Magnetic Circular Dichroism Studies on the Mononuclear Ferrous Active Site of Phthalate Dioxygenase from *Pseudomonas cepacia* Show a Change of Ligation State on Substrate Binding†

George T. Gassner,‡ David P. Ballou,‡ Gregory A. Landrum,§ and James W. Whittaker*,§

Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109, and Department of Chemistry, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213

Received October 7, 1992; Revised Manuscript Received March 2, 1993

ABSTRACT: Phthalate dioxygenase from *Pseudomonas cepacia* contains a mononuclear ferrous center that is strictly required for catalytic oxygen activation. The spectroscopic characterization of this iron site and its ligand interactions has been complicated in the past by interference from a Rieske-type binuclear (2Fe–2S) cluster in the enzyme, which dominates the absorption spectra and is superimposed in X-ray absorption spectra for the mononuclear site. We have used low-temperature, variable magnetic field circular dichroism spectroscopy to selectively detect the ligand field spectra of the paramagnetic mononuclear ferrous active site in the presence of the diamagnetic exchange-coupled Rieske center and observe spectral changes associated with substrate binding. The perturbations of the d → d spectra for the mononuclear ferrous site reflect a decrease in coordination number from six to five on binding substrate. This structural change suggests that displacement of an iron ligand prepares the ferrous center for dioxygen activation.

The utilization of dioxygen in biology has led to the evolution of diverse catalytic structures for overcoming the kinetic barriers to reactions involving dioxygen. This has been demonstrated recently with the molecular characterization of a wide range of O_2-metabolizing enzymes, many of which are now known to be metalloenzymes containing complexes of Cu (Lontie, 1984), Fe (Que, 1980), or Mn (Britt et al., 1991). These highly evolved biological metal complexes are active sites for dioxygen chemistry, typically functioning by binding and activating dioxygen in preparation for its insertion into unactivated C–H or C–C bonds. There are two broad classes of O_2-reactive metalloenzymes: those containing binulcear and those containing mononuclear metal centers. The former class is represented by the binuclear cuprous active site in tyrosinase (Lerch, 1976) and the binuclear ferrous complex in methane monooxygenase (Fox et al., 1988), both forming two-electron redox centers as required for dioxygen activation. However, mononuclear active-site structures appear to predominate in O_2 activation. In addition to the well-known heme-containing proteins such as cytochrome P-450, there is a large group of oxygenases that use a mononuclear ferrous center for catalysis (Martinez et al., 1991; Dix et al., 1987; Fitzpatrick, 1989; Kivirikko et al., 1989; Hagedorn et al., 1988). Within the latter class further structural distinctions can be drawn based on the nature of the additional redox cofactor or cosubstrate required for activity. We can thus distinguish categories such as Fe-pterin [phenylalanine (Martinez et al., 1991) and tyrosine (Dix et al., 1987; Fitzpatrick, 1989) hydroxylases], α-ketoglutarate-coupled Fe [prolyl-4-hydroxylase (Kivirikko et al., 1989)], and the ferrous center associated with a Rieske-type binuclear (2Fe–2S) iron–sulfur cluster (Batie et al., 1987). This latter structure motif is represented in the phthalate dioxygenase of *Pseudomonas cepacia*, an enzyme serving as a prototype for this class of bacterial oxygenase, which is of growing interest with regard to environmental manangement and bioremediation chemistry (Hagedorn et al., 1988).

Phthalate dioxygenase (PDO)[1] catalyzes the first step in the biodegradation of phthalate through dihydroxylation of the aromatic ring system, converting it to the *cis*-4,5-dihydrodiol. This is subsequently transformed through dehydrogenation to form the diphenol, 4,5-dihydroxyphthalate, which is consumed in the catechol cleavage pathway (Hagedorn et al., 1988). Phthalate dioxygenase has been biochemically characterized and is known to be a 200-kDa tetrameric protein containing three Fe atoms per subunit. Previous spectroscopic studies have shown that two of these Fe atoms are combined in a Rieske-type Fe–S center (Gurbiel et al., 1989), while the third is bound in non-heme, non-sulfur coordination (Batie et al., 1987; Ballou & Batie, 1989; Batie & Ballou, 1990). In addition to PDO, and NADH-dependent iron–sulfur flavoprotein reductase is required for efficient substrate turnover. The crystal structure of this reductase has just been completed (Correll et al., 1992). Previous studies directed at probing the structure and interactions of the Rieske (2Fe–2S) cluster using ENDOR (Cline et al., 1985; Telser et al., 1987; Gurbiel et al., 1989) and X-ray absorption spectroscopy (XAS) (Tsang et al., 1989) have shown that the cluster has bisthiolate ligation to one Fe and bisimidazole coordination to the other, providing the first structural assignment for a Rieske center. Subsequently, the structure has been confirmed for the Rieske-containing protein from *Rhodobacter capsulatus* (Gurbiel et al., 1991).

