Rates of the Phthalate Dioxygenase Reaction with Oxygen Are Dramatically Increased by Interactions with Phthalate and Phthalate Oxygenase Reductase†

Michael Tarasev, Frank Rhames, and David P. Ballou*

Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0606

Received May 10, 2004; Revised Manuscript Received July 6, 2004

ABSTRACT: The phthalate dioxygenase system, which catalyzes the dihydroxylation of phthalate to form its *cis*-dihydrodiol (DHD), has two components: phthalate dioxygenase (PDO), a multimer with one Rieske-type [2Fe-2S] and one Fe(II) center per monomer, and phthalate dioxygenase reductase (PDR), which contains flavin mononucleotide (FMN) and a plant-like ferredoxin [2Fe-2S] center. PDR is responsible for transferring electrons from NADH to the Rieske center of PDO, and the Rieske center supplies electrons to the mononuclear center for the oxygenation of substrate. Reduced PDO (PDO_{red}) that lacks Fe(II) at the mononuclear metal site (PDO-APO) reacts slowly with O_2 (1.4×10^{-3} s^{-1} at 125 μM O_2 and 22 °C), presumably in a direct reaction with the Rieske center. Binding of phthalate and/or PDR_{ox} to reduced PDO-APO increases the reactivity of the Rieske center with O_2. When no PDR or phthalate is present, the oxidation of the Rieske center in native PDO_{red} [which contains Fe(II) at the mononuclear site] occurs in two phases (∼1 and 0.1 s^{-1} at 125 mM O_2, 23 °C), both much faster than in the absence of Fe(II), presumably because in this case O_2 reacts at the mononuclear Fe(II). Addition of PDR_{ox} to native PDO_{red} resulted in a large fraction of the Rieske center being oxidized at 5 s^{-1}, and the addition of phthalate resulted in about 70% of the reaction proceeding at 42 s^{-1}. With both PDR_{ox} and phthalate present, most of the PDO_{red} (approximately 80−85%) oxidizes at 42 s^{-1}, with the remaining oxidizing at ∼5 s^{-1}. Thus, the binding of phthalate or PDR_{ox} to PDO_{red} each results in greater reactivity of PDO with O_2. The presence of both the substrate and PDR was synergistic, making PDO fully catalytically active. A model that explains the observed effects is presented and discussed in terms of PDO subunit cooperativity. It is proposed that, during oxidation of reduced PDO, each of two Rieske centers on separate subunits transfers an electron to the Fe(II) mononuclear center on a third subunit. This explanation is consistent with the observed multiphasic kinetics of the oxidation of the Rieske center and is being further tested by product analysis experiments.

Aromatic compounds are among the most abundant anthropogenic pollutants. Many of them constitute major health hazards due to both their toxicity and their resistance to natural catabolic processes. Possibly, because many such xenobiotics have not been in the environment for very long, appropriate metabolic pathways for their biodegradation have not yet fully evolved, and existing ones are often incomplete or inefficient (*1*). As the catabolic potential of existing life forms seems to be inadequate to deal with many of the aromatic compounds that man has made, researchers turn to the development of genetically engineered species with improved biodegradative capacities (*2*, *3*). However, before optimal strategies to design tailored biocatalysts can be developed, it is necessary to comprehend in detail the structural and functional relationships and the metabolic mechanisms involved in degrading aromatic compounds. This work describes studies of an oxygenase that is involved in such transformations, the phthalate dioxygenase system (PDS)[1] from *Burkholderia cepacia* DB01.

PDS catalyzes the first step in the breakdown of the aromatic compound phthalate to form *cis*-4,5-dihydro-4,5-dihydroxyphthalate (DHD). This is converted by the next enzyme in the metabolic pathway to 4,5-dihydroxyphthalate and then to protocatechuate (3,4-dihydroxybenzoate), a common intermediate in aromatic metabolism. Phthalate dioxygenase (PDO) is part of a Rieske non-heme iron dioxygenase system that uses a type IA electron transfer system (*4*). PDO has been extensively characterized and is a model for many of the properties and the mechanisms of other *cis*-diol forming dioxygenases (*5*). This two-component system comprises monomeric phthalate dioxygenase reductase (PDR), an enzyme that contains both FMN and a

† Financial support was received from NIH Grant GM20877 (to D.P.B.).

* To whom correspondence should be addressed. E-mail: dballou@umich.edu. Phone: 734-764-9582. Fax: 734-763-4581.

[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; $PDO\text{-}APO^F$, apoprotein prepared according to the stringent procedure (1.9 Fe per monomer); $PDO\text{-}APO^P$, apoprotein prepared by a shorter procedure (2.2 Fe per monomer); PDR, phthalate dioxygenase reductase; DHD, *cis*-4,5-dihydrodiol phthalate; FAS, ferrous ammonium sulfate; NDO, naphthalene dioxygenase; NDF, naphthalene dioxygenase ferredoxin; NDS, naphthalene dioxygenase system; BZDO, benzoate dioxygenase; MMO, methane monooxygenase; AntDO, anthranilate dioxygenase; NMRD, nuclear magnetic relaxation dispersion; MCD, magnetic circular dichroism.

10.1021/bi0490587 CCC: $27.50
Published on Web 09/14/2004

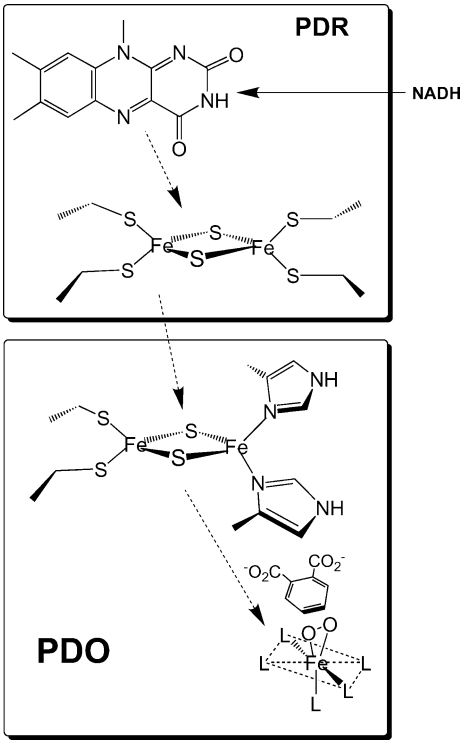

FIGURE 1: Phthalate dioxygenase system. This figure shows various redox cofactors and the general pathway for electron transfer. The ligands are represented by L. Because no X-ray structure of PDO is available, assignment of ligands and geometry are not specified. However, the X-ray structure of NDO shows that the mononuclear site can be described as a two-His one-carboxylate facial triad (*34*). In PDO likely ligands are the conserved residues, His181, His186, and Asp358 or Asp365. Other studies have indicated that one or two waters are also ligated in various states of PDO (*30*).

ferredoxin [2Fe-2S] center and which accepts electrons from NADH, and a multimeric dioxygenase (presumably an α_4 tetramer).[2] Each subunit of PDO contains a Rieske [2Fe-2S] center and a mononuclear Fe(II) site (Figure 1). The mononuclear Fe(II) is essential for catalytic activity (*6*, *7*) and is proposed to be the site of oxygen binding and activation and substrate hydroxylation. Figure 1 shows oxygen binding side-on, which is consistent with recent X-ray structural studies on the related enzyme naphthalene dioxygenase (NDO) (*8*). This geometry also would appear to be optimal for dioxygenation of the phthalate with both atoms of molecular oxygen being incorporated into the ring.

