Chemistry of the Catalytic Conversion of Phthalate into Its *cis*-Dihydrodiol during the Reaction of Oxygen with the Reduced Form of Phthalate Dioxygenase†

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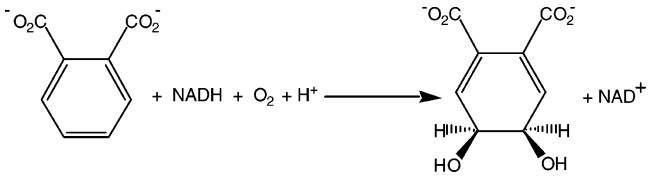
ABSTRACT: The phthalate dioxygenase system, a Rieske non-heme iron dioxygenase, catalyzes the dihydroxylation of phthalate to form the 4,5-dihydro-*cis*-dihydrodiol of phthalate (DHD). It has two components: phthalate dioxygenase (PDO), a multimer with one Rieske-type [2Fe−2S] and one mononuclear Fe^{II} center per monomer, and a reductase (PDR) that contains flavin mononucleotide (FMN) and a plant-type ferredoxin [2Fe−2S] center. This work shows that product formation in steady-state reactions is tightly coupled to electron delivery, with 1 dihydrodiol (DHD) of phthalate formed for every 2 electrons delivered from NADH. However, in reactions of reduced PDO with O_2, only about 0.5 DHD is formed per Rieske center that becomes oxidized. Although the product forms rapidly, its release from PDO is slow in these reactions with oxygen that do not include reductase and NADH. EPR data show that, at the completion of the oxidation, iron in the mononuclear center remains in the ferrous state. In contrast, naphthalene dioxygenase (NDO) [Wolfe, M. D., Parales, J. V., Gibson, D. T., and Lipscomb, J. D. (2001) *J. Biol. Chem*. 276, 1945−1953] and benzoate dioxygenase (BZDO) [Wolfe, M. D., Altier, D. J., Stubna, A., Popescu, C. V., Münck, E., and Lipscomb, J. D. (2002) *Biochemistry*, *41*, 9611−9626], related Rieske non-heme iron dioxygenases, form 1 DHD per Rieske center oxidized, and the mononuclear center iron ends up ferric. Thus, both electrons from reduced NDO and BZDO monomers are used to form the product, whereas only the reduced Rieske centers in PDO become oxidized during production of DHD. This emphasizes the importance of PDO subunit interaction in catalysis. Electron redistribution was practically unaffected by the presence of oxidized PDR. A scheme is presented that emphasizes some of the differences in the mechanisms involved in substrate hydroxylation employed by PDO and either NDO or BZDO.

The phthalate dioxygenase system (PDS)[1] from *Burkholderia cepacia* DB01 belongs to a family of Rieske non-heme iron oxygenases that catalyze many metabolically important reactions in the aerobic degradation pathways of aromatic compounds by microorganisms. The phthalate dioxygenase reaction (Scheme 1), which is typical of this whole family of dioxygenases, is a 4-electron reduction of oxygen with two electrons coming from NADH and two from the substrate.

This two-component system consists of phthalate oxygenase reductase (PDR), which contains FMN and ferredoxin [2Fe−2S] centers, and the multimeric phthalate oxygenase (PDO), which contains one Rieske [2Fe−2S] center and one mononuclear Fe^{II} site per monomer. PDS is classified as a Rieske non-heme iron dioxygenase (*4*) with a type IA electron-transfer system (*5*). PDS does not contain a separate ferredoxin, which is a small electron-transfer protein found in type-II and type-III dioxygenase electron-transfer systems. The reaction in PDS starts with the transfer of a hydride from the reduced pyridine nucleotide to the FMN of PDR. The Rieske center of phthalate dioxygenase (PDO) sequentially accepts electrons from the reductase [2Fe−2S] center and transfers them to the iron mononuclear center of PDO during catalysis. This mononuclear Fe^{II} is essential for catalytic activity (*6*, *7*) and is proposed to be the site of oxygen binding, activation, and substrate hydroxylation. In the process of the reaction, the dioxygen molecule is cleaved and its atoms are inserted into the aromatic nucleus to produce a *cis*-dihydrodiol (DHD) (Scheme 1).

Scheme 1: Catalytic Pathway for the Phthalate Hydroxylation



Questions remain about what electron-transfer pathways are utilized in the enzymatic reaction and what regulatory mechanisms affect their efficiency. For example, is it required that both electrons necessary for product formation come from Rieske center electrons, or can the second electron be

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[1] Abbreviations: PDS, phthalate dioxygenase system; PDO, phthalate dioxygenase; PDO-Fe^{II}, enzyme as isolated supplemented with Fe^{II}; PDO-APO, phthalate dioxygenase that lacks iron in the mononuclear center; PDO-APO^F, apoprotein prepared according to the stringent procedure (1.9 Fe/monomer); PDR, phthalate dioxygenase reductase; DHD, *cis*-4,5-dihydrodiol of phthalate; NDO, naphthalene dioxygenase; NDF, naphthalene dioxygenase ferredoxin; NDS, naphthalene dioxygenase system; BZDO, benzoate dioxygenase.

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obtained from the reduced mononuclear iron center? If both electrons for DHD formation must be provided by Rieske centers, do the centers in the multimeric PDO cooperate or must PDR be involved to facilitate electron transfer? Is there subunit cooperativity within the PDO multimer that is important for catalytic activity? As suggested by the oxidation kinetics of PDO, which seem to support the existence of two electron-transfer pathways for the oxidation of Rieske centers, the subunit cooperation is essential for the product formation (*3*).

The overall dioxygenation can be divided conceptually into a reductive reaction followed by the reaction with oxygen that leads to oxygenation of the substrate. Studies of the oxidative half-reaction are useful for answering questions such as those proposed above. Recent studies of the oxidative reactions have shown that in naphthalene 1,2-dioxygenase (NDO) (*8*) and in benzoate dioxygenase (BZDO) (*9*) both the Rieske and the reduced mononuclear centers are oxidized rapidly to provide electrons for product formation. Thus, on completion of the oxidation, the mononuclear iron is in the ferric state. This allows these systems to produce one product molecule for each Rieske center that is oxidized.

Hydroxylation of phthalate catalyzed by PDO is in many respects similar to the catalysis performed by NDO and BZDO. However, as we report here, in the oxidative half-reaction of PDO, cooperation of two Rieske centers and of an Fe^{II} mononuclear center are necessary for product formation. In contrast to NDO and BZDO, only about 0.5 molecules of product are formed per oxidized Rieske center, and at the end of the oxidation reaction, iron in the mononuclear center is in the ferrous state rather than in the ferric state as found with NDO and BZDO. Comparisons of similarities and differences in the enzyme oxidation and substrate hydroxylation reactions in these related enzymes are likely to lead to a better understanding of the role that structural elements play in the enzyme activity.