The mononuclear ferrous ion in PDO is absolutely required for catalytic activity. Its extraction from the substrate-free

† We would like to gratefully acknowledge support for this work from National Institutes of Health Grants GM-20877 (to D.P.B.) and GM-46749 (to J.W.W.).

* To whom correspondence should be addressed.
‡ University of Michigan.
§ Carnegie Mellon University.

[1] Abbreviations: PDO, phthalate dioxygenase; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; MCD, magnetic circular dichroism; NIR, near infrared; XAS, X-ray absorption spectroscopy; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; NADH, nicotinamide adenine dinucleotide, reduced.

0006-2960/93/0432-4820$04.00/0

enzyme results in loss of activity, which can be restored by adding back ferrous ion. Other divalent metal ions (e.g., Co^{2+}, Mn^{2+}) also form tight complexes but do not restore activity. The motif of a mononuclear ferrous center associated with a Rieske-type (2Fe–2S) cluster is also represented in other enzymes catalyzing aromatic dihydroxylation reactions: benzene dioxygenase (Geary et al., 1990), naphthalene dioxygenase (Zylstra & Gibson, 1989), and toluene dioxygenase (Ensley & Gibson, 1983). The nature of the mononuclear iron site and possibilities of electronic interactions between it and binuclear metal centers in catalysis can be effectively addressed by spectroscopic methods. The mononuclear site is of particular interest since it may be involved in the generation of free radical intermediates in its catalytic mechanism.

EXAFS studies indicate that the binding of substrate to PDO leads to a decrease in average coordinate bond length to the mononuclear metal from 2.12 to 2.07 Å (Tsang et al., submitted for publication), consistent with a change from hexacoordinate to pentacoordinate ligation. EXAFS experiments on PDO containing Fe^{2+} or Co^{2+} at the mononuclear site provide similar conclusions, with the clearest results obtained for the Co-PDO. However, these results for Fe-PDO are complicated by the superposition of EXAFS for all three iron sites and the well-known limitations of XAS in defining ligation number. We have now used low-temperature, variable magnetic field MCD spectroscopy to probe the electronic and geometric structure of the mononuclear metal center of PDO, taking advantage of the unique selectivity of low-temperature MCD spectra for the paramagnetic components of a sample. Unlike conventional EPR and ENDOR experiments, this approach is not limited to Kramers ground states, permitting detection of the non-Kramers Fe^{2+} ion, thus providing important information on coordination number and ligation geometry. Using MCD spectroscopy, we have confirmed that the dramatic structural change in this metal center occurring as a result of substrate binding is a *decrease* in coordination number from six to five, which appears to represent displacement of a metal ligand by a noncoordinating substrate. This change is reflected in characteristic perturbations of both excited-state spectra and ground-state properties detected in saturation magnetization MCD studies.