The reductive portion of the PDS-catalyzed reaction has been the subject of several studies (*9*−*12*). The first three articles focus on the mechanism of reducing PDR by NADH, and the last reference reports on the electron transfer from reduced PDR to oxidized PDO. At the time of those publications very little was known about how PDO brought about the activation of oxygen and the hydroxylation of phthalate. It has been shown previously that formation of the PDO−PDR complex has little effect on the reduction of PDR by NADH (*12*). However, when phthalate and a divalent metal such as Fe(II) or Co(II) are bound at the mononuclear site of PDO, the redox potential of the Rieske center is decreased by ~80 mV, and paradoxically, the rate of intermolecular electron transfer from fully reduced PDR to PDO_{ox} is increased 1000-fold (*12*). It was proposed that structural changes occur on binding metal and substrate at the mononuclear site that are important for electron transfer (*12*).

In the present study, we have investigated the effects of binding of iron, substrate, and reductase on the kinetics of the reaction of the reduced Rieske centers of PDO with oxygen.

MATERIALS AND METHODS

PDO and PDR were isolated from *B. cepacia* DB01 and purified as described previously (*5*). As isolated, PDO contained 2.8 ± 0.2 Fe per monomer. Preparation of PDO-APO^F, a modified enzyme that lacks the iron in the mononuclear center, was achieved by repeated cycles involving dilution with 0.1 M HEPES, pH 8, containing 5 mM EDTA, followed by concentration using a Centricon-30. Finally, the sample was dialyzed against the same buffer and then against EDTA-free buffer. Such samples typically contained 1.9 ± 0.2 Fe per monomer. A less stringent preparation (PDO-APO^P) involved passing PDO through a G-25 column equilibrated with 0.1 M HEPES, pH 8, and containing 5 mM EDTA, followed by incubating the solution for 1 h at 4 °C in 10 mM EDTA, and finally, removing EDTA by passing the sample through the G-25 column equilibrated with the same buffer without EDTA. The latter PDO-APO samples contained 2.2 ± 0.2 Fe per monomer.

PDO was reconstituted with iron by adding anaerobically a 100-fold excess of $Fe(NH_4)_2(SO_4)_2$ (FAS). 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 [PDO-Fe(II)] that contained 3.0 ± 0.1 Fe per monomer was immediately used in the experiments. In experiments where PDO-Fe(II) was used without phthalate, fully reconstituted samples of PDO-Fe(II) were passed through a G-25 column equilibrated with the phthalate-free buffer, further diluted 5−20-fold by the same buffer, and anaerobically supplemented with 20 μM FAS. Iron content in PDO was determined by the standard ferrozine assay (*13*) or by atomic absorption spectroscopy using a Perkin-Elmer 3300 atomic absorption spectrometer equipped with HGA-600 graphite furnace or, in some cases, by inductively coupled plasma high-resolution mass spectrometry (ICP) using a Finnigan element instrument from Thermo Finnigan Co.

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 differences between oxidized and reduced PDO monomers and PDR, respectively. PDO activity was determined in steady-state assays by monitoring the change in absorbance at 340 nm due to consumption of NADH. Reaction mixtures contained 0.2 μM PDR, 2 mM phthalate, and 100−250 μM NADH in 0.1 M HEPES, pH 8. The reaction was initiated by the addition of 0.2 μM PDO. When necessary, FAS (10−20 μM) was added. Note that the measured activity does not represent maximum PDO activity but rather the activity under the above conditions at 1:1 PDO:PDR stoichiometry. These conditions were chosen because they are practical and reproducible.

Samples of reduced PDO (20−40 μM before mixing) for reaction with O_2 were vacuum/gas exchanged (Ar) in

[2] Recent preliminary ultracentrifugation data (J. Bolin, personal communication) indicate the possibility that PDO might be in equilibrium between hexamer and tetramer forms.

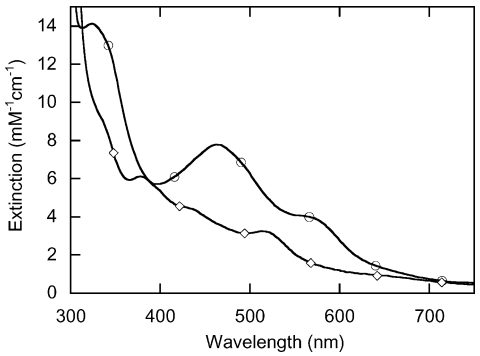

FIGURE 2: Visible spectra of oxidized (○) and reduced (◇) phthalate dioxygenase. Conditions were PDO (115 μM) in 0.1 M HEPES and 2 mM phthalate, pH 8.

tonometers (about 10 times) and overlaid with purified argon. Enzyme reduction was achieved by anaerobic photoreduction (*14*) in the presence of 0.03−1 μM 5-deazariboflavin (gift from Dr. V. Massey) and 1−5 mM glycine or by anaerobic titration with sodium dithionite. The reactions with oxygen were the same with either method of reduction. In the experiments with the PDO-APO^F that was not reconstituted, 0.5−1 mM EDTA was added to the solution to safeguard against the contamination of the sample with adventitious iron. Kinetic data were acquired using a Kinetic Instruments, Inc., stopped-flow spectrophotometer in single-wavelength mode. Reactions were observed at 575 and at 465 nm, wavelengths that correspond to the maxima in the Rieske center visible absorption spectrum of PDO_{ox} (Figure 2). Data collection and analysis were performed using program A, which employs the Marquardt−Levenberg algorithm (*15*) and was developed in our laboratory by C.-J. Chiu, R. Chang, J. Dinverno, and Dr. D. P. Ballou, University of Michigan. Rates and amplitudes obtained from curve fitting (mean values from at least five independent experiments) were within 15% of each other unless specified otherwise. Experiments were performed in 0.1 M HEPES or in 0.1 M potassium phosphate buffer, pH 8.0, at 22 °C. The oxidation state of the enzyme during titrations was monitored using a Shimadzu UV 2051PC spectrophotometer. EPR studies were performed using a Brucker EMX EPR spectrometer equipped with a Brucker 4102-ST general purpose cavity. Data were collected at 15 K with a modulation amplitude of 10 G, microwave frequency of 9.426 GHz, and power of 10 mW.

Standard iron solutions for atomic absorption spectroscopy were from Aldrich Chemical Co. All other chemicals were of analytical grade and were used without further purification.

RESULTS

PDO Steady-State Catalytic Activity. Native PDO (~2.8 Fe per monomer) activity was 5.5 ± 0.3 s^{-1} as measured by steady-state assays (Materials and Methods). Reconstitution with Fe(II) [referred to as PDO-Fe(II); ~3 Fe per monomer] resulted in only a slight increase in the measured activity (5.9 ± 0.1 s^{-1}). By contrast, the turnover rate under the same conditions for PDO-APO^F (~1.9 Fe per monomer) was 0.05 ± 0.02 s^{-1}, less than 1% that of fully reconstituted PDO-Fe(II). This small residual activity was assumed to be due to traces of PDO-Fe(II) in which the mononuclear iron had not been removed. We did not check to see if this residual NADH consumption led to *cis*-dihydrodiol formation. Reconstitution of PDO-APO^F with Fe(II) increased the activity to a maximum of only 2.9 s^{-1} [similar to BZDO (*16*); no increase in PDO activity was observed when Fe(III) was used, presumably because of the poor solubility of ferric iron]. Typically, in assays involving reconstitution, a 50-fold excess of FAS relative to PDO was in the reaction mixture, and no further increase of activity was observed when up to a 500-fold excess of FAS was added. In the presence of phthalate and a stoichiometric amount of added Fe(II), the PDO-APO^F was found to contain 2.9 ± 0.2 Fe per monomer; i.e., it was fully populated. This species behaved essentially the same as when a 50-fold excess of FAS was used. Regardless of the method of iron reconstitution, it was not possible to reconstitute PDO-APO^F to the full activity of the original native enzyme. Thus, despite fully populating the mononuclear center, the enzyme regained only about 50% of its original oxygenase activity as measured by steady-state assays, suggesting that the rigorous procedure necessary to fully deplete PDO of the mononuclear center iron resulted in some damage to the enzyme. Similarly, the reconstitution of apo-BZDO (1.9 Fe per $\alpha\beta$)[3] with Fe(II) resulted in enzyme with ~2.5 total Fe per $\alpha\beta$ (*16*). Although this reconstituted BZDO [as well as the apo-BDZO with Fe(II) present in the assay] had catalytic properties nearly identical to those of BZDO as isolated, seemingly indicating full restoration of the enzymatic activity, it should be noted that BZDO as isolated contained only ~2.7 Fe per $\alpha\beta$. Thus, it is impossible to tell whether Fe(II) reconstitution of the apo-BZDO actually restored full catalytic activity or, similar to PDO-APO^F, resulted in the enzyme with some mononuclear centers that, although repopulated with iron, remained catalytically inactive.