MATERIALS AND METHODS

PDO and PDR were isolated from *B. cepacia* DB01 and purified as described previously (*10*). Concentrations of enzymes were determined spectrophotometrically using $\Delta\epsilon_{575} = 2.38\ mM^{-1}\ cm^{-1}$ and $\Delta\epsilon_{466} = 17.54\ mM^{-1}\ cm^{-1}$ for the extinction difference between oxidized and reduced PDO and PDR, respectively. PDO activity was determined in steady-state assays by monitoring the change in absorbance at 340 nm caused by consumption of NADH. Reaction mixtures contained 0.2 μM PDR, 2 mM phthalate, and 100−250 μM NADH in 0.1 M HEPES at pH 8. The reaction was initiated by the addition of 0.2 μM PDO. When necessary, $Fe(NH_4)_2(SO_4)_2$ (FAS, 10−20 μM) was added. Note that the activity measured in this manner does not represent the maximum PDO activity but rather the activity at 1:1 PDO/PDR stoichiometry under these specific conditions. These conditions were chosen because they are practical and reproducible. As isolated, PDO contained 2.8 ± 0.2 iron per monomer and its activity was 5.5 ± 0.3 s^{-1}. PDO was reconstituted with iron by incubating stock enzyme for 5 min with a 100-fold excess of FAS under aerobic conditions. The excess iron was removed by one passage through a desalting Hi-Trap column (Pharmacia Biotech) equilibrated with the appropriate buffer containing 1 mM phthalate. Iron-reconstituted PDO that contained 3.0 ± 0.1 Fe per monomer (activity 5.9 ± 0.1 s^{-1}) was immediately used in the experiments. PDO-APO^F, which lacks iron in the mononuclear center, was prepared by extensive dialysis against HEPES buffer containing 5 mM EDTA as fully described in ref *3* and contained 2.0 ± 0.2 Fe per monomer. Its activity was 0.05 ± 0.02 s^{-1}. Reconstitution of PDO-APO^F with Fe^{II} resulted in an enzyme that contained at least 3 iron per monomer and increased the activity to a maximum of 2.9 ± 0.1 s^{-1} with no higher activity observed under any experimental conditions.[2] Partial reconstitution of PDO with Fe^{II} was accomplished by adding the appropriate amount of an anaerobically prepared FAS solution to an anaerobic PDO-APO sample. Iron content in PDO was determined by the standard ferrozine assay (*11*) or by atomic absorption spectroscopy using a Perkin−Elmer 3300 Atomic Absorption spectrometer equipped with a HGA-600 graphite furnace.

PDO samples for oxidative half-reaction studies (20−40 μM before mixing for stopped-flow experiments and 60−400 μM before mixing for product analysis and EPR studies) were vacuum/gas-exchanged (Ar) in tonometers (about 10 times) and overlaid with ~2 psi of purified argon. Enzyme reduction was achieved by photoreduction (*12*) of the anaerobic enzyme in the presence of 0.03−1 μM 5-deazariboflavin (a generous gift from Dr. Massey, University of Michigan) and 1−5 mM glycine or by titration with an anaerobic sodium dithionite solution. Results did not depend on the method of reduction. In the experiments with the unreconstituted PDO-APO, 0.5−1 mM EDTA was added to the solution to complex any adventitious iron that was present. Experiments were performed in 0.1 M HEPES or in 0.1 M potassium phosphate buffer at pH 8.0 and 22 °C. The oxidation state of the enzyme was monitored using a Shimadzu UV 2051PC spectrophotometer.

In experiments for product analysis in the oxidative half-reactions, reduced PDO was diluted 1:1 with 100% oxygen-equilibrated buffer, with or without PDR being present. Thus, the oxygen concentration right after mixing was ~600 μM. Aliquots were taken for analysis immediately after mixing and periodically until about 1 h after initiation of the reaction. Aliquots obtained before adding oxygenated buffer (both before and after the reduction) were used to provide baseline values for product formation. Aliquots were removed from the reduced sample in an anaerobic glovebox to avoid oxygen contamination. Reaction aliquots were quenched by filtering the enzyme from the solution with Microcon-YM30 concentrators (Amicon, Co.) or by heating for 3 min at 90 °C. No additional product or substrate was released when heated at 95 °C for up to 10 min. Heating resulted in total denaturation of the enzyme and released all bound substrate and product. Likewise, treatment with HCl released the same amount of product. Moreover, nearly the same quantity of DHD was released after more than 1 h (see Figure 1). Similar denaturation was required to release the product from NDO (8). Precipitates from denatured samples were removed by spinning for 2 min in a tabletop centrifuge. The supernatant was then filtered through the Microcon-30 concentrator. Samples were frozen at 77 K until product

[2] This is probably due to partial disruption of the mononuclear center during preparation of PDO-APO^F. The Rieske centers are apparently not affected significantly.

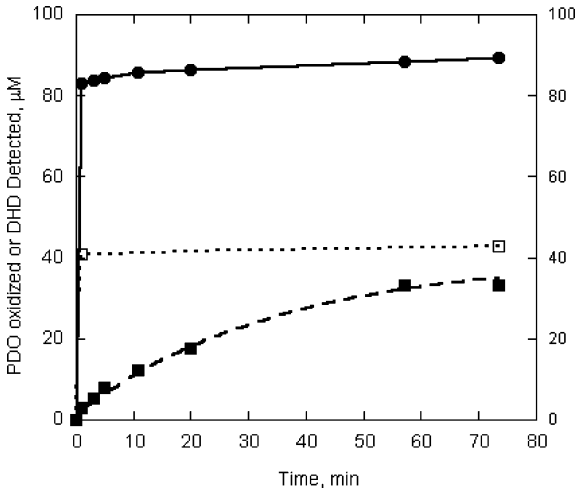


FIGURE 1: Product formation in the reaction of 178 μM of reduced PDO (before mixing, based on Rieske center concentration) with oxygen in the presence of 1 mM phthalate. The reaction was initiated at time zero by the addition of oxygenated buffer (625 μM O_2 after mixing) containing 1 mM phthalate. Oxidation of the PDO Rieske center (●), DHD formation as observed by quenching the reaction by filtering out the enzyme (■), and product formation after quenching the reaction by heat denaturation (○).

analysis was carried out. Control experiments using previously extracted DHD verified that no DHD deterioration occurs during the freeze/thaw and/or heating of samples up to 95 °C for up to 5 min.

Product analysis was performed with a Waters HPLC system equipped with a Waters 441 detector ($\lambda = 250$ nm) using an Aminex HPX-87H column running isocratically in 8 mM H_2SO_4 at 60 °C with a 0.75 mL/min flow rate. Data recording and analysis were performed with a PowerChrom integrator from ADInstruments Ltd. For EPR studies, an aliquot of anaerobically prepared fully reduced PDO sample (about 300 μM) was removed from a titration cuvette and frozen in liquid nitrogen in the glovebox. The cuvette was resealed, removed from the glovebox, opened to air, and mixed with the equal volume of the aerated buffer (250 μM O_2 before mixing). Thus, after the initial burst of PDO oxidation, the rates of oxidation of the Rieske centers were determined by the diffusion of oxygen into the solution. Samples were periodically removed for analysis by EPR and HPLC. PDO oxidation was spectrally monitored. This process made it possible to correlate the oxidation of the Rieske centers (and possibly of the mononuclear iron) using both optical and EPR spectroscopy with the formation of the product. EPR studies were performed using a Brucker EMX spectrometer equipped with a Brucker 4102-ST general purpose cavity. Data were collected at 15 K (9 K for the NO experiment) with a modulation amplitude of 10 G, microwave frequency of 9.426 GHz, and microwave power of between 0.1 and 20 mW. Iron standard solutions for atomic absorption were from Aldrich. All other chemicals were of the analytical grade and used without further purification.