EXPERIMENTAL PROCEDURES

Phthalate dioxygenase was prepared as previously described (Batie & Ballou, 1990), except that the final DEAE chromatography step was deleted. PDO thus obtained was > 95% homogeneous. PDO (2 mL) samples were dialyzed against two changes of 2 L of 5 mM EDTA and 50 mM HEPES, pH 8, for a total of 24 h to prepare enzyme that contained an intact Rieske cluster but was devoid of metal at the mononuclear metal binding site. EDTA was removed by gel filtration over a Bio-Rad DG-10 desalting column. The enzyme sample was exchanged into 50 mM HEPES in D_2O (pD = 8) by serial dilution and ultrafiltration with a Centricon 30 microconcentrator. After this procedure, the sample was approximately 95% D_2O. The D_2O-exchanged protein was made anaerobic by purging with argon and then reconstituted with iron in the form of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in D_2O using a 10% excess of metal. Cobalt-substituted PDO derivatives were prepared in an analogous fashion, using $CoCl_2$ in D_2O. The substrate complex was prepared by adding phthalate from a neutralized 176 mM stock solution in D_2O to the enzyme sample. For MCD spectroscopy, these anaerobic protein samples were made 50% in glycerol-d_3 and injected into a sample holder previously purged with argon. The MCD sample cell was formed from a pair of quartz disks separated by a 3-mm-thick rubber gasket in which a hole had been bored to hold the protein solution. Optical-quality glasses were prepared by slowly cooling these samples, and each sample was checked for depolarization by comparing CD spectra of a 0.12 M nickel tartarate solution placed before and after the sample (Browett et al., 1983). In all cases less than 2% depolarization was observed. The MCD instrumentation used in these experiments (Aviv Model 41DS spectropolarimeter equipped with an Oxford SM4-6T magnetocryostat for low-temperature, variable magnetic field perturbations) has been described in detail elsewhere (Whittaker & Whittaker, 1991). For NIR MCD experiments, a liquid N_2-cooled InSb photovoltaic photodiode with a 1-mm-square active area was used for signal detection. Saturation magnetization experiments were performed by setting the sample temperature and ramping the magnetic field of a SM4-6T Oxford Instruments superconducting magnetocryostat at a sufficiently slow rate to avoid hysteretic artifacts.

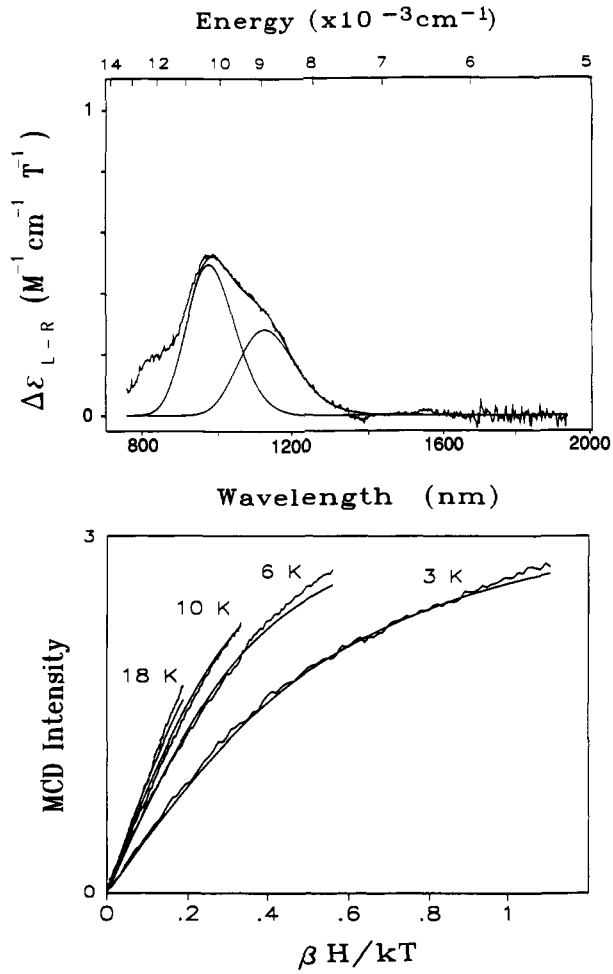


FIGURE 1: Substrate-free Fe^{2+}-phthalate dioxygenase, 1.4 mM active sites after dilution with glycerol. (Top) MCD spectrum recorded at 4.5 K. The smooth lines reflect Gaussian deconvolution of the spectrum and the sum of components. (Bottom) Saturation magnetization curves recorded at 1000 nm. The smooth curves represent optimized fits to the experimental data, providing estimates of the ground-state parameters.