Consistent with the iron content (about 2.2 Fe per monomer), PDO-APO^P steady-state turnover activity was 1.5 ± 0.2 s^{-1}, which was ~25% of its original activity (compared to <1% exhibited by the PDO-APO^F). Reconstitution of PDO-APO^P resulted in full restoration of the activity (5.8 ± 0.2 s^{-1}). Apparently, in contrast to the more stringent procedure for preparing PDO-APO^F, this procedure did not lead to significant deterioration of the enzyme and could be useful for incorporating ^{57}Fe for future Mössbauer spectroscopic studies.

Kinetics of the Reaction of Reduced Forms of PDO-APO with O_2. The absorption spectrum of PDO in the visible region is dominated by the absorption of the Rieske center (Figure 2, circles), with only small perturbations (≤1%) deriving from the mononuclear center iron or from the binding of substrate. When reduced, the enzyme is significantly bleached in the visible region (*5*) (Figure 2, diamonds). The peaks at 465 and 575 nm of oxidized PDO were used to monitor the oxidation state of the Rieske center of PDO. With native PDO, the reactions of oxygen with reduced PDO are expected to occur at the mononuclear iron center where oxygenation of the substrate takes place. Therefore, the Rieske centers primarily become oxidized by transferring electrons to the mononuclear iron. By contrast, reduced PDO-APO^F reacts with O_2 very slowly, presumably in a direct reaction between the Rieske center and O_2. The measured oxidation rates for PDO-APO^F are denoted by k_1 in Table 1 for a series of oxygen concentrations, and a typical reaction

[3] BZDO consists of three ($\alpha\beta$) dimers each containing one Rieske and one Fe mononuclear center.

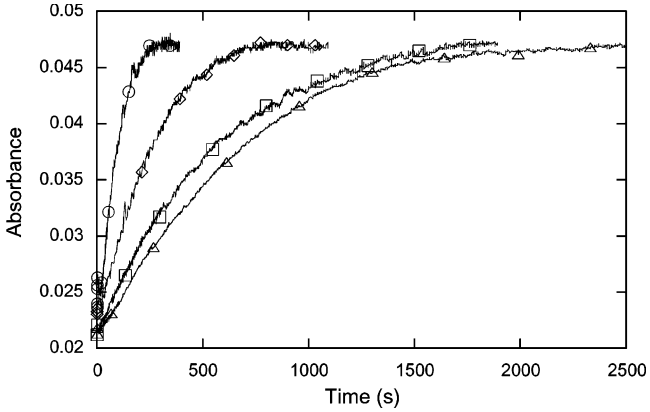

FIGURE 3: Oxidation of reduced PDO-APO^F by oxygen. PDO-APO^F (10 μM) was mixed with 125 μM O_2 in 0.1 M HEPES, pH 8.0, with and without phthalate and/or PDR_{ox} at 22 °C. Key: (△) PDO-APO^F; (□) PDO-APO^F + phthalate (1 mM); (◇) PDO-APO^F + PDR_{ox} (10 μM); (○) PDO-APO^F + phthalate (1 mM) + PDR_{ox} (10 μM). All concentrations are those after mixing in the stopped-flow spectrophotometer. Data were recorded at 575 nm.

Table 1: Apparent Rate Constants for the Oxidation of the Reduced Rieske Center of PDO[a]

	PDO-APO^F	PDO-Fe(II)	
oxygen, μM	k_1, s^{-1}	k_2, s^{-1}	k_3, s^{-1}
62	7.5×10^{-4}	0.09	0.81
125	1.4×10^{-3}	0.08	1.1
312	1.8×10^{-3}	0.18	2.3
625	2.6×10^{-3}	0.27	2.7

[a] Conditions are as described in Materials and Methods and in Figure 2. No phthalate or PDR_{ox} was present in these experiments.

curve is shown in Figure 3 for 1.25×10^{-4} M oxygen (triangles). The half-time for reoxidation of PDO-APO^F is 495 s when using 1.25×10^{-4} M oxygen. Although reduced iron−sulfur centers will react with oxygen, we cannot rule out the possibility that the rates we observed with PDO-APO^F were actually due to traces of iron bound at the mononuclear site. If this is true, it would imply that significant subunit-to-subunit and multimer-to-multimer interactions occur to permit delivery of electrons to the rare active mononuclear centers. Much higher electron transfer rates (see below) were observed in the presence of added Fe(II). If adventitious iron is involved, the observed rates should be considered as upper limits for the oxidation of the Rieske center. The addition of either phthalate or oxidized reductase to PDO-APO^F resulted in slightly increased oxidation rates, with oxidation also proceeding in single phases (Table 2 and Figure 3, squares and diamonds). In the presence of both reductase and the substrate, the oxidation occurred in a single exponential process that was nearly 10-fold faster than that of PDO-APO^F alone (Figure 3, circles). Again, this was probably not due to adventitious iron from the reductase because added iron causes much greater increases in the rate of oxidation (see below). These observations suggest that the binding of phthalate and/or reductase cause structural changes at the Rieske center that promote its reaction with O_2.

Kinetics of the Reaction of Oxygen with Reduced Forms of PDO That Contain Fe(II) at the Mononuclear Site. The reaction of O_2 with reduced native PDO-Fe(II) (activity = 5.5−5.9 s^{-1}), with no PDR or phthalate present, occurs in two phases, which are both faster than those for any of the PDO-APO^F species (k_2 and k_3 in Tables 1 and 2 and Figure 3, circles). At all oxygen concentrations used, the faster phase of the oxidation contributed about 40% to the total absorption change.[4] Less active preparations of PDO (steady-state activity of reconstituted enzyme ∼4.5−5 vs 5.9 s^{-1} for the fully active PDO) exhibited smaller fractions in the fast phase (20−30%). This is consistent with the faster phase being due to enzyme with fully competent mononuclear sites, with the remainder oxidizing via electron transfer to nearby centers. The rates of both phases of this reaction were dependent on oxygen concentration and reached limiting values with k_{max} of 3.9 ± 0.5 s^{-1} and 0.51 ± 0.25 s^{-1} and apparent K_D values for oxygen of 260 ± 100 and 620 ± 550 μM, respectively. Figure 4 shows traces of reactions of oxygen with reduced forms of both reconstituted and apoPDO^F and with additions of PDR_{ox} and/or phthalate and highlights the differences in these reactions. We assume that the reactions with oxygen in the absence of phthalate lead to superoxide, but no evaluation of this possibility was made.