RESULTS

Oxidized PDO samples contained less than 0.02 DHD/Rieske center as analyzed by HPLC (see the Materials and Methods). No additional product formation was detected, even in the presence of oxygen, phthalate, oxidized PDR, and excess Fe^{II}. Thus, similar to NDO (*8*) and BDZO (*9*), free Fe^{II} is not a suitable electron donor for product formation in the phthalate dioxygenase system. Addition of NADH to the reaction mixture containing PDO, PDR, and phthalate initiated the normal tightly coupled catalytic conversion of phthalate into its dihydrodiol. In this reaction, one molecule of product was formed for every 2 electrons delivered by NADH to the phthalate dioxygenase system. The catalytic reaction remained tightly coupled, even when using native enzyme that had not been supplemented with Fe^{II}.

Product Analysis of the Oxygen Half-Reaction of PDO-Fe^{II}. In the presence of oxygen and phthalate, reduced Rieske centers in the PDO-Fe^{II} multimer undergo rapid oxidation with the concomitant development of the product. DHD formation could be observed with or without phthalate dioxygenase reductase being present. As was previously reported with NDO (*8*), very little product could be observed initially in the supernatant when the reaction was quenched by filtering out the enzyme. The product was released slowly from PDO, so that by 60 min the total amount of the product released into the filtrate approached 0.5 (0.48 ± 0.03) DHD/Rieske center that had been oxidized (Figure 1). There were two phases to the increase in DHD content in the filtrate, with rates of 0.03 ± 0.02 and 0.0006 ± 0.0004 s^{-1}. These rates are extremely slow when compared to the rates of oxidation of Rieske centers in PDO-Fe^{II} (1 and 0.1 s^{-1} in the absence and up to ~40 s^{-1} in the presence of phthalate) (*3*). Thus, even the faster phase (0.03 s^{-1}) of dissociation of the PDO−DHD complex that accounts for 55 ± 10% of the total product release is unlikely to be related to any electron transfers from the Rieske centers and is obviously not relevant for normal catalysis in the presence on PDR and NADH (turnover rate of 5.5−6 s^{-1} under similar conditions).

Oxidation of PDO as isolated (no extra Fe^{II} added) exhibited higher contributions (up to 25%) from slower oxidation rates of the Rieske center. This was likely due to oxidation of Rieske centers in subunits of PDO that were not linked to active Fe^{II} mononuclear centers. For such samples, 20−25% less DHD formed during the single turnover reaction than observed for PDO-Fe^{II}. Product release from the enzyme remains as slow as observed with PDO-Fe^{II}.

Quenching the reaction by filtering out the enzyme allows only for the determination of product that is free in solution. DHD that is formed but is bound to PDO stays in the concentrate along with the enzyme and thus will remain undetected. To determine the amount of DHD bound to the enzyme, we quenched the oxidation of PDO by heating the sample, as described in the Materials and Methods, both immediately after adding oxygen to the mixture and after 1 h of incubation. The amount of DHD detected after heat denaturation (0.48 ± 0.04 molecules of DHD per Rieske center oxidized) remained constant throughout the course of the reaction (Figure 1 and Table 1), indicating that oxygenation was essentially finished before the first aliquot was quenched for product analysis (30−50 s after the addition of oxygen). It can be noted that the Rieske centers of PDO-Fe^{II} are nearly fully oxidized by this time (about 90−95% oxidation). This result concurs with the fast oxidation rates of PDO-Fe^{II} observed in stopped-flow experiments (*3*).

Addition of oxidized reductase to PDO results in an increased rate of oxidation of the Rieske center, and the

Table 1: DHD Production in Oxidative Half-Reactions of PDO-Fe^{II} and PDO-APO^F at Different Levels of Reconstitution

	Fe/Rieske (± 0.1)[e]	Rieske center oxidation[a] (%)	reconstituted mononuclear sites (%) (± 10%)	DHD yield/Rieske experiment[b] (± 0.02)	DHD yield/Rieske model[c]
PDO-Fe^{II}	3	97	100	0.48	0.5
PDO-Fe^{II} + PDR_{ox}	3	98	100	0.48	n/a
PDO-APO^F	2	20	0	>0.01	0
PDO-APO^F + Fe^{II}	2.25	47	25	0.18	0.22
PDO-APO^F + Fe^{II}	2.5	73	50	0.38	0.38
PDO-APO^F + Fe^{II}	3[d]	88	100	0.48	0.5
PDO-APO^F + Fe^{II} + PDR_{ox}	2.25	65	25	0.23	n/a

[a] Relative percentages of the Rieske centers that were oxidized within 1 min after the reaction was initiated. [b] The ratio of DHD produced to Rieske center oxidized, as determined by HPLC analysis of the heat-denatured sample obtained within 1 min after initiation of the reaction. [c] Results for the tetrameric PDO with all fully active mononuclear sites presented. See the text for details. [d] Addition of a 50-fold excess of FAS (over the stoichiometric amount) to the reaction mixture did not further improve the activity of reconstituted PDO-APO^F and did not affect the amount of DHD produced. [e] The error margin given in the Material and Methods is higher because of the inclusion of the dispersion between different preparations.

presence of both PDR and the substrate has a synergistic effect (*3*). However, the total amount of product formed during the oxidation of reduced PDO-Fe^{II} did not change in the presence of PDR_{ox} (Table 1). As in the absence of the reductase, DHD formation was essentially complete before the first sample could be quenched (30−40 s). Addition of PDR_{ox} had no effect on the rates of DHD release from PDO.

State of the Mononuclear Center during Oxidation of PDO-Fe^{II}. Two electrons are needed for the oxygenation of phthalate to form its *cis*-4,5-dihydrodiol. In oxidative half-reactions with no external sources of electrons available, both electrons must be supplied from within the reduced multimer. Potentially, one electron could come from a reduced Rieske center and the other from the Fe^{II}, thus resulting in the mononuclear center containing Fe^{III} at the conclusion of the reaction. In such a case, one DHD could form per Rieske center being oxidized. This was indeed found to be the case for the related enzymes, naphthalene dioxygenase (*8*) and benzoate dioxygenase (*9*). Alternatively, if the mononuclear iron ends up as Fe^{II}, both electrons must ultimately come from reduced Rieske centers, so that only one DHD could form per two reduced Rieske centers oxidized. From the results presented above (∼0.5 mol of DHD/mol of Rieske centers oxidized), this appeared to be the case for PDO.