RESULTS

The near infrared magnetic circular dichroism (NIR-MCD) spectrum for substrate-free Fe^{2+} reconstituted PDO is shown in Figure 1 (top). This spectrum was extracted from low-

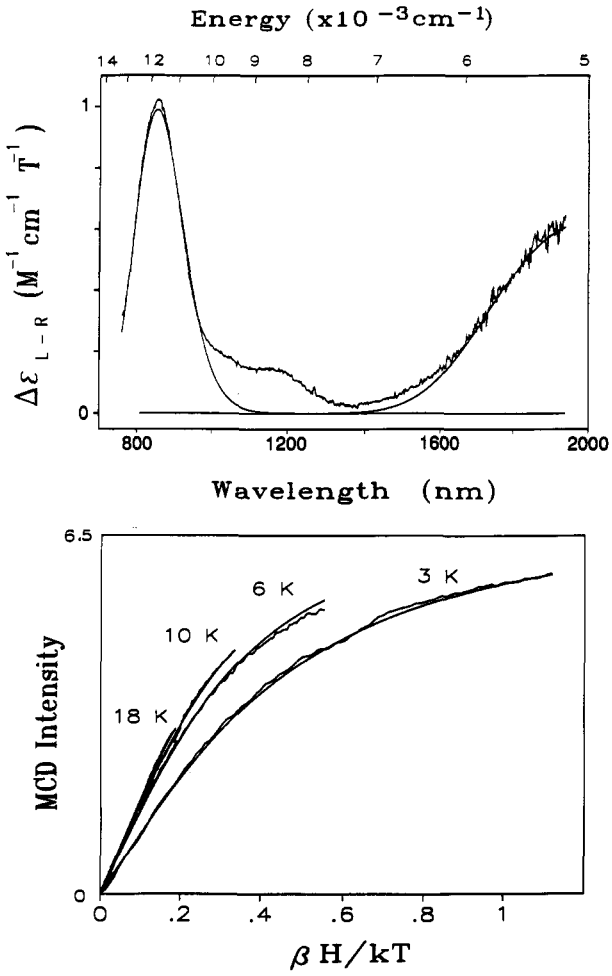


FIGURE 2: Substrate-bound Fe^{2+}-phthalate dioxygenase, 1.4 mM active sites after dilution with glycerol. (Top) MCD spectrum (4.5 K). Smooth curves are Gaussian fits to the spectrum as in Figure 1. (Bottom) Saturation magnetization curves recorded at 850 nm. The smooth curves represent optimized fits to the experimental data, providing estimates of the ground-state parameters.

temperature (4.5 K), high magnetic field (5.0 T) data by subtraction of the zero field (CD) baseline. The spectrum shown therefore represents the pure MCD component of the electronic transition, induced by the magnetic field. The temperature-dependent intensity of these signals identifies them as MCD C-terms arising within a paramagnetic ground state (Schatz & McCaffery, 1969). The essential features in the spectrum are a relatively weak, broad feature centered near 1000 nm, with partial resolution of a shoulder to lower energy, and a second feature near 830 nm of lower intensity. This minor feature has been consistently observed in three independently prepared samples of the substrate-free enzyme. Gaussian-resolved components of the broad feature near 1000 nm were separated in the corresponding energy spectrum and transformed to the wavelength scale in Figure 1 (top) to illustrate the deconvolution of a pair of bands within this broad feature. The sum of these features is also shown to demonstrate the quality of the fit.

In saturation magnetization experiments, the observation of MCD in transitions to excited states provides information on the nature of the electronic ground state of a paramagnetic complex. Magnetization curves for substrate-free Fe^{2+}-PDO are shown in Figure 1 (bottom). Each curve represents the magnetization profile obtained at a single temperature between 3 and 18 K. The upper limit of each curve corresponds to a magnetic field strength of 5.0 T, and both these data (Figure

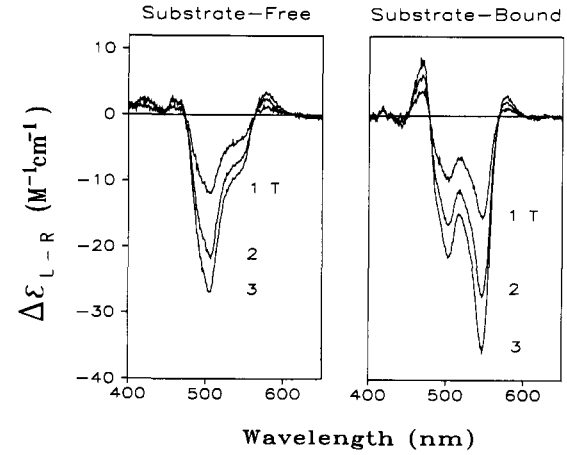


FIGURE 3: Co^{2+}-phthalate dioxygenase, 1.2 mM after dilution with glycerol. (Left) MCD spectra of substrate-free enzyme recorded at 4.5 K. (Right) MCD spectra of substrate-bound complex recorded at 4.5 K.