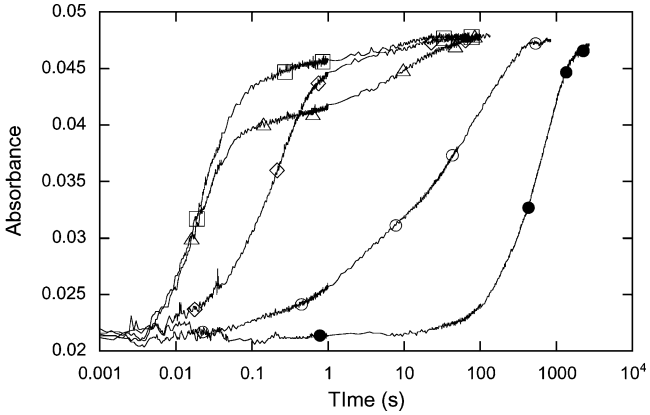

FIGURE 4: Oxidation of reduced forms of PDO by oxygen. Reduced forms of PDO (10 μM) were mixed with 125 μM O_2 in 0.1 M HEPES, pH 8.0, with and without phthalate and/or PDR_{ox} at 22 °C. Key: (●) PDO-APO^F; (○) PDO-Fe(II); (◇) PDO Fe(II) + PDR_{ox} (10 μM); (△) PDO Fe(II) + phthalate (1 mM); (□) PDO Fe(II) + phthalate (1 mM) + PDR_{ox} (10 μM). The time axis is displayed logarithmically to show the widely different rates. Data were recorded at 575 nm.

A small (∼10%) contribution of a much slower phase (∼4×10^{-3} s^{-1}) was observed for some preparations [Table 2 under PDO-Fe(II)], and this is probably due to incomplete reconstitution of the mononuclear center of PDO in the absence of substrate. Consistent with this notion, less active preparations of PDO exhibited larger contributions from this slow phase. Oxidation of PDO (as isolated) was similar to that of PDO-Fe(II) but had a slightly smaller contribution from the fast phase and larger amplitudes in the slow phases (up to 40−45% contribution from the slow phase k_1 typical of the PDO-APO^F). Thus, the slowest phases are likely due to defective or incompletely reconstituted mononuclear centers.

Addition of phthalate caused about 70% of the Rieske centers to oxidize at ∼40 s^{-1} (k_5 in Table 2 and Figure 4, triangles). The remaining centers oxidized in two phases that

[4] The relative magnitudes of the kinetic phases were determined by fitting the data to a parallel reaction model assuming that the electron transfers from the Rieske centers are independent of each other. However, it is possible that the faster oxidation step must precede the slower one. If so, a consecutive reaction model would be more appropriate. This would lead to different contributions from the specific kinetic phases of the reaction without affecting the rates of electron transfer themselves.

Table 2: Observed Rate Constants (k) and Magnitudes of the Phases (A) for the Oxidation of Reduced Rieske Centers of PDO in Reactions with Oxygen[a]

	Fe per Rieske center	k_1, s^{-1} (A_1, %)	k_2, s^{-1} (A_2, %)	k_3, s^{-1} (A_3, %)	k_4, s^{-1} (A_4, %)	k_5, s^{-1} (A_5, %)
PDO-APO^F	1.9 ± 0.2	1.4×10^{-3} (100)				
PDO-APO^F + phthalate	1.9 ± 0.2	1.8×10^{-3} (100)				
PDO-APO^F + PDR_{ox}	1.9 ± 0.2	3.9×10^{-3} (100)				
PDO-APO^F + phthalate + PDR_{ox}	1.9 ± 0.2	1.25×10^{-2} (100)				
PDO-APO^F + Fe(II)	2.9 ± 0.2		0.06 (15)	0.8 (85)		
{PDO-APO^F + phthalate} + Fe(II)	2.9 ± 0.2	3×10^{-3} (20)	0.1 (20)	1.2 (15)		48 (45)
{PDO-APO^F + Fe(II)} + phthalate	2.9 ± 0.2		0.3 (70)			35 (30)
PDO-APO^P	2.2 ± 0.2	3.7×10^{-3} (50)	0.08 (30)	0.9 (20)		
PDO-Fe(II)	3.0 ± 0.1	$(5 \times 10^{-3})^b$	0.08 (60)	1.1 (40)		
PDO-Fe(II) + phthalate	3.0 ± 0.1		0.1 (10)	1.8 (20)		41 (70)
PDO-Fe(II) + PDR_{ox}	3.0 ± 0.1		0.08 (15)	1.1 (45)	4.7 (40)	
PDO-Fe(II) + phthalate + PDR_{ox}	3.0 ± 0.1		0.14 (10)		5.5 (15)	40 (75)

[a] Reactions were carried out in the stopped-flow instrument as described in Materials and Methods. Numbers in table are the average of at least five measurements. The oxygen concentration was 125 μM after mixing. Absolute magnitudes of the phases are reproducible to within 5%. Conditions were as in Figures 2 and 3. See text for details. [b] Phase present in some PDO-Fe(II) samples. Its appearance will reduce the contribution from the k_3 fast oxidation phase.

were similar to those observed without the substrate present. When reduced PDO-Fe(II) in the presence of oxidized PDR, but in the absence of phthalate, was reacted with oxygen, ~40% of the Rieske centers oxidized at 5 s^{-1} (k_4 in Table 2 and Figure 4, diamonds). The remaining centers oxidized in two slower phases (k_2 and k_3) similar to the slow phases observed without the reductase present. Addition of both phthalate and oxidized PDR resulted in most of the reaction (~90%) occurring in the two fastest phases (k_4 and k_5) with only about 10% occurring at 0.14 s^{-1} (Figure 4, squares, and Table 2). The relative contributions of the fast phases vary slightly between different PDO preparations with up to 85% of the Rieske centers oxidizing at ~40 s^{-1} in some samples.

When fully reconstituted with iron, PDO-APO^F in the presence of phthalate reacted with oxygen in a manner similar to that of PDO-Fe(II), but with a smaller contribution (30−50% vs 70%) from the fast phase (k_5) (Table 2). This is consistent with PDO-APO^F having a large fraction of defective mononuclear centers.

In addition to the effects described above, the distribution of the kinetic phases in reconstituted PDO-APO^F also depended on the method of reconstitution of the mononuclear center. When PDO-APO^F containing Fe(II) was mixed in the stopped-flow instrument with the phthalate-containing aerobic buffer [Table 2, row {PDO-APO^F + Fe(II)} + phthalate], in addition to the slow oxidation phase typical for the enzyme with the inactive mononuclear site, two faster phases were observed. However, when Fe(II) was anaerobically added to PDO-APO^F that had been preincubated with phthalate [row {PDO-APO^F + phthalate} + Fe(II) in Table 2] and then was mixed with aerobic buffer in the stopped-flow instrument, three faster phases were observed. Thus, PDO-APO^F that had been preincubated with phthalate can be reconstituted to form a more active enzyme than when the order of addition is reversed, probably due to the documented cooperativity between the binding of phthalate and Fe(II) (*12*). In general, comparisons of kinetics between PDO-Fe(II) and reconstituted PDO-APO^F are somewhat complicated because the latter could not be completely restored to full activity. The fraction of the more gently prepared iron-depleted enzyme (PDO-APO^P) that retains some mononuclear iron (20−25%; see above) exhibited both of the oxidation rates observed in nonreconstituted as well as in reconstituted PDO. Therefore, while a significant fraction of the Rieske centers in PDO-APO^P (about 50%) oxidized at the slow rate characteristic of PDO-APO^F, the remaining 50% reacted with oxygen similarly to the Rieske center in PDO-Fe(II) (Table 2). Contrary to PDO-APO^F, reconstitution of the PDO-APO^P enzyme with ferrous iron resulted in full restoration of the enzymatic activity.