To unambiguously determine the state of mononuclear iron on DHD formation, PDO samples were quenched by freezing at various times during the course of the oxidation reaction, EPR spectra were recorded (Figure 2), and the product was analyzed by HPLC as described in the Materials and Methods. Oxidized Rieske centers are EPR-silent because of antiferromagnetic coupling of their two ferric irons. However, the EPR spectra of reduced PDO exhibit a strong rhombic signal centered near 350 mT ($g_{ave} \sim 1.89$), which is due to the reduced Rieske center of the protein. Thus, it was possible to monitor the reduction state of the Rieske center. Mononuclear centers containing Fe^{II} are also EPR silent; however, with the iron in the ferric form, they exhibit a signal around 150 mT ($g \sim 4.3$). In the reaction of reduced PDO-Fe^{II} with oxygen, the signal from the reduced Rieske centers decreased as the sample was oxidized.[3] However, no significant amount of ferric iron was detected in the sample at any time during or after the reaction (Figure 2). Note, that trace 1 in Figure 2 shows that 15 μM Fe^{III} is easily detectable under the conditions used. The PDO concentration in the presented experiment was 167 μM, and 82 μM product was produced at the end of the reaction. The rate of oxidation of the Rieske centers, determined from the decrease in the $g_{ave} \sim 1.89$ signal, correlated to the rate obtained from the spectrophotometric measurements of the same sample (data not shown). This overall reaction was slow to reach the completion because the concentration of the oxygen added was less than that of the PDO and the full oxidation required O_2 to diffuse into the solution.

In some Fe^{II}-reconstituted PDO samples, low levels of Fe^{III} were detected (as determined by a small signal at 150 mT). Similar signals were observed previously (*3*) and were attributed to adventitious Fe^{III} bound to the enzyme. Such signals were practically undetectable at microwave power levels below 1 mW. Quantitatively, this ferric iron amounted to less than 10% of the iron in the mononuclear center, and its concentration did not change during the experiment.

We also confirmed the ferrous state of iron in the mononuclear center at the conclusion of the reaction by incubating the reacted sample with nitric oxide, as suggested by a reviewer. This resulted in the development of a signal at ∼150 mT characteristic of an Fe^{II}−NO complex (trace 6 of Figure 2), similar to the one observed previously for the oxidized PDO samples (*3*). PDO obtained at the conclusion of the reaction retained catalytic activity, as verified by the steady-state assays, similar to that of the PDO before the reaction.

Product Formation during Oxidative Half-Reaction of PDO-APO^F with and without Extra Iron Added. No measurable DHD production was observed in oxidative half-reactions of PDO-APO^F in which the mononuclear center had not been reconstituted with Fe^{II} (Table 1). Even prolonged incubation (up to 1 h) did not result in any significant increase in DHD either released into the solution or bound to the enzyme. Addition of Fe^{II} to PDO-APO^F significantly increased the rates of oxidation of the Rieske center (*3*) and also enabled the formation of the product. The total amount of the product formed depended on the relative content of the reconstituted mononuclear centers in PDO-APO^F (Table 1). For the reaction of oxygen with reduced fully reconstituted PDO-APO^F (at least 3 Fe/monomer), about one DHD was formed per two Rieske centers oxidized, which is the same as for the PDO-Fe^{II}, even

[3] In the first 30 s after mixing 300 μM PDO solution with the aerated buffer (250 μM O_2), 91 μM PDO was oxidized and 46 μM of DHD was produced.

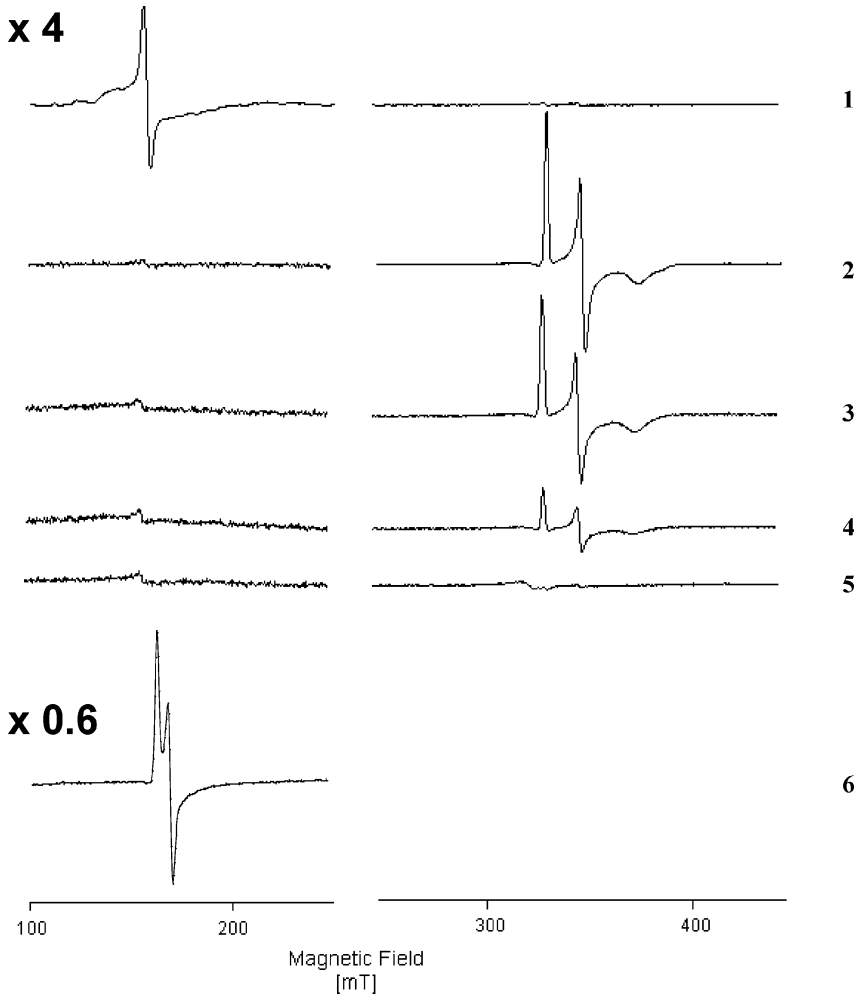


FIGURE 2: EPR spectra of PDO (as isolated) in 0.1 M HEPES at pH 8.0 containing 0.5 mM phthalate. Fully oxidized PDO (80 μM after mixing) with 15 μM adventitious Fe^{III} (1) and fully reduced PDO (167 μM after mixing) before (2) and 30 s (3), 500 s (4), and 4300 s (5) after the initiation of the oxidation reaction. (6) Sample as in (5) after vacuum-Ar exchange and incubation for 8 min with NO. The conditions are the same as described in the Materials and Methods. Microwave power was 10 mW.

though the catalytic activity of PDO-APO^F, as measured by the steady-state assay, was only about 50% of that of PDO-Fe^{II} (2.9 s^{-1} versus 5.9 s^{-1}). Significantly smaller amounts of DHD were observed when PDO-APO^F was only partially reconstituted with iron (Table 1). At all levels of reconstitution, product formation occurred before the first sample was quenched (30−50 s after the addition of oxygen), as was the case in PDO-Fe^{II}. No additional DHD formation was observed in PDO-APO^F that was fully reconstituted with iron, even 1 h after the initiation of the reaction (same as for PDO-Fe^{II}, Table 1). In PDO-APO^F samples that were only partially (50 or 25%) reconstituted with Fe^{II}, incubation for 1 h resulted in a small ~10% increase in the total DHD produced. This was probably due to slow redistribution of electrons between the Rieske centers in different monomers of the PDO multimer. In both fully and partially reconstituted PDO-APO^F, release of DHD was slow with the rates similar to these observed in PDO-Fe^{II}.

Addition of oxidized reductase to partially (25%) reconstituted PDO resulted in only a slight increase (0.23 versus 0.19 DHD/Rieske center oxidized) of the total amount of DHD produced (Table 1). Prolonged incubation did not result in any significant increase of the total DHD produced (an additional 0.03 DHD/Rieske center was formed, which is within the margin of experimental uncertainty). The rates of product release were slow and similar to those observed in the absence of the reductase.