1, bottom) and the corresponding saturation magnetization data for the substrate-bound enzyme (Figure 2, bottom) reflect a temperature-dependent nesting of the curves when plotted in terms of the saturation parameter, $\beta H/kT$. For substrate-free Fe^{2+}-PDO this trend extends to the highest temperature recorded (18 K) with the magnetization curves showing only slight saturation, which would appear as an approach to an asymptotic limiting MCD intensity.

The corresponding NIR-MCD spectra for the PDO with both phthalate and mononuclear Fe^{2+} ion bound is shown in Figure 2 (top). The spectra are distinctly different from those observed in the absence of substrate, in terms of both transition energies and MCD intensities. The spectra of the substrate-bound enzyme are dominated by a relatively strong positive MCD C-term near 830 nm and a new feature arising in the IR that reaches a maximum at the limit of the spectrum near 2000 nm. Analysis of the energy spectrum shows that each of the dominant features in this spectrum has the simple Gaussian band shape of an isolated transition. The integrated intensity of these two features combined is approximately 4 times that observed for the pair of bands near 1000 nm in the substrate-free enzyme, reflecting an increase in total intensity of the MCD spectrum for the ferrous complex in the presence of substrate. In addition to these major features, a pair of bands remains near 1000 nm with approximately 30% the intensity observed in the substrate-free sample. These features are progressively eliminated by the addition of phthalate to the sample, but residual signals are observed even for samples containing saturating levels of phthalate (10 times K_d; K_d-(phthalate) = 300 μM). The same distribution between species in the presence and absence of substrate is observed when substoichiometric Fe^{2+} is added. Saturation magnetization curves obtained for the substrate-bound form of the enzyme in MCD also reflect a change in the enzyme, as shown in Figure 2 (bottom). The curves over the same temperature range as in Figure 2 (top) now nearly coincide above 6 K. No MCD intensity is observed in the spectral features associated with the Rieske (2Fe–2S) cluster present in the enzyme in any of these samples.

Reconstitution of apo-PDO with Co^{2+} results in the formation of a catalytically incompetent complex with Co substituting for Fe in the divalent metal binding site. At low temperature, temperature-dependent MCD spectra (C-terms) are observed in the visible spectral region, near 500 nm (Figure 3, left). For the substrate-free enzyme, a negatively signed

MCD band is observed at 505 nm. On addition of substrate, the spectrum is perturbed and an overall increase in intensity is observed. Two resolved negatively signed MCD bands at 500 and 550 nm are found for the substrate-bound complex, with a greater intensity appearing to lower energy band. These dominant spectral features appear to be associated with weaker positive MCD intensity near 480 and 590 nm. Evaluation of saturation magnetization curves for the substrate-bound Co^{2+} enzyme by the intercept method (Thomson & Johnson, 1980) yields an estimate of the ground-state g-value ($g_{iso} \approx 4.5$).

DISCUSSION

The presence of the Rieske center in phthalate dioxygenase complicates the spectroscopic analysis of the relatively weakly absorbing mononuclear metal center, since features associated with the binulcear cluster dominate throughout the near UV–visible–NIR spectral ranges. However, the oxidized Rieske center is an antiferromagnetically coupled binuclear ferric cluster with a diamagnetic ($S_T = 0$) ground state, and therefore it does not appear in paramagnetic MCD spectra, which selectively probe the mononuclear ferrous site. This selective probe permits observation of the effects of substate binding at the mononuclear metal center. The binding of phthalate to PDO is cooperative with the binding of Fe^{2+} at the mononuclear site; the K_d of the phthalate-bound form of the enzyme for Fe^{2+} is $<10^{-11}$ M. In the absence of substrate, the K_d of the mononuclear iron is approximately 25 nM and this Fe^{2+} site is sensitive to air oxidation, presumably because the metal ion dissociates and reacts with oxygen. Thus, the presence of the substrate greatly decreases the rates for both loss of metal from the mononuclear site and its oxidation. Dialysis of PDO against EDTA in the absence of phthalate permits the removal of ferrous iron. Several other divalent ions, which can serve as probes of the metal binding site, also bind similarly but do not restore activity.