Reaction of NO with the Mononuclear Fe(II) as a Model for Oxygen Binding. NO mimics many of the binding properties of O_2 to iron centers and thus can often be used as a model for some of the properties of how oxygen interacts with the iron-containing centers (*17*). The advantage of using NO as a probe for oxygen binding is that the formation of the nitrosyl complex NO−Fe(II) at the mononuclear site gives rise to a distinct $S = {}^3/_2$ EPR spectrum (*18*, *19*). Studies of NO binding to the mononuclear iron centers in NDO (*20*) and BZDO (*16*) show that this binding is dependent on the state of the enzyme. When the Rieske center is reduced, NO binds to the mononuclear iron only when substrate is present. Very similar results are also seen with PDO. By contrast to the reduced form of PDO, when the Rieske center of PDO is oxidized, NO binds to the mononuclear Fe(II) both in the presence and in the absence of the substrate to give rise to EPR signals with g values of 4.2 and 3.9 that can be attributed to an $S = {}^3/_2$ system (Figure 5). The small $g >$ 4.5 signal was present in all of the samples and can be attributed to a small population (~5%) of adventitious ferric species bound to the enzyme. This iron does not appear to interact with NO. As reported with NDO and BZDO (*16*, *20*, *21*), when the Rieske center in PDO is reduced, NO binding, as evidenced by the changes in the EPR spectra (Figure 6), occurs only in the presence of the substrate (phthalate). Thus, the oxidation state of the Rieske center, as well as the binding of substrate to the mononuclear iron, regulates NO binding to the mononuclear site iron. Similar patterns of regulation with oxygen would be expected to greatly reduce the formation of active oxygen species when substrate is not present by ensuring that O_2 would not be bound when the Rieske center is reduced unless the substrate is already bound where it can be hydroxylated.

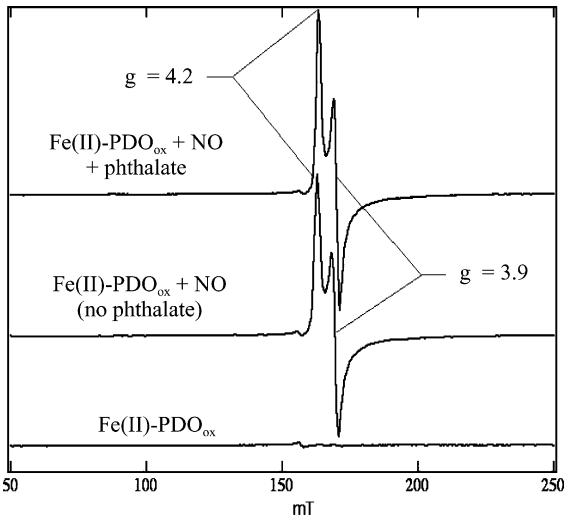

FIGURE 5: EPR spectra of PDO-Fe(II) (100 μM) incubated with NO in the presence and absence of 5 mM phthalate. The Rieske center is oxidized. Conditions were as described in Materials and Methods.

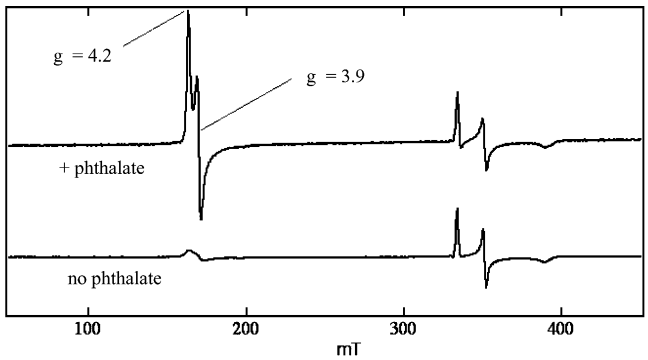

FIGURE 6: EPR spectra of reduced PDO-Fe(II) (100 μM) incubated with NO in the presence and absence of 5 mM phthalate. Conditions were as described in Materials and Methods.

DISCUSSION

The current studies provide further evidence that the reaction of oxygen with native PDO occurs at the mononuclear Fe(II) site. In addition, these studies show that electron transfer from the Rieske [2Fe-2S] centers to the mononuclear iron centers can be quite fast and, as discussed below, cooperative between the PDO monomers. Importantly, electron transfer is also significantly affected by the presence of PDR and phthalate.

PDO-APO Reactions with Oxygen. The observed very slow single exponential kinetics of the reaction of O_2 with reduced PDO-APO^F are consistent with the reaction occurring directly at the Rieske [2Fe-2S] center. The presence of PDR_{ox} and/or phthalate seems to induce changes in the Rieske center that make it more reactive with oxygen. However, nuclear magnetic relaxation dispersion studies have shown that phthalate binds at the mononuclear site (*22*), and EPR studies (*23*) imply that it binds at least 12 Å from the nearest Rieske center. Indeed, the crystal structure of the related enzyme, NDO, shows that its Rieske centers are ~45 Å from the mononuclear Fe(II) sites of the same subunit, although only ~12 Å from the mononuclear Fe(II) in the adjacent subunit (*24*). It is therefore surprising that the binding of phthalate so far away affects the reaction of the reduced Rieske [2Fe-2S] center with oxygen. However, also consistent with the fact that binding of components distant to the Rieske center can affect its properties are results showing that binding of phthalate to either PDO-APO or native PDO decreases the redox potential of the quite distant Rieske center (*12*). Binding of PDR_{ox} to PDO-APO^F has a slightly larger effect on the oxidation kinetics of the Rieske center than does phthalate (Figure 2), and binding of both phthalate and PDR_{ox} resulted in a further increase of the rate of oxidation of reduced PDO-APO^F, with the reaction still proceeding in a single phase. Redox potentials alone are not always sufficient to predict the rate of long-range electron transfers, because such transfers are often gated by other events (e.g., proton uptake, protein matrix conductivity changes between donor and acceptor sites, or conformational changes) (*20*, *21*, *25*−*28*). The present data further suggest that changes in the driving force induced by the binding events may be less important than the corresponding structural changes for facilitation of electron transfer.

Reconstituted Reduced PDO Reactions with Oxygen. Incorporation of Fe(II) into the mononuclear centers results in significantly (at least 100-fold) increased rates of oxidation of the reduced Rieske centers in PDO. Presumably, when reconstituted with Fe(II), electron transfer between the Rieske and mononuclear centers permits the Rieske centers to be oxidized primarily via reactions of oxygen with the mononuclear Fe(II). The observed kinetics are largely biphasic with small contributions of a third, ultraslow phase (k_1) that reflects incomplete reconstitution of the mononuclear centers of PDO-Fe(II) in the absence of the substrate. Multiphase oxidation of the Rieske centers in the absence of the substrate was also observed previously with NDOS and AntDO (*20*, *21*). In both of these cases the rates of the phases were about 2 orders of magnitude smaller than the corresponding turnover numbers for these enzymes. This is consistent with the slow rates representing direct oxidation of the Rieske centers. In the absence of the substrate the conformational state of PDO is apparently not optimized for turnover; however, even in this state the rate of the Rieske center oxidation ($k_3 = 1.1\ s^{-1}$) is not drastically less than the rate for turnover observed under our conditions ($5.5-6\ s^{-1}$). The kinetics of NDO reoxidation might be expected to be simpler than those for PDO because the oxidation of one Rieske center is sufficient for converting naphthalene into its dihydrodiol product. The second electron needed for product formation is provided through the oxidation of the mononuclear Fe center to the Fe(III) state (*20*). In contrast, the mononuclear iron in PDO has been found to be in the Fe(II) form at the completion of product formation, so that the mononuclear iron does not supply any net electrons for forming product. (These differences in mechanism between the NDO and PDO systems will be elaborated in detail in a future publication.) Thus, the second electron for product formation in PDO must derive from a second Rieske center. The more complex kinetics of the reaction of reduced PDO-Fe(II) with O_2, even in the absence of the substrate or reductase, probably reflects the required cooperation between Rieske centers located on different subunits of the enzyme. Similar assumptions were made for AntDO (*21*).