Modeling of Product Formation if Fe^{II} Reconstituted PDO. In the head-to-tail (Rieske center-to-mononuclear site) subunit arrangement, observed in the $\alpha_3\beta_3$ hexamer of NDO (*13*), each mononuclear site is paired with the nearby Rieske center on the adjacent subunit. Only one Rieske center is located within efficient electron-transfer distance of the mononuclear site, and thus, in a single oxidation reaction, the second electron must be provided by the oxidation of the iron in the mononuclear center (thereby, this iron must be found in the Fe^{III} state at the end of the reaction), with the resultant stoichiometry of the product formation of 1 product per Rieske center oxidized. A similar head-to-tail arrangement of subunits for PDO would prohibit catalysis as two Rieske centers must deliver electrons to the mononuclear site for substrate hydroxylation. One way to bring to two Rieske centers within the range of the efficient electron transfer to the mononuclear iron is to fold the head-to-tail arrangement to form a structure shown in Figure 3.

The Berkeley Madonna version 8.0.1 program was used to model the fractional reconstitution of the mononuclear centers with different levels of added Fe^{II}. The amount of product produced in each particular case was calculated on the basis of the following assumptions: (i) Only one active

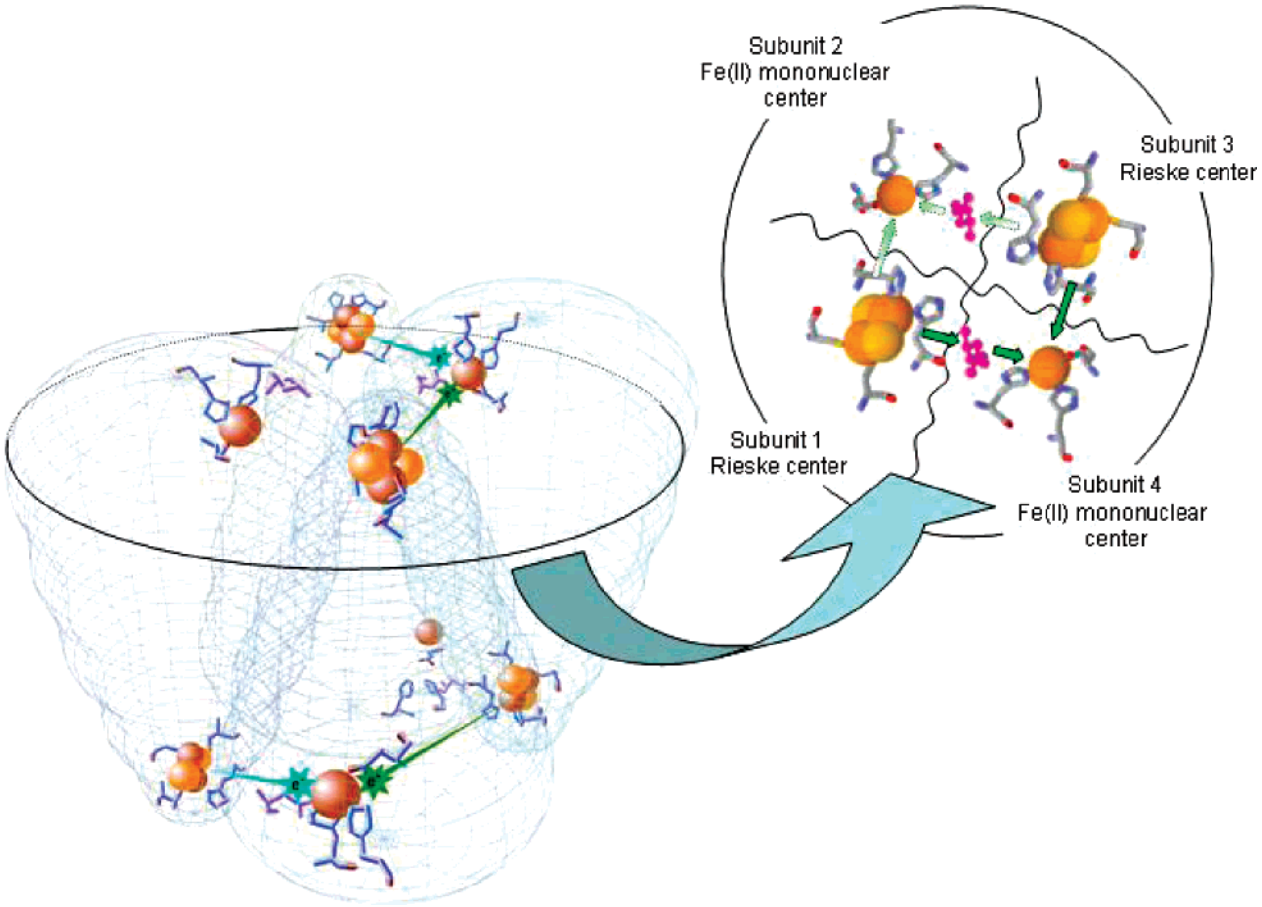


FIGURE 3: Model of electron transfers in PDO multimer with the top view magnified. Monomers in the PDO tetramer are arranged side by side in alternate directions, with each Rieske center capable of delivering electrons to two mononuclear centers located nearby on either of two other subunits. Each Rieske center is linked to one mononuclear site via a bridging aspartate (red). In a single turnover reaction, to form a DHD, iron in the mononuclear center must receive electrons from two Rieske centers (green arrows on the top view). Only one active mononuclear site is necessary for rapid oxidation of both Rieske centers (dark green arrows or light green arrows). As discussed previously (*3*), Rieske and mononuclear centers on the same subunit are located about 40 Å apart, making a fast electron transfer between them improbable.

mononuclear center per two Rieske centers is necessary for DHD formation (no electrons are provided by the Fe^{II} incorporated into the mononuclear center), and if an active site *is* available, two Rieske centers *would* cooperate to irreversibly provide one electron each for DHD formation; (ii) Electron transfer is slow between individual multimeric enzyme molecules as well as between the Rieske and mononuclear centers within a given monomer; (iii) Fe^{II} preferentially binds at the mononuclear centers rather than adventitiously at other regions of PDO; (iv) All mononuclear centers can bind Fe^{II}; (v) There is no significant cooperativity in iron binding at the mononuclear centers and no preferential binding of oxygen to a given mononuclear iron. These seem reasonable because, in the presence of phthalate, Fe^{II} does bind tightly to the PDO (K_d in the picomolar range) with practically no observed cooperativity (unpublished data). Most of the other assumptions naturally follow from the results presented above and the model proposed previously (*3*) (Figure 3).

