116*, 3078−3086.

(9) Edmonson, D. E. *Biochem. Soc. Trans.* **1985**, *13*, 593−600.

0002-7863/95/1517-1807$09.00/0

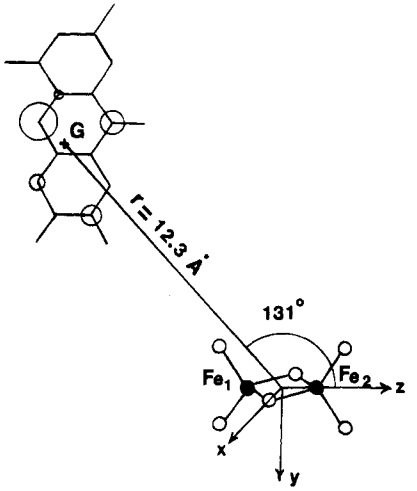

Figure 1. Schematic representation of the prosthetic groups in phthalate dioxygenase reductase according to the X-ray crystal structure. The spin density expected for a neutral semiquinone radical is represented on the flavin ring.

account through a local spin model in which the interactions between all paramagnetic sites are explicitly considered.[8] One original prediction of this model is the existence of magic configurations for which the dipolar terms cancel. In the case of a $[2Fe-2S]^+$ cluster interacting with a mononuclear center or a radical M, this occurs when the angle between the $\overrightarrow{Fe(II)\,Fe(III)}$ and $\overrightarrow{OM}$ vectors is close to 135°, O being the center of the cluster.[8] Since the angle θ between $\overrightarrow{Fe_1\,Fe_2}$ and $\overrightarrow{OG}$ is equal to 131° in PDR (Figure 1), this effect accounts for the absence of dipolar coupling on the EPR spectrum provided that Fe_1 is the reducible iron.

This interpretation is borne out by the numerical calculation of the EPR spectra corresponding to the arrangement depicted in Figure 1 for the two possible valence assignments Fe_1 = Fe(II) and Fe_1 = Fe(III). The magnetic parameters of the $[2Fe-2S]^+$ cluster and of the semiquinone species, **g** values and line-width parameters, were first determined so as to reproduce the iron–sulfur and radical components of the published spectrum of NADH-reduced PDR at 20 K.[1] The shape of the radical component was well-simulated by using an isotropic **g** value equal to 2.008 and a gaussian line shape with a derivative peak-to-peak line width equal to 1.9 mT, typical of neutral flavin semiquinone radicals.[10] However, the magnitude of the radical component was markedly substoichiometric, a feature that is also apparent on the published spectra of other class I aromatic dioxygenases.[3,4] This effect may arise from saturation effects or from the presence of a substoichiometric amount of the semiquinone species in the sample, which may originate from a change of the redox equilibrium induced by the freezing process.[11] The best simulation of the EPR spectrum of PDR obtained with the program DIPLOC[8] for an infinite distance is represented in Figure 2a, and the corresponding values of the magnetic parameters are reported in Table 1. The interaction spectra calculated by using these parameters and the structural arrangement depicted in Figure 1 are represented in parts b and c of Figure 2 for the assignments Fe_1 = Fe(II) and Fe_1 = Fe(III), respectively. In these calculations, the magnetic axes of the $[2Fe-2S]^+$ cluster were those indicated in Figure 1. Although dipolar effects are barely detected in Figure 2b, which

(10) Eriksson, L. E. G.; Ehrenberg, A. *Biochim. Biophys. Acta* **1973**, *293*, 57–66.

(11) Williams–Smith, D. L.; Bray, R. C.; Barber, M. J.; Tsopanakis, A. D.; Vincent, S. P. *Biochem. J.* **1977**, *167*, 593–600.