The absence of MCD intensity in the spectral features of the Rieske center strongly limits the extent to which the mononuclear ferrous center and the (2Fe–2S) cluster can be electronically coupled in either substrate-free or substrate-bound complexes. This result is consistent with previous EPR experiments that suggested a minimum separation of 10 Å between the Rieske and mononuclear iron centers (W. R. Dunham, C. Batie, and D. Ballou, unpublished results). Further experiments specifically exploring the Rieske center and its interactions in a variety of enzyme complexes are currently in progress.

Fe^{2+} Spectra. The selectivity of low-temperature, high magnetic field MCD measurements for paramagnetic components of a complex sample is well illustrated in these studies. Here the relatively weak spectral features of a mononuclear high-spin ferrous ion are detected in MCD in the presence of strong absorption features arising from a diamagnetically coupled binuclear ferric (2Fe–2S) cluster (the Rieske center), which dominates the absorption and CD spectra for the PDO enzyme. The observed MCD spectra (Figures 1 and 2) can be assigned as the highest energy spin-allowed ligand field (d → d) transitions for a high-spin ($S = 2$) paramagnetic d^6 metal ion. The temperature-dependent MCD [Figures 1 and 2 (bottom)] identifies these spectra as arising from a paramagnetic ground state, and the temperature-dependent saturation behavior is characteristic of a non-Kramers (even electron, integer spin) metal complex exhibiting large zero-field splittings. For a d^6 Fe^{2+} metal complex, the highest energy spin-allowed ligand field bands occur in the near infrared region of the spectrum and reflect the d-orbital

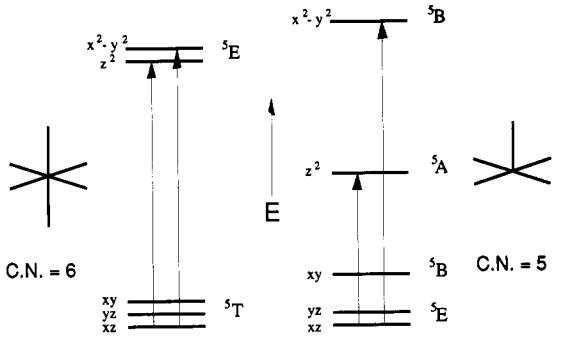


FIGURE 4: State splittings and spectroscopic transitions for six- and five-coordinate ferrous complexes predicted by ligand field theory.

splittings in the metal complex. These ligand field (d → d) transitions for the Fe^{2+} ion are relatively weak in absorption. Extinctions in the range of 1–10 M^{-1} cm^{-1} are typical, with the larger intensity associated with low-symmetry complexes. Orbital splittings in the ferrous complex are reflected in the excited-state splittings as illustrated in Figure 4 for six- and five-coordinated metal ion. Orbital labels are given in this figure to provide a connection to the discussion of orbital splittings below. These labels correspond to the spatial symmetry of the many-electron ferrous electronic state, given by the doubly-occupied orbital. The d-orbital energies and splittings in complexes are dominated by coordination number but also relate to the geometry of the site. For six-coordinate, roughly octahedral metal complexes (Figure 4, left), the highest energy spin-allowed transitions correspond to an electronic excitation into d-orbitals ($d_{x^2-y^2}$, d_{z^2}), which are strongly antibonding with respect to metal–ligand interactions. The near-degeneracy of these two highest-lying d-orbital excited states results in the observation of two overlapping but partly resolved transitions in the NIR (ca. 1000 nm) exhibiting a small splitting of approximately 1000 cm^{-1} and relatively low intensity (Whittaker & Solomon, 1988). Removal of a ligand to form the five-coordinate complex (Figure 4, right) results in a large axial perturbation of the metal ion, reflected in a large splitting in the excited-state spectra and an increase in absorption intensity as the orbital selection rule (Laporte's rule) is relaxed by the elimination of inversion symmetry in the complex. Thus, fundamental information on coordination number and geometry is available in the spectra; two overlapping spectral features near 1000 nm reflect octahedral six-coordination, while distinct features near 1000 and 2000 nm are characteristic of five-coordination for the ferrous ion.