The fractional concentration of PDO with iron incorporated in none, one, or two mononuclear centers with different levels of added iron can be calculated. For example, results obtained for the tetrameric structure (Figure 3) under the assumption listed above are shown in Table 2. On the basis of the obtained distribution profile, we then calculated the maximum amount of DHD that can be produced depending on the number of Rieske centers available to provide electrons for catalysis and the number of available iron-containing mononuclear centers. The quantities of DHD expected for this model at different levels of PDO reconstitution are shown in the last column of Table 1. An excellent correlation exists between the predicted and experimentally observed amounts of DHD. Data presented in the table are based on the additional assumption (vi) that all mononuclear centers are active. This assumption appears reasonable considering the time scale of the single-turnover experiments. Models that stipulate a significant part of the mononuclear centers in a totally inactive state (i.e., they do not participate in the catalysis at all) failed to predict the experimentally observed amounts of DHD.[4] Previously published data indicate that PDO is most probably an α_4 tetramer (3); however, unconfirmed data exists (see footnote 1 in ref *5*) that the enzyme

[4] The cases modeled included (a) a random distribution of the fully catalytically inactive mononuclear centers (as opposed to centers that are catalytically active but can only slowly accept electrons from the Rieske centers) with and without Fe^{II} preferably binding to the fully active mononuclear centers and (b) a nonrandom distribution of the fully catalytically inactive mononuclear centers with one active and one inactive center per every two Rieske centers, again, with and without Fe^{II} preferably binding to the fully active mononuclear centers.

Table 2:

percent of the mononuclear sites reconstituted with Fe^{II}		number of mononuclear centers containing the "active unit"[a] (percent of the total)			percentage of "active units" containing at least one mononuclear site reconstituted with Fe^{II} (percent of the total)	predicted percentage of product under the assumptions (DHD/Rieske)[b] (%)
		0 atoms of Fe^{II}	1 atom of Fe^{II}	2 atoms of Fe^{II}		
100	tetramer	0	0	100	100	50
50	tetramer	25	50	25	75	38
25	tetramer	56	38	6	44	22

[a] In a tetrameric (α_4) PDO, the "active unit" would consist of two Rieske centers (units 1 and 3 on the top view, Figure 3) and three mononuclear centers (units 2 and 4). If the enzyme would be a hexameric (α_6) enzyme, the "active unit" would consist of three Rieske centers (units 1, 3, and 5, figure not shown) and three mononuclear centers (units 2, 4, and 6). [b] When none of the mononuclear center contains Fe^{II}, no DHD is produced. If the "active unit" contains one or two mononuclear centers reconstituted with Fe^{II}, one molecule of DHD is produced (because there are only two electrons available for product formation).

can exist in a higher aggregate states (α_6 or α_8). Similar to the case with the tetramer, in a NDO-like head-to-tail arrangement of subunits, only one Rieske center will be available for electron transfer to any given mononuclear site. Again, this will result in 1:1 product/oxidized Rieske center stoichiometry, if the mononuclear center can supply the second electron necessary for catalysis (at the end of the reaction, iron is in the Fe^{III} state), or no product at all, if the second electron had to be provided from the second Rieske center (at the end of the reaction, iron remains in the Fe^{II} state). For a hexamer in an arrangement similar to that for the tetramer (Figure 3), three Rieske centers and three mononuclear sites are "nearby" and, depending on the specifics of the structure, either two or three Rieske centers are available for electron transfer to a given mononuclear center. If the electrons for catalysis are provided only by the Rieske centers, at the end of the reaction, one of the centers would remain uncoupled from DHD formation because no second electron would be available. In a fully Fe reconstituted enzyme, such an arrangement would result in 30% less in product formation as compared to the "tetramer" model (about 0.33 DHD/Rieske as opposed to 0.5 DHD produced/Rieske oxidized predicted for the tetramer and observed in experiments). In the case of partially reconstituted PDO, the predicted amount of DHD produced would also deviate significantly from that predicted for the tetramer and that experimentally observed. Similar models can be constructed for the octameric PDO structure; however, in the simplest case, product formation in the octamer would be indistinguishable from that in the tetramer (octomer taken as an aggregate of two identical tetramers).

DISCUSSION

Studies of the oxidative half-reaction are useful for resolving questions related to the mechanisms of effective catalysis in complex systems. Recent studies of NDO (*8*) and BZDO (*9*) using this methodology revealed that with both of these enzymes the reduced Rieske center, as well as the reduced mononuclear center, can contribute electrons to produce *cis*-dihydrodiol products, even in the absence of the reducing system components of these systems. During catalysis, one product is formed for one Rieske center oxidized, with the second electron necessary for dihydrodiol formation being provided by the oxidation of the iron in the mononuclear center. Addition of reduced naphthalene dioxygenase ferredoxin (NDF), the small electron-transfer component of the NDO system, resulted in the formation of an additional product, with 1 product forming per 2 electrons delivered to the NDO molecule. Moreover, addition of oxidized NDF to reduced NDO resulted in a slight increase in total product formation, probably because of stimulated redistribution of electrons in the NDO system (*8*). Thus, in the presence of NDF, subunits that do not have competent mononuclear centers are still able to contribute electrons to competent subunits.

As mentioned previously, hydroxylation of phthalate catalyzed by PDO in many respects resembles the catalysis performed by NDO and by BZDO. Similar to the results reported previously for the NDO and BZDO (*8*, *9*), the oxygenase component of the phthalate dioxygenase system alone is capable of catalyzing the dihydroxylation of the aromatic substrate with consequent formation of their *cis*-dihydrodiols. Similarly, in the PDS, the reductase (PDS does not contain ferredoxin) is not necessary for oxygen activation and substrate hydroxylation. However, the overall product yield per Rieske center oxidized in oxidative half-reactions of PDO is approximately half of that observed in the naphthalene and benzoate dioxygenase systems. As shown by the EPR experiments at the completion of the reaction with PDO, the iron in the mononuclear center is found to be in the ferrous state. This does not imply that the mononuclear iron in PDO cannot be transiently oxidized during catalysis; however, it limits the net phthalate dihydroxylation to one DHD formed per two Rieske centers that are oxidized. Similar studies using EPR showed that in NDO and BZDO the mononuclear iron was in the ferric state.

Because the mononuclear iron is not oxidized, only reduced Rieske centers of PDO provide the net reducing equivalents. Addition of oxidized reductase to the reaction mixture does not affect the amounts of the product formed, although, as shown previously, it does affect the rates of the oxidation of the Rieske centers (*3*). When PDR_{ox} is added to partially reconstituted PDO, a small increase in product yield (about 10% relative to the maximum amount of DHD possible) is observed. The total amount of the product formed accounted for only a small fraction of electrons still available from the Rieske centers. It is possible that this small fraction was due to some contamination from the reductase preparation. Thus, in PDO, redistribution of electrons among the subunits is not significantly induced by the oxidized reductase at any appreciable rate or extent. This conforms with the observation that during stop-flow experiments no rereduction of PDR was observed when PDR_{ox} was mixed with reduced PDO (*3*).

It can be noted that, in the experiments with the NDO system (*8*), the mononuclear sites of NDO were not fully populated (as evidenced by the increase in product formation on the addition of Fe^{II} to the reaction, up to 0.99 from 0.85 DHD/Rieske center oxidized). Addition of oxidized NDF to that enzyme apparently induced some electron redistribution between the subunits of NDO with the total increase in *cis*-diol formation of about 10% of the total product formed (up to 0.96 from 0.85 DHD/Rieske center oxidized). The experiments with partially reconstituted PDO show that at maximum $\leq$10% of all of the electrons still available from the unreacted Rieske centers are shuttled to the active sites with the help of PDR_{ox}. Thus, the electron redistribution in the multimer facilitated by the respective reductive partners could be similar in both NDS and PDS. In the PDO system, it appears to be very inefficient. Further studies are necessary to determine whether that will be also true in NDS.