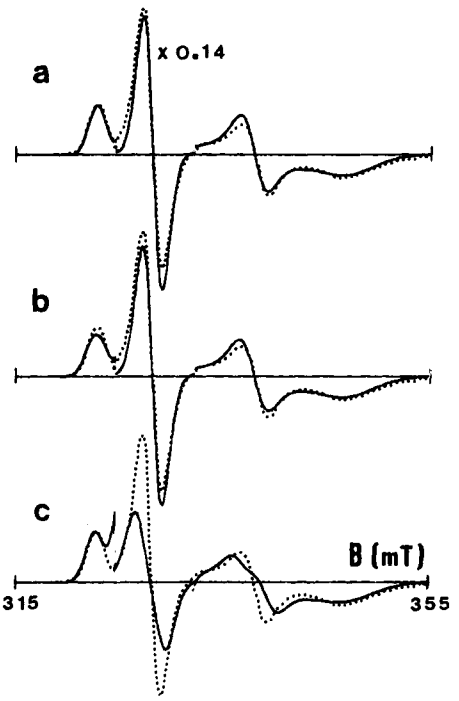

Figure 2. EPR spectra calculated with the magnetic parameters reported in Table 1 for a microwave frequency equal to 9.224 GHz; (a) $r = \infty$, (b) structural arrangement of Figure 1 and Fe_1 = Fe(II), and (c) structural arrangement of Figure 1 and Fe_1 = Fe(III). The spectrum represented in dotted lines was obtained by digitization of the experimental spectrum of PDR at 20 K reported in ref 1. For clarity, the radical signal was multiplied by 0.14 on all the calculated spectra.

Table 1. Magnetic Parameter Values Used in the Calculation of the Spectra Represented in Figure 2

	[2Fe–2S]	FMN
Fe(III) site	$g_{1x} = 2.015$, $g_{1y} = 2.034$, $g_{1z} = 2.030$	$g = 2.008$
Fe(II) site	$g_{2x} = 2.103$, $g_{2y} = 2.097$, $g_{2z} = 2.021$	
	$\sigma_x = 1.5 \times 10^{-2}$, $\sigma_y = 5 \times 10^{-3}$, $\sigma_z = 4.3 \times 10^{-3}$	$\sigma = 8 \times 10^{-3}$

corresponds to the magic magnetic configuration, they give rise in Figure 2c to a large broadening of the radical signal whose peak-to-peak line width becomes equal to 3.3 mT, together with a significant distortion of the iron–sulfur component. Very similar spectra were obtained by rotating the magnetic axes with respect to (x, y, z), the only difference being the shape of the distorted iron–sulfur component in Figure 2c. We have also examined if the EPR spectrum of PDR might result from a cancellation of the dipolar and exchange effects by introducing a finite value of the exchange parameter J_{AB} in the spin Hamiltonian corresponding to the arrangement depicted in Figure 1 and the assignment Fe_1 = Fe(III). No cancellation effect could be observed for any value of J_{AB}. Similar calculations carried out with the assignment Fe_1 = Fe(II) indicated that the magnitude of J_{AB} is less than 0.5 mT.

Discussion and Conclusion

The absence of magnetic coupling effects on the EPR spectrum of NADH-reduced PDR appears at first sight surprising if one considers the proximity of the paramagnetic centers revealed by the X-ray crystal study.[5] Apparently, this cannot be attributed to a large conformational change induced by the reduction since an analysis of the NADH:PDR^{2-} crystal structure at 2.7 Å resolution indicated only minor variations with respect to the oxidized form.[5]

An interesting model was proposed 20 years ago by Leigh to explain how the dipolar coupling between a radical species

and a fast-relaxing species could lead to a large decrease in amplitude of the radical signal without apparent broadening.[12] If this model could be applied to PDR, it might account for the experimentally observed effects (Figure 2a). However, this model is only valid within the Redfield limit, which requires that the spin−lattice and spin−spin relaxation times of the fast-relaxing species be much shorter than the spin−spin relaxation time of the slow-relaxing species; T_{1f} and $T_{2f} \ll T_{2s}$. Although relaxation times have not been measured in PDR, it is easy to realize that these relations cannot be obeyed by considering T_1 and T_2 values determined for similar systems at 20 K, the temperature at which the EPR spectrum of PDR was recorded.[1] At this temperature, T_1 and T_2 are equal to 2×10^{-5} and 10^{-7} s, respectively, for the typical [2Fe−2S] ferredoxin from *Spirulina maxima*,[13] while T_2 is equal to 10^{-7} s for the semiquinone flavin of flavocytochrome *b*2 from *Hansenula anomala*.[14] In fact, the Redfield limit could be reached in PDR if the temperature was sufficiently high that the $[2Fe-2S]^+$ EPR signal was significantly broadened due to the shortening of T_1, which is not the case at 20 K. As a matter of fact, if the expressions given in ref 12 are applied as they are to PDR by taking $T_{1f} = 10^{-4}$ s and $r = 12.3$ Å, they predict that the amplitude of the radical signal should decrease beyond detection.