Spectra for PDO complexes appear to conform to these limiting cases, with the overlapping pair of bands near 1000 nm having low intensities and small splittings associated with the substrate-free form (Figure 1), while the somewhat more intense features of the substrate-bound form exhibit a large splitting (approximately 6000 cm^{-1}) (Figure 2). These dramatic spectral differences reflect structural differences in the two complexes and allow us to assign a roughly octahedral geometry to the substrate-free native enzyme and a penta-coordinate structure to the ferrous ion in the substrate complex. In view of the facts that the affinity of the enzyme for ferrous ion is so high (vide supra) and that samples containing substoichiometric iron exhibit the same perturbation, it is unlikely that these changes are associated with an unbound Fe fraction. Moreover, the MCD intensity of hexaaquo ferrous complexes is extremely weak.

MCD saturation magnetization data can be interpreted in terms of ground-state spin Hamiltonian parameters for the

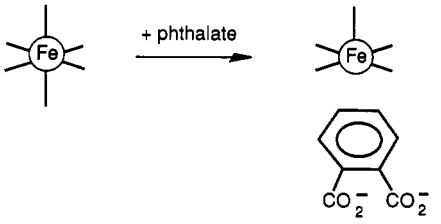
two complexes, providing electronic structural information complementing that available in the excited-state spectra. A detailed evaluation of saturation magnetization MCD gives estimates of ground-state zero-field splittings and *g*-values corresponding to the type of data accessible from magnetic susceptibility or EPR experiments, with a site selectivity based on the resolution of the electronic spectra (Schatz et al., 1978). Thus, MCD saturation behavior of a particular spectral component reflects the ground-state properties of the species giving rise to that transition. For both substrate-free and substrate-bound Fe-PDO complexes we observe a dramatic temperature dependence of the MCD saturation magnetization curves obtained on excitation within absorption bands for the mononuclear Fe^{2+} sites. This temperature dependence results from the Boltzmann thermal population over electronic sublevels of the paramagnetic high-spin ($S = 2$) ferrous ground state. Spin Hamiltonian parameters have been evaluated from this data on the basis of the theory of MCD intensity arising within a non-Kramers doublet lowest in the ground state for both complexes. This analysis, based on a model for *x,y*-polarized MCD intensity induced by the $g_{\parallel}$ Zeeman term within a non-Kramers doublet ground state, has proven effective in evaluating ground-state parameters for ferrous proteins and inorganic complexes (Whittaker & Solomon, 1988). For the substrate-free PDO, a large initial splitting ($\delta = 9$ cm^{-1}) is observed, associated with a ground-state Zeeman term $g = 9.2$. Both the large splitting and the *g*-value exceeding 8.0 reflects near-degeneracies of low-lying orbital states characteristic of a slightly distorted octahedral complex. The initial splitting evaluated for the substrate-bound complex is relatively small ($\delta = 5.9$ cm^{-1}), associated with a smaller ground-state *g*-value ($g = 7.8$). These differences in ground-state properties are consistent with a change in coordination number from 6 to 5 on binding substrate, the reduction in the ground-state orbital momentum on lifting degeneracies being reflected in the *g*-value (Whittaker & Solomon, 1988).

Careful examination of the spectra (Figures 1 and 2) reveals that species are present in addition to the majority forms. In the substrate-free complex, a relatively weak feature is observed near 830 nm, at approximately the same energy as that recorded for the majority species in the substrate-bound form. The intensity of this feature is less than 10% of that observed for the substrate-saturated complex. A minority species is also present in the spectrum of the substrate-bound PDO, contributing a broad and weak feature near 1000 nm, similar to that recorded for the unliganded enzyme form. These features, amounting to approximately 30% of the intensity of the features observed for the substrate-free form, are still observed at the highest phthalate concentrations where the substrate concentration is expected to be saturating ($>10K_d$). While a definitive explanation is not possible on the basis of the available data, the occurrence of both types of signal in each enzyme form suggests the possibility that two ligation environments are in equilibrium in the native enzyme. Substrate binding perturbs this equilibrium and shifts the balance between iron ligation states. This implies a nonexclusive coupling between substrate binding and change in iron site geometry that is more likely to occur for a noncoordinating than for a coordinating substrate.