Effects of Phthalate on Oxidation Kinetics. Addition of phthalate to the reaction mixture induces dramatic increases in the oxidation rates of the Rieske centers. Thus, it appears that substrate binding near to the Fe(II)-containing mono-

nuclear center can provide a regulatory mechanism for the Rieske center oxidation. In the absence of phthalate, reactions of reduced PDO with oxygen are greatly attenuated so that oxidation to produce H_2O_2 and other reactive oxygen species is minimized. This binding of phthalate apparently makes electron transfer pathways to the mononuclear iron−oxygen complex more efficient from both PDO subunits involved. This is indicated by the magnitude of the fast phase (k_5) of electron transfer and the decreased contributions from both slower phases (k_2 and k_3). The reaction of oxygen with reduced NDO, AntDO, or BZDO is similarly stimulated by the presence of their substrates (*16*, *20*, *21*). In all cases, the addition of the appropriate substrate resulted in the development of a fast phase (or multiple fast phases in AntDO) that was significantly (10−500-fold) faster than the overall turnover under the same conditions. Moreover, the rates of electron transfer from the Rieske center in BZDO depend on the nature of the substrate (*16*), once more underlining the importance of the substrate-induced modification of the enzyme structure for Rieske center oxidation. The phthalate-dependent fast oxidation phase in PDO (k_5) is also nearly 10-fold faster than the turnover number under these conditions (42 vs 5−6 s^{-1}) and is therefore competent to be a required step in catalysis. This fast phase of the Rieske center oxidation in PDO is somewhat slower than the rates of oxidation of the Rieske center observed in NDO (at least 350 s^{-1} in the presence of naphthalene) (*20*) and in BZDO (260 s^{-1} in the presence of benzoate) (*16*). Similar to PDO, only a fraction of the reduced Rieske centers in these proteins were oxidized fast (57% for NDO and 36% for BZDO vs ∼70% on average for PDO).

As shown by MCD (*29*), NMRD (*30*), and X-ray absorption spectroscopy (*7*) studies, phthalate binding near the mononuclear center displaces a weakly coordinated water molecule to effect a change from six- to five-coordinate iron. Thus, the apparent increase in the rates of Rieske center oxidation on phthalate binding may represent the preparation of the iron for oxygen binding, and this constitutes a substrate-induced activation of the mononuclear center for catalysis. One of the manifestations of this activation would be to enable the binding of NO, as described above, and thus most probably O_2, to the Fe(II) of the mononuclear center (with the Rieske center of PDO in the reduced state). The mechanism of how this occurs remains to be solved.

Effect of PDR_{ox} on Rieske Center Oxidation. The other fast phase in the oxidation of the Rieske center (∼4.7 s^{-1}) observed in PDO in the presence of oxidized PDR is also fast enough to be relevant for catalysis. Kinetic studies also showed that the extent of the oxidation of the Rieske cluster in NDO could be increased by inclusion of the oxidized ferredoxin component (NDF) (*20*). Moreover, a greater yield of product was realized, even though no additional electrons had been contributed to the system. NDF apparently facilitates electron transfer between the Rieske centers, allowing for oxidation of the Rieske centers in inactive NDO subunit pairs, i.e., in subunits that lack mononuclear iron. In NDO, the results can be explained simply by NDF acting as an electron shuttling agent. A similar redistribution of electrons between subunits was postulated for the AntDO hexamer (*21*). However, binding of oxidized PDR to reduced PDO under anaerobic conditions did not result in any oxidation of Rieske centers and the consequent reduction of PDR. Moreover, binding of PDR_{ox} to PDO-Fe(II) not merely effected electron redistribution between the enzyme subunits (although this cannot be excluded) but also brought about a large increase in the rate of electron transfer between the Rieske and mononuclear centers. Can the effect of oxidized PDR on PDO be the result of extremely fast electron shuttling between the Rieske centers similar to that proposed for the NDS? In the absence of reductase and phthalate ∼60% of the oxidation of the Rieske centers in PDO occurs at 0.08 s^{-1}, while the remainder occurs at ∼1.1 s^{-1} (Table 2). Therefore, if the only function of the reductase were to rapidly shuttle electrons from "inactive" subunits to Rieske centers of active subunits, we would expect that most of the oxidation of the Rieske centers would occur at ∼1 s^{-1}. Instead, the presence of oxidized reductase leads to the development of a new faster phase (∼5 s^{-1}) comprising ∼40% of the total change (Table 2), and the loss of most of the slow (∼0.08 s^{-1}) phase. Thus, oxidized reductase, although not directly involved in transferring electrons in our experiments, nevertheless induces changes in PDO that result in a significant acceleration of the oxidation of the Rieske centers. The binding of PDR (oxidized) to PDO clearly enhances the electron transfer rate from the Rieske centers to the mononuclear center and may also stimulate the reactivity of the mononuclear iron with oxygen. In contrast to the studies presented here, it can be noted that during turnover it is likely that reduced rather than oxidized PDR is the species that influences the reaction of oxygen with PDO and that can modify the PDO−PDR interaction. The effects of reduced PDR could be different from those of oxidized PDR. The effect of PDR on the oxidation of PDO may be similar to that observed with component B in the methane monooxygenase system (*28*, *31*). Although component B of the methane monooxygenase system, like PDR_{ox}, does not carry electrons, binding of component B to the hydroxylase component causes changes that accelerate and potentially regulate the catalysis. In particular, the regulatory effect manifests in component B accelerating intermolecular electron transfer from MMO reductase to MMO hydroxylase.

Combined Effect of PDR_{ox} and Phthalate on Rieske Center Oxidation. Binding of both phthalate and oxidized PDR to the reduced PDO resulted in fast oxidation of most of the Rieske centers (75% on average and as much as 85% in some preparations). Thus, the presence of both the substrate and the oxidized PDR appears to induce structural changes in PDO that are synergistic in making the enzyme fully catalytically active.

A Model for Biphasic Kinetics in the Oxidation of the Rieske Centers in PDO. The observed biphasic kinetics of the reaction of O_2 with reduced PDO-Fe(II) indicates that either there are two subpopulations of PDO, each with different rates of reaction with oxygen, or there are two electron transfer pathways available between the Rieske and the mononuclear centers. The reaction of oxygen with reduced PDO that has mononuclear centers that are only partially repopulated with Fe(II) (for example, nonreconstituted PDO-APO^P) exhibits phases characteristic of both PDO-APO^F and PDO-Fe(II). Rieske centers that lack efficient access to mononuclear iron undergo slow oxidation typical of the PDO-APO^F, whereas Rieske centers that utilize normal electron transfer pathways to the mononuclear sites can

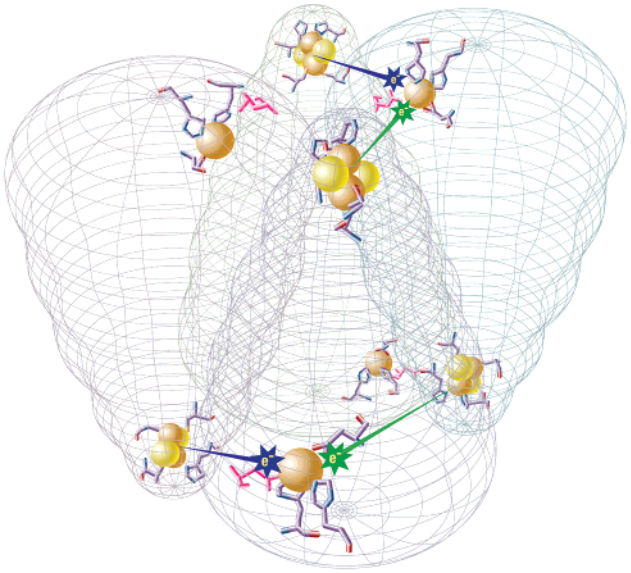

FIGURE 7: Model of electron transfers in the PDO multimer. Blue arrows indicate the faster electron transfer via the bridging Asp178 (red residue), and green arrows indicate the slower electron transfer path. See text for details.

oxidize similarly to those observed in PDO-Fe(II). This allows for differentiating between whether the biphasic oxidation kinetics in PDO-Fe(II) are caused by enzyme inhomogeneity or by the existence of different electron transfer pathways.