In fact, at 20 K, the spin−lattice relaxation time of the $[2Fe-2S]^+$ cluster is sufficiently long that a splitting should be observed on both the $[2Fe-2S]^+$ and the flavin semiquinone EPR signals. If the $[2Fe-2S]^+$ cluster could be considered as a point dipole, the absence of dipolar interaction effects might be attributed to a peculiar arrangement in which the intercenter vector makes an angle of 54° with the three magnetic axes of the $[2Fe-2S]^+$ cluster. However, we have shown previously that polynuclear clusters cannot be approximated by point dipoles due to the delocalization of their magnetic moment imposed by the internal spin coupling.[8] When this delocalization is taken into acount in a system comprising a $[2Fe-2S]^+$ cluster interacting with a mononuclear complex or a radical species, a magic magnetic configuration is predicted for the arrangement depicted in Figure 1 and the assignment Fe_1 = Fe(II) (Figure 2b).

The peculiar situation encountered in phthalate dioxygenase reductase illustrates nicely the interest of a local spin model. This model explains the lack of dipolar coupling effects on the EPR spectrum of the NADH-reduced enzyme and demonstrates unambiguously that Fe_1 is the reducible site of the [2Fe−2S] center. This confirms an earlier proposal that was based on the comparison between the structures of plant ferredoxins and of the [2Fe−2S] domain of PDR.[5] Our calculations also indicate that the magnitude of the exchange parameter J_{AB} is much smaller than that determined for other electron transfer metalloproteins in which the interacting paramagnetic centers are separated by similar or even larger distances.[7,8,15] Therefore, the superexchange paths connecting the $[2Fe-2S]^+$ and flavin groups are not particularly efficient in PDR. This result is not surprising if one considers that these paths probably involve relatively weak interdomain bonds, like direct and water-mediated hydrogen bonds as well as van der Waals contacts.[5] Since these paths are expected to contribute to the electron transfer pathways between these redox centers,[16] this suggests that the electronic factor of the electron transfer rate is not very large. At the present time, only a lower limit equal to 200 s^{-1} has been evaluated for this rate.[1]

Note Added in Proof. A recent study (Gassner, G.; Wang, L.; Batie, C.; Ballou, D. P. *Biochemistry* **1994**, *33*, 12184−12193) has shown that the rate of the electron transfer from the flavin to the [2Fe−2S] center is only 35 s^{-1} in PDR, in agreement with our prediction concerning the weak efficiency of the electron transfer pathways.

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(12) Leigh, J. S. *J. Chem. Phys.* **1970**, *52*, 2608−2612.
(13) Gayda, J. P.; Bertrand, P.; Deville, A.; More, C.; Roger, G.; Gibson, J. F.; Cammack, R. *Biochim. Biophys. Acta* **1979**, *581*, 15−26.
(14) Bertrand, P.; Janot, J. M.; Benosman, H.; Gayda, J. P.; Labeyrie, F. *Eur. Biophys. J.* **1987**, *14*, 273−278.
(15) Guigliarelli, B.; Guillaussier, J.; More, C.; Setif, P.; Bottin, H.; Bertrand, P. *J. Biol. Chem.* **1993**, *268*, 900−908.
(16) Bertrand, P. In *Structure and Bonding*; Palmer, G., Ed.; Springer-Verlag: Berlin, 1991; Vol. 75, pp 1−47.