Co^{2+} Spectra. Cobalt-substituted PDO provides additional information on the effect of substrate binding on the mononuclear metal center. Reconstitution of the apoenzyme with Co^{2+} forms an enzyme complex in which cobalt substitutes for ferrous iron. Although the Co form is catalytically inactive, the metal is bound tightly and cooperatively with substrate in a manner analogous to the Fe^{2+} form. The cobaltous (Co^{2+}) ion exhibits a paramagnetic quartet ($S = {}^3/_2$) ground state in the high-spin d^7 configuration. Saturation magnetization data on the Co^{2+}-substituted phthalate dioxygenase indicate a ground-state *g*-value near 4 associated with the C-term MCD intensity, supporting a high-spin (quartet) ground-state assignment in this complex. This indicates a weak field coordination environment lacking a strong tetragonal perturbation. The ligand field spectra of high-spin cobaltous ions have been used extensively to probe divalent metal binding sites in alcohol dehydrogenase, blue copper proteins, and other biological metal complexes (Kaden et al., 1974; Holmquist et al., 1975; Kaden, 1974; McMillin et al., 1974), providing essential calibration for our studies on Co-substituted PDO. Characteristic spectra are observed for six- and five-coordinate Co^{2+} coordination complexes, permitting structural insights to be obtained from these optical spectra by empirical correlations. MCD spectroscopy provides a powerful tool in these studies, permitting the spectra of the paramagnetic Co^{2+} ion to be detected in the presence of intense absorption arising from the diamagnetic, coupled binuclear Rieske (2Fe–2S) complex. The observed spectra shown in Figure 3 clearly demonstrate a perturbation of the Co center on formation of the substrate complex. The spectrum of the Co site in the native Co-PDO is characteristic of six-coordinate high-spin Co^{2+} in a roughly octahedral geometry (Kaden et al., 1974; Holmquist et al., 1975; Kaden, 1974; McMillin et al., 1974). On addition of substrate, the spectra change. The $d \rightarrow d$ spectra for Co in the substrate complex appear to include features characteristic of both five- and six-coordinate species, consistent with partial conversion to a form with lower ligation number for the metal center. Thus, both Fe^{2+} and Co^{2+} reconstituted enzyme forms appear to undergo a ligation change at the mononuclear metal center on binding substrate.

The nature of the change in ligation of the ferrous center is quite interesting. Normally substrate binding in a metalloenzyme active site results in either no change or an increase in coordination number of the active-site metal ion, as a result of conservative substitution for labile ligands of the resting metal center. For PDO, however, binding substrate actually *decreases* the coordination number for the metal center, converting a six-coordinated ferrous complex into a five-coordinate one, apparently by displacing a labile ligand. Binding of a variety of substrates to PDO can occur in the absence of divalent metal, although ca. 1000-fold more weakly than in its presence. Moreover, there is an absolute requirement for two *o*-carboxyl or other negatively charged groups on the substrate. Previous studies have provided an interpretation in terms of phthalate–enzyme interactions that do not involve direct coordination to Fe but rather binding by Coulomb interactions to positively charged amino acid residues in the protein. Substrate binding does perturb the mononuclear ferrous site, however, as reflected in the altered ligation environment for the mononuclear iron. Optical absorption and EPR measurements show that the binuclear Rieske center is also perturbed on forming the substrate complex, but these spectral changes appear to be relatively minor and do not reflect dramatic changes in ligation state for the Rieske center, as indicated by the X-ray absorption data previously reported for the PDO Rieske complex (Tsang et al., 1989). The displacement of a metal ligand from the monoferrous site, indicated by these spectral studies, is shown in Scheme I. It was previously observed (unpublished results of C. J. Batie and D. P. Ballou) that bound substrate greatly increases the half-life for dissociation of the mononuclear ferrous iron. The

Scheme I



ligation changes that occur on binding of phthalate, converting the mononuclear ferrous site from hexacoordinate to a higher affinity pentacoordinate form, thus minimize the loss of mononuclear iron to the solution where it can rapidly oxidize to form highly insoluble ferric hydroxide. The perturbation of the Fe^{2+} site may relate mechanistically to the preparation for reaction with O_2, as the displacement of a metal ligand on binding a substrate levels the reduced metal ion coordinately unsaturated, opening a potential binding site for dioxygen.

ACKNOWLEDGMENT

G.T.G. and D.P.B. would like to acknowledge useful discussions with Dr. James E. Penner-Hahn.

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