In the PDO-APO^{P} multimer, 75−80% of all PDO monomers lack iron at the mononuclear sites. If there are two subpopulations of the enzyme with different electron transfer rates, in the absence of substrate or PDR, 75−80% of the Rieske centers would be oxidized directly by molecular oxygen slowly at about 4×10^{-3} s^{-1}, and the remaining 20−25% would be oxidized more rapidly through the mononuclear center at ~1 and ~0.1 s^{-1}, as seen with the reconstituted enzyme. On the other hand, if there are two possible electron transfer pathways between the Rieske and mononuclear centers, we would expect a much higher contribution of the faster phases because those Rieske centers with inactive mononuclear sites could still reduce adjacent mononuclear centers (25% reconstitution will then result in 50/50 distribution of slow PDO-APO^{F}-type oxidation and faster PDO-Fe(II)-type oxidation). Results of the experiments with the PDO-APO^{P} (Table 2) are consistent with the model postulating the existence of two different electron transfer pathways (Figure 7).

The NDO structure (*32*) indicates that the Rieske center of one subunit delivers electrons to the Fe(II) site of the adjacent subunit via a bridging aspartate (Asp205). This role for Asp205 was corroborated by studies involving the Asp205 mutant forms of NDO (*24*). Moreover, the equivalent mutant forms of anthranilate dioxygenase (Asp218Ala) (*21*) and toluene dioxygenase (Asp219Ala) (*33*), which are also Rieske dioxygenases, have very little catalytic activity. The precise reasons for the lack of activity of the mutant enzymes are not known; however, it was proposed that electron transfer from the Rieske center to the mononuclear center on the adjacent subunit is blocked (*24*). In contrast to NDO, the reduced Rieske center of the analogous Asp218Ala mutant form of the AntDO was reoxidized rapidly by oxygen (presumably via a reaction at the mononuclear site). This implies that electron transfer from the Rieske center to the mononuclear center was also rapid in this mutant form (*21*). It was therefore postulated that Asp218 in AntDO is important for maintenance of the protonation state and reduction potential of the enzyme, as well as for dihydroxylation of substrate. Thus, it is not clear what the exact roles of the bridging aspartate are. However, this aspartate clearly is important for catalysis.

The structure of NDO (1NDO) shows that His104, a Rieske center ligand, is in van der Waals contact with His213, a ligand to the mononuclear Fe(II) on the adjacent subunit. Both histidines also appear to be in van der Waals contact with the bridging aspartate Asp205. Thus, it is possible that electron transfer could be occurring through a histidine-to-histidine pathway between the Rieske and the mononuclear center as well as via the bridging aspartate residue. Small conformational changes in enzyme quaternary structure could result in the disruption of this contact so that only the bridging aspartate would provide an essential electron transfer path. Such variation in the assembly of the subunits within the multimer could possibly account for the existing discrepancy in the roles ascribed to the bridging aspartate (*16*, *20*, *21*).

The crystal structure of PDO is not yet available; however, PDO and NDO have considerable similarities in the sequences of their Rieske center and mononuclear domains. Surprisingly, there is very little sequence similarity anywhere else. Importantly, the ligands of the Rieske center, which include two histidines (His72 and His92 in PDO), as well as two cysteines (Cys70 and Cys89 in PDO), are conserved. Also conserved are His181 and His186 in PDO, which are probably the histidines ligating the iron in the mononuclear center (*23*) and are probably part of the two-His one-carboxylate "facial triad" that is observed in many other Fe(II)-containing oxygenases (*34*). Moreover, the aspartate responsible for bridging the Rieske center with the adjacent mononuclear site (on the adjoining subunit, as discussed above) in NDO (Asp205) is also conserved (Asp178 in PDO). Thus, it is reasonable to propose similar electron transfer pathways in the two enzymes.

As proposed above, in PDO two subunits appear to cooperate in delivering electrons to one of the mononuclear centers. A mechanism involving consecutive reduction of the iron mononuclear center by two Rieske centers from different monomers within the hexamer was proposed for the AntDO (*21*). In PDO the faster phase (k_3) could involve electron transfer from the Rieske center on the adjacent unit, and the slower phase (k_2) could involve electron transfer from the more distant Rieske center of its own subunit. Alternatively, two Rieske centers could provide the electrons for the catalysis occurring at the mononuclear center located on the third subunit (Figure 7). In any case, contributions from the two electron transfer pathways should result in equal magnitude of the relevant kinetic phases. However, our data show a 40/60% split between the fast and the slow phases (k_3 and k_2 in Table 2), possibly indicating a disruption in cooperativity in some PDO multimers.

The binding of phthalate to the mononuclear site of PDO in the absence and, to an even greater extent, in the presence of PDR$_{ox}$ enabled the majority (up to 85%) of the Rieske centers to oxidize at about 40 s^{-1}. It seems unlikely that such a high electron transfer rate could be achieved over the

distance of 35−45 Å, which on the basis of the NDO structure likely separates the Rieske center from the mononuclear Fe(II) center of the same subunit. Thus, even if a Rieske center oxidation rate (e.g., 0.1 s^{-1}) would be attributed to the intrasubunit electron transfer, it is highly unlikely that the binding of substrate would allow for the electron transfer rates to increase to 40 s^{-1}.

The model in Figure 7 shows the Rieske centers of two PDO subunits adjacent to the mononuclear center of another subunit. This model implies that an asymmetry might exist in the electron transfer pathways available for Rieske center oxidation. One such pathway would involve direct electron transfer between the Rieske and mononuclear Fe(II) centers ligands, while the other would utilize the bridging aspartate. The observed rates (k_2 and k_3) may differ from each other either because the two Rieske centers that contribute the electrons are not symmetrical with respect to the mononuclear center and/or because the contributions of the first and second electrons in the dioxygenation reaction are to different states of the mononuclear iron. Phthalate-induced activation of the Fe(II) mononuclear cluster would have to induce conformational changes either within the mononuclear site or in the quaternary structure of the PDO multimer to increase the rates of electron transfers from both adjacent Rieske centers. On the basis of the presented model, one would expect that in the presence of phthalate 100% of the Rieske centers in PDO-Fe(II) would be oxidized fast. It is difficult to explain why 30% (on average) of the Rieske centers are oxidized at rates similar to that of the PDO-Fe(II) without the substrate. Possibly, some of the PDO monomers are damaged and are incapable of substrate-induced activation. This might be similar to the damage observed in PDO-APO^F preparation that, even though fully reconstituted with iron (∼3 Fe/monomer), exhibited only half of its original activity.

The proposed model implies that oxidation of two Rieske centers is necessary for the development of one DHD molecule, as would be required with PDO because the mononuclear iron is Fe(II) at the completion of the reaction. Thus only 0.5 molecule of product would be produced per oxidized Rieske center. This is in fact what happens with PDO (unpublished data, manuscript in preparation). This stoichiometry would contrast with the results obtained in both NDO and BZDO systems (*16*, *20*) where essentially one molecule of product forms per each Rieske center oxidized (per Fe-containing mononuclear center) and the mononuclear iron is in the Fe(III) state at the completion of the reaction.