It has been shown that in the reaction of oxygen with reduced PDO the product forms fast but remains bound to the enzyme. DHD release from PDO is slow and is apparently unrelated to the slow oxidation observed for a small fraction of reduced Rieske centers (*3*). This was probably the major reason for the underestimated product yield determined in a much earlier study of the PDS (*6*). Tight product binding at the completion of the oxidation reaction was also observed with the NDO and BZDO systems (*8*, *9*). The ability of PDO to sustain high turnover rates in steady-state turnover clearly indicates that a mechanism exists to rapidly dissociate DHD from PDO to enable the next cycle in turnover. In the cases of the NDO and BZDO systems, which leave the iron in the ferric state at the end of the oxidation reaction, rereduction of the mononuclear ferric iron may serve to trigger product release, as indeed seems to be the case in NDO (*8*). However, because no Fe^{III} was detected in our experiments, we can rule out rereduction of the mononuclear center iron being the triggering event for product ejection from PDO. Therefore, some other event must facilitate DHD release. It could be proton release/uptake events necessary for restoration of the protonation state of the enzyme after catalysis or conformational changes associated with the rereduction of the Rieske center.

A comparison of the activity of PDO-Fe^{II} and reconstituted PDO-APO^F determined from the steady-state reaction and from the reaction of reduced PDO with oxygen (*3*) reveals an apparent discrepancy. In steady-state reactions, fully reconstituted PDO-APO^F exhibited an apparent turnover number of only half of that of the unmodified enzyme (up to 2.9 s^{-1} as compared to 5.9 s^{-1} observed with PDR-Fe^{II}). However, the amount of product generated in the course of the single reoxidation reaction was about the same for both PDO-Fe^{II} and reconstituted PDO-APO^F. This apparent contradiction can be resolved, considering the differences in these experiments. Reduction of PDO by PDR as well as the rereduction of PDR by NADH[5] are processes at least as fast as intramolecular electron transfer in PDO, and therefore, it is unlikely that Rieske center rereduction by PDR is the rate-limiting step in the catalysis. With about half of the mononuclear centers in the multimer PDO-APO^F inactivated,[6] the activity, as measured by NADH oxidation, is only about $^1/_2$ of that of PDO-Fe^{II} and all of the flux of reducing equivalents is mediated by the remaining active mononuclear centers, which are fully coupled for generating the product. In reactions of oxygen with reduced PDO (the oxidative half-reaction), the electron supply is limited to electrons available from the reduced Rieske centers (mononuclear centers contribute no net electrons). Thus, one active mononuclear center per two Rieske centers is sufficient to generate the maximum amount of product that can be produced from the available electron supply.

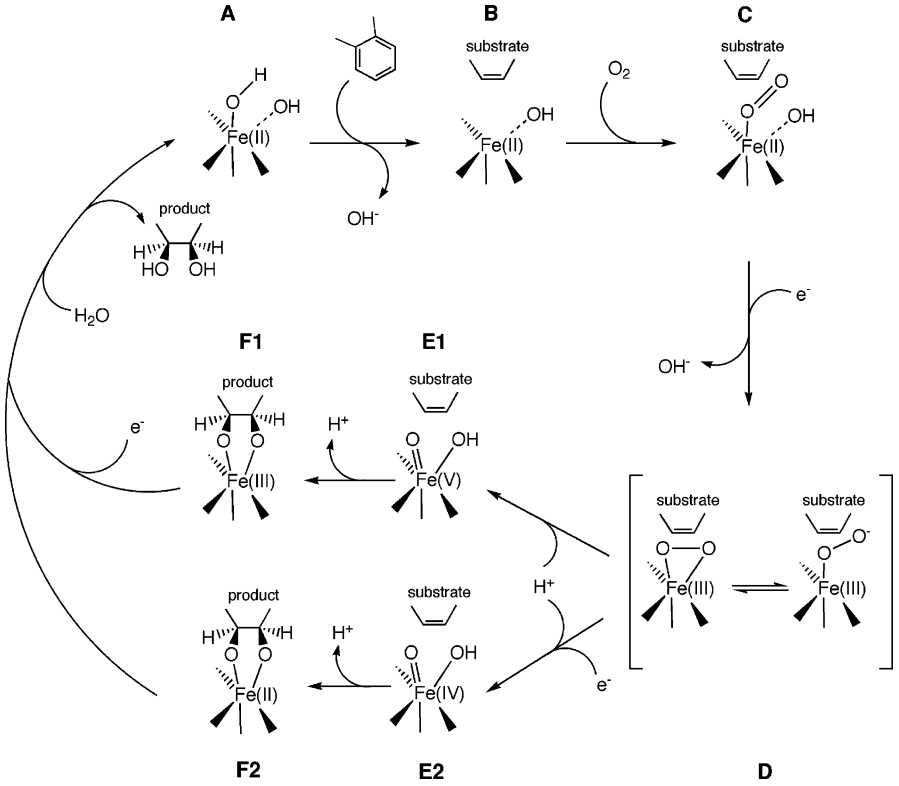
This correlates with the model for electron transfers in PDO presented earlier (*3*). The model (Figure 3) proposed a side-by-side arrangement of the monomers within the PDO tetramer with the subunits stacked in alternate directions (Rieske center of one subunit facing the mononuclear center of another). Electron transfer to the mononuclear center from the Rieske center of the same subunit [$\sim$35$-$45 Å away, based on the similarity with the NDO structure (*13*)] is unfavorable. However, in this model, each mononuclear center is adjacent to two Rieske centers of other subunits (see the explanation to Figure 3). Note that this arrangement is asymmetrical, because only one of the Rieske centers is likely to be linked to the mononuclear site via a bridging aspartate analogous to the arrangement proposed for NDO (*14*), anthranilate (*15*), toluene (*16*), and biphenyl (*17*) dioxygenases (see ref *3* for a full discussion). This model, which was introduced to account for the multiphase kinetics observed for the PDO reaction with oxygen, also predicts the observed stoichiometry of DHD production (0.5 DHD per Rieske oxidized) in the oxidative half-reaction. The model was further tested by simulating the product formation in the reaction of oxygen with PDO partially reconstituted with Fe^{II}. Amounts of DHD produced that are predicted by the simulation based on the model described above and shown in Figure 3 correlate with those experimentally observed. They are consistent with the enzyme being either an α_4 or α_8 multimer and appear to be inconsistent with PDO being an α_6 hexamer.

The overall pathway for catalysis is presented in Scheme 2. The cycle consists of substrate binding, oxygen activation, and substrate oxygenation. The initial change in coordination from a 6-coordinate Fe^{II} resting state (A) to a 5-coordinate substrate-bound state (B) was shown previously (*18*$-$*20*)**.** It remains to be determined whether this coordination change

[5] The slowest rate in reduction of PDR by NADH is the semiquinone formation by the intramolecular electron transfer from reduced FMN to the oxidized [2Fe$-$2S] center, which is gated by the release of NAD, as shown by Gassner and Ballou (*1*, *2*). Electron transfer from fully reduced PDR to the oxidized Rieske center of PDO occurs at about 200 s^{-1} and is apparently slow compared to the rate of intramolecular electron transfer from FMN to [2Fe$-$2S] in PDR during its oxidation (*1*).