REFERENCES

1. Gibson, D. T., and Parales, R. E. (2000) Aromatic hydrocarbon dioxygenases in environmental biotechnology, *Curr. Opin. Biotechnol. 11*, 236−243.
2. Furukawa, K. (2000) Engineering dioxygenases for efficient degradation of environmental pollutants, *Curr. Opin. Biotechnol. 11*, 244−249.
3. Petrounia, I. P., and Arnold, F. H. (2000) Designed evolution of enzymatic properties, *Curr. Opin. Biotechnol. 11*, 325−330.
4. Batie, C. J., Ballou, D. P., and Correll, C. C. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) pp 543−556, CRC Press, Boca Raton, FL.
5. Batie, C. J., and Ballou, D. P. (1990) Phthalate dioxygenase, *Methods Enzymol. 188*, 61−70.
6. Ballou, D., and Batie, C. (1988) Phthalate oxygenase, a Rieske iron-sulfur protein from *Pseudomonas cepacia*, *Prog. Clin. Biol. Res. 274*, 211−226.
7. Tsang, H. T., Batie, C. J., Ballou, D. P., and Penner-Hahn, J. E. (1996) Structural characterization of the mononuclear iron site in *Pseudomonas cepacia* phthalate DB01 dioxygenase using x-ray absorption spectroscopy, *J. Biol. Inorg. Chem. 1*, 24−33.
8. Karlsson, A., Parales, J. V., Parales, R. E., Gibson, D. T., Eklund, H., and Ramaswamy, S. (2003) Crystal structure of naphthalene dioxygenase: side-on binding of dioxygen to iron, *Science 299*, 1039−1042.
9. Ballou, D. P., Gassner, G. T., Wang, L., Batie, C., Gatti, D., Dunham, W. R., and Sands, R. H. (1994) in *Flavins and Flavoproteins 1993* (Yagi, K., Ed.) pp 639−647, Walter de Gruyter and Co., New York.
10. Gassner, G., Wang, L., Batie, C., and Ballou, D. P. (1994) Reaction of phthalate dioxygenase reductase with NADH and NAD: kinetic and spectral characterization of intermediates, *Biochemistry 33*, 12184−12193.
11. Gassner, G. T., and Ballou, D. P. (1995) Preparation and characterization of a truncated form of phthalate dioxygenase reductase that lacks an iron-sulfur domain, *Biochemistry 34*, 13460−13471.
12. Gassner, G. T. (1995) Mechanistic studies of the phthalate dioxygenase system, Ph.D. Thesis, University of Michigan.
13. Davis, M. D., Kaufman, S., and Milstien, S. (1986) A modified ferrozine method for the measurement of enzyme-bound iron, *J. Biochem. Biophys. Methods 13*, 39−45.
14. Massey, V., and Hemmerich, P. (1977) A photochemical procedure for reduction of oxidation-reduction proteins employing deazariboflavin as catalyst, *J. Biol. Chem. 252*, 5612−5614.
15. Bevington, P. R. (1969) in *Data Reduction and Error Analysis for the Physical Sciences*, pp 235−242, McGraw-Hill, New York.
16. Wolfe, M. D., Altier, D. J., Stubna, A., Popescu, C. V., Munck, E., and Lipscomb, J. D. (2002) Benzoate 1,2-dioxygenase from *Pseudomonas putida*: single turnover kinetics and regulation of a two-component Rieske dioxygenase, *Biochemistry 41*, 9611−9626.
17. Orville, A. M., and Lipscomb, J. D. (1997) Cyanide and nitric oxide binding to reduced ptotocatechuate 3,4-dioxygenase: insight into the basis for order-dependent ligand binding by intradiol catecholic dioxygenases, *Biochemistry 36*, 14044−14055.
18. Arciero, D. M., Lipscomb, J. D., Huynh, B. H., Kent, T. A., and Munck, E. (1983) EPR and Mossbauer studies of protocatechuate 4,5-dioxygenase. Characterization of a new Fe2+ environment, *J. Biol. Chem. 258*, 14981−14991.
19. Chen, V. J., Orville, A. M., Harpel, M. R., Frolik, C. A., Surerus, K. K., Munck, E., and Lipscomb, J. D. (1989) Spectroscopic studies of isopenicillin N synthase. A mononuclear nonheme Fe2+ oxidase with metal coordination sites for small molecules and substrate, *J. Biol. Chem. 264*, 21677−21681.
20. Wolfe, M. D., Parales, J. V., Gibson, D. T., and Lipscomb, J. D. (2001) Single turnover chemistry and regulation of O2 activation by the oxygenase component of naphthalene 1,2-dioxygenase, *J. Biol. Chem. 276*, 1945−1953.
21. Beharry, Z. M., Eby, D. M., Coulter, E. D., Viswanathan, R., Neidle, E. L., Phillips, R. S., and Kurtz, D. M., Jr. (2003) Histidine ligand protonation and redox potential in the Rieske dioxygenases: role of a conserved aspartate in anthranilate 1,2-dioxygenase, *Biochemistry 42*, 13625−13636.
22. Tierney, D. L., Gassner, G. T., Luchinat, C., Bertini, I., Ballou, D. P., and Penner-Hahn, J. E. (1999) NMR characterization of substrate binding in the phthalate dioxygenase system, *Biochemistry 38*, 11051−11061.
23. Coulter, E. D., Moon, N., Batie, C. J., Dunham, W. R., and Ballou, D. P. (1999) Electron paramagnetic resonance measurements of the ferrous mononuclear site of phthalate dioxygenase substituted with alternate divalent metal ions: direct evidence for ligation of two histidines in the copper(II)-reconstituted protein, *Biochemistry 38*, 11062−11072.
24. Parales, R. E., Parales, J. V., and Gibson, D. T. (1999) Aspartate 205 in the catalytic domain of naphthalene dioxygenase is essential for activity, *J. Bacteriol. 181*, 1831−1837.
25. Gassner, G. T., and Ballou, D. P. (1997) in *Flavins and Flavoproteins* (Stevenson, K., Massey, V., and Williams, C. H. J., Eds.) pp 909−912, University of Calgary Press, Calgary.
26. Karpefors, M., Adelroth, P., Zhen, Y., Ferguson-Miller, S., and Brzezinski, P. (1998) Proton uptake controls electron transfer in cytochrome *c* oxidase, *Proc. Natl. Acad. Sci. U.S.A. 95*, 13606−13611.

27. Adelroth, P., Karpefors, M., Gilderson, G., Tomson, F. L., Gennis, R. B., and Brzezinski, P. (2000) Proton transfer from glutamate 286 determines the transition rates between oxygen intermediates in cytochrome *c* oxidase, *Biochim. Biophys. Acta 1459*, 533−539.
28. Liu, Y., Nesheim, J. C., Lee, S. K., and Lipscomb, J. D. (1995) Gating effects of component B on oxygen activation by the methane monooxygenase hydroxylase component, *J. Biol. Chem. 270*, 24662−24665.
29. Gassner, G. T., Ballou, D. P., Landrum, G. A., and Whittaker, J. W. (1993) 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, *Biochemistry 32*, 4820−4825.
30. Bertini, I., Luchinat, C., Mincione, G., Parigi, G., Gassner, G. T., and Ballou, D. P. (1996) NMRD studies on phthalate dioxygenase: evidence for displacement of water on binding substrate, *J. Biol. Inorg. Chem.*, 468−475.
31. Gassner, G. T., and Lippard, S. J. (1999) Component interactions in the soluble methane monooxygenase system from *Methylococcus capsulatus* (Bath), *Biochemistry 38*, 12768−12785.
32. Kauppi, B., Lee, K., Carredano, E., Parales, R. E., Gibson, D. T., Eklund, H., and Ramaswamy, S. (1998) Structure of an aromatic-ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase, *Structure 6*, 571−586.
33. Jiang, H., Parales, R. E., Lynch, N. A., and Gibson, D. T. (1996) Site-directed mutagenesis of conserved amino acids in the alpha subunit of toluene dioxygenase: potential mononuclear non-heme iron coordination sites, *J. Bacteriol. 178*, 3133−3139.
34. Hegg, E., and Que, L. J. (1997) The 2-His-1-carboxylate facial-triad—an emerging structural motif in mononuclear non-heme iron(II) enzymes, *Eur. J. Biochem. 250*, 625−629.

BI0490587