[6] In the presence of the substrate in iron-reconstituted PDO-APO^F, only 30$-$45% (depending on the method of reconstitution) of the Rieske centers oxidize rapidly ($\sim$40 s^{-1}), whereas 70$-$75% oxidize rapidly in PDO-Fe^{II}. Remaining Rieske centers of the reconstituted PDO-APO^F are oxidized at much slower rates (0.1$-$1 s^{-1}) that are characteristic of the substrate-free enzyme (*3*). Apparently, these centers are still capable of accepting electrons from the Rieske centers as evidenced by the absence of the ultra slow (about 10^{-3} s^{-1}) phase typical for the unreconstituted PDO-APO^F. As mentioned previously, PDO-APO^F, even when fully reconstituted with iron, has a steady-state turnover number of 2.9 s^{-1}, as opposed to 5.9 s^{-1} observed for PDO-Fe^{II}. Inactivation of the mononuclear centers in PDO-APO^F that manifests itself in slow electron-transfer rates and lower steady-state turnover number probably reflects the inability of the mononuclear centers to take on the active conformation induced by the phthalate binding nearby.

Scheme 2: Possible Reaction Pathway of PDO and NDO[a]



[a] See the text for details.

on binding of substrates is a general property of Rieske oxygenases or a mechanism utilized only in a few particular cases. This coordination change has been demonstrated for several mononuclear oxygenases (*21*). It was shown by NMR relaxation studies that the loss of a ligand, most likely H_2O or OH^-, opens a site for oxygen to bind (*22*) and form the species C. Formation of the $Fe^{III}-$peroxide (species D) as an intermediate during substrate hydroxylation was proposed for several systems, including putidamonooxin (*23*, *24*), NDO (*8*, *25*), BZDO (*9*), and PDO (*26*). This intermediate can be envisioned as either an $\eta 1$ or $\eta 2$ complex as shown in species D. Recent X-ray crystallographic studies show oxygen bound to NDO in a side-on manner (*27*). The peroxo intermediate experimentally detected for a number of non-heme iron systems (i.e., refs *28* and *29*) apparently has a weakened O−O bond that is activated for cleavage (*30*). For the NDO system, it was proposed (*6*) that the Fe−peroxy complex that is formed (species D) either attacks the substrate directly or undergoes prior O−O bond scission. In catalysis, heterolytic cleavage of the O−O bond in species D could generate a strongly oxidizing high-valent species E1 or E2, similar to the proposed oxygenating compound-I-like species in cytochrome P450. Pathway 1 (E1 and F1) involving the formation of the formal Fe^V iron-oxo species was proposed for NDO (*8*) and BZDO (*9*). This pathway, which is based on similar chemistry to that for P450 enzymes, is especially attractive for these two enzymes, because it accounts for a ferric species at the end of the reaction, which is in agreement with the existing data on NDO and BZDO catalysis.

Another attraction of this pathway, as was suggested by Wolfe et al. (*8*), arises from the fact that NDO is similar to P450 enzymes in that it is able to catalyze a wide range of oxygenation reactions, probably because of the development of the strongly oxidizing species E1. However, the precise nature of the active intermediate in NDO is still a subject of debate. While there are numerous studies that indicate the involvement of the ferric-(hydro) peroxy species in catalysis (*25*, *31*−*36*), at this time, there is no experimental evidence supporting its ability to directly hydroxylate nonactivated aromatic substrates. Similarly, while the formal $Fe^V{=}O$ species is well-documented in the heme systems, where a more accurate description is $[Fe^{IV}{=}O + Por^{+\cdot}]$ (*37* and references within), there is no experimental evidence showing that a similar active intermediate can indeed be attained in a non-heme ligand environment. A recent theoretical study (*38*) indicates that the formation of an iron-oxo (Fe^V) species prior to substrate hydroxylation is energetically unfavored but that direct attack on the substrate by the $Fe^{III}-$OOH species, resulting in the formation of epoxide as an intermediate, seems to provide the lowest energy barrier for *cis*-dihydrodiol formation. The results of Chen et al. (*31*), while supporting the participation of the high-valent iron-oxo species in the non-heme iron catalysis, do not support the direct attack by $Fe^{III}-$OOH. Isotope-labeling studies showed that ^{18}O from $H_2{}^{18}O$ was incorporated into the alcohol products, thus suggesting that a species such as $Fe^V{=}O$ that could exchange with the solvent was involved in the catalytic pathway, at least for some types of catalysts. Hydroxylation solely by the $Fe^{III}-$OOH species was inferred for the other types of oxygenation, with the differentiation based on the spin state of iron in the $Fe^{III}-$OOH intermediate (*32*). Similarly, the study of olefin epoxidation and cis-dihydroxylation catalyzed by similar non-heme iron catalysts also support the importance of the iron spin state in the catalysis mechanism (*33*). Formation of low-spin $Fe^{III}-$OOH intermediates, whose O−O bonds are weakened by the $S = {}^1/_2$

iron center, would promote the catalysis through the higher valence iron state. On the other hand, catalysts that give rise to the high-spin $Fe^{III}-OOH$ intermediates generate *cis*-diol products via an $Fe^{III}-\eta^2-OOH$ species that, as pointed out by Chen et al. (31), may or may not isomerize into a *cis*-$OH-Fe^{V}=O$ species in the course of catalysis. This approach seems to correlate with the conclusions of Lehnert et al. (*39*) who also showed different reactivities of the model non-heme iron complexes that depended on the spin state of the $Fe^{III}-$alkylperoxo complex. In particular, the high-spin state has an energy barrier for O−O homolysis (weak Fe−O and strong O−O bonds). This barrier is absent in the low-spin complex (strong Fe−O and weak O−O bonds). The spin state of iron in the proposed but as yet not experimentally observed $Fe^{III}-$hydroperoxo intermediate in Rieske dioxygenases remains to be determined, but Chen et al. (*33*) argue that a formal $Fe^{V}=O$ species can form at the Rieske dioxygenase mononuclear site and may be involved in the cis-dihydroxylation.

In contrast to NDO, delivery of the second electron from the Rieske center is essential for the *cis*-diol formation in the PDO system. In this case, a high-valent iron-oxo (Fe^{IV}) species could be accessible via a low-energy barrier (*38*). This suggests the pathway via E2 and F2 that involves the formation of an Fe^{IV} intermediate after the second electron transfer to the mononuclear center. This pathway would lead to a final species that contains iron in the ferrous form, which fits the experimental data for PDO catalysis. A similar pathway involving the generation of the formal Fe^{IV} species in PDO was proposed much earlier by J. Groves and D. Ballou (*26*). As was pointed out (*8*), this pathway would lead to a species with lower oxidizing capacity, which may partially account for the tighter substrate selectivity observed in PDO.

Thus, it seems that the difference in mechanisms employed for product hydroxylation by NDO and BZDO, as compared to PDO, arises from the difference in timing of the electron transfers to the mononuclear center. In NDO and BZDO, the second electron transfer is delayed until the very end of catalysis, which leads to the putative development of a more oxidizing species (formally Fe^{V}), with the catalysis proceeding by pathway 1. In PDO, the second electron transfer might occur rapidly, allowing for the formation of the Fe^{IV} species as shown in pathway 2.

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