Substitutions of the "Bridging" Aspartate 178 Result in Profound Changes in the Reactivity of the Rieske Center of Phthalate Dioxygenase†

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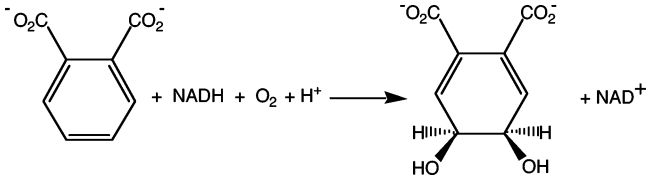
ABSTRACT: Phthalate dioxygenase (PDO) and its reductase (PDR) are parts of a two-component Rieske oxygenase system that initiates the aerobic breakdown of phthalate by forming *cis*-4,5-dihydro-4,5-dihydroxyphthalate. Aspartate D178 in PDO, which lies between the Rieske [2Fe-2S] center of one subunit and the mononuclear center of the adjacent subunit, is highly conserved among the Rieske dioxygenases. The analogous aspartate has been implicated in electron transfer in naphthalene dioxygenase and in substrate binding and oxygen reactivity in anthranilate dioxygenase. Substitution of D178 with alanine or asparagine in PDO resulted in proteins with significantly increased Fe(II) dissociation constants. The rates of oxidation of the reduced Rieske centers in D178A and D178N were decreased by more than 10^4-fold; only part of the loss of activity can be attributed to depletion of iron from the mononuclear centers. Reduction of PDO by reduced PDR was also slower in the D178A and D178N variants. Observed decreases in turnover rates of D178A and D178N compared to that of wild-type (WT) PDO ($>10^2$-fold) can be ascribed to the cumulative effect of the low intrinsic iron content of the D178A and D178N mutants and the combination of the decreased rates of Rieske center reduction and oxidation. The coupling of dihydrodiol formation approached 100% in WT PDO but was only ∼16% in D178A and ∼7% in D178N. In single-turnover experiments, very small amounts of DHD were produced by D178A and D178N "as purified". The presence of saturating amounts of ferrous ion improved coupling to nearly 100% for the D178N variant but only slightly improved coupling for D178A. Thus, although hydroxylation is still possible in the variants, the reactions are largely uncoupled due to slow intramolecular electron transfer rates and the apparent weak binding of iron at the mononuclear centers.

The phthalate dioxygenase system (PDS),[1] a two-component enzyme system in *Burkholderia cepacia* DB01, initiates the aerobic breakdown of phthalate via formation of *cis*-4,5-dihydro-4,5-dihydroxyphthalate (Scheme 1). PDS comprises the monomeric phthalate dioxygenase reductase (PDR), an enzyme that contains both FMN and a plant-type [2Fe-2S] ferredoxin center, and a multimeric dioxygenase (PDO),[2] with each subunit containing a Rieske [2Fe-2S] center and a ferrous mononuclear center. FMN in PDR accepts a hydride from NADH and rapidly shuttles an electron to the [2Fe-2S] ferredoxin center (*1*). Reduced PDR reduces the Rieske centers in PDO that, in turn, transfer the electrons to the iron mononuclear centers to be used in catalysis.

Scheme 1: Catalytic Pathway for the Phthalate Hydroxylation



Crystal structures of oxygenases for naphthalene (NDO) (*2*), biphenyl (BPDO) (*3*), nitrobenzene (NBDO) (*4*), cumene (CumDO) (*5*), carbazole 1,9a-dioxygenase (CarDO) (*6*), and 2-oxoquinoline-8-monooxygenase (OMO) (*7*) show that the mononuclear iron and Rieske center on the same subunit are

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[1] Abbreviations: PDS, phthalate dioxygenase system; PDO, phthalate dioxygenase; WT, enzyme with no substitutions in the sequence; PDO WT-Fe(II), phthalate dioxygenase with the mononuclear center containing Fe(II); PDO WT-APO, WT phthalate dioxygenase that lacks iron in the mononuclear center; PDO WT-APO[20], WT phthalate dioxygenase with 20% of the mononuclear centers containing Fe(II); PDR, phthalate dioxygenase reductase; $FMNH^-$, fully reduced form of flavin; FMN^{sq}, semiquinone form of flavin; FMN^{ox}, oxidized form of flavin; DHD, *cis*-4,5-dihydrodiol of phthalate; FAS, ferrous ammonium sulfate; NDO, naphthalene dioxygenase; NDF, naphthalene dioxygenase ferredoxin; NDS, naphthalene dioxygenase system; TDO, toluene dioxygenase; AntDO, anthranilate dioxygenase; BPDO, biphenyl dioxygenase; BZDO, benzoate dioxygenase; NBDO, nitrobenzene dioxygenase; CarDO, carbazole 1,9a-dioxygenase; CumDO, cumene dioxygenase; OMO, 2-oxoquinoline 8-monooxygenase; ICP, inductively coupled plasma high-resolution mass spectrometry.

[2] Recent gel filtration experiments indicate that the molecular mass of the PDO multimer is ∼250 kDa; however, the difference between that result and ∼200 kDa, which can be attributed to a tetramer (α_4), and ∼300 kDa, which can be attributed to a hexamer (α_6), is not statistically significant. Results from analytical centrifugation experiments are consistent with PDO being a hexamer or a dynamic equilibrium between tetrameric and octameric forms. Preliminary gas-phase electrophoretic mobility molecular analysis and electrospray ionization mass spectrometry experiments (J. A. Loo, personal communication) indicated that at neutral pH the molecular mass of both WT and the mutant PDO forms is ∼290 kDa, consistent with a hexameric structure with no evidence of other multimeric forms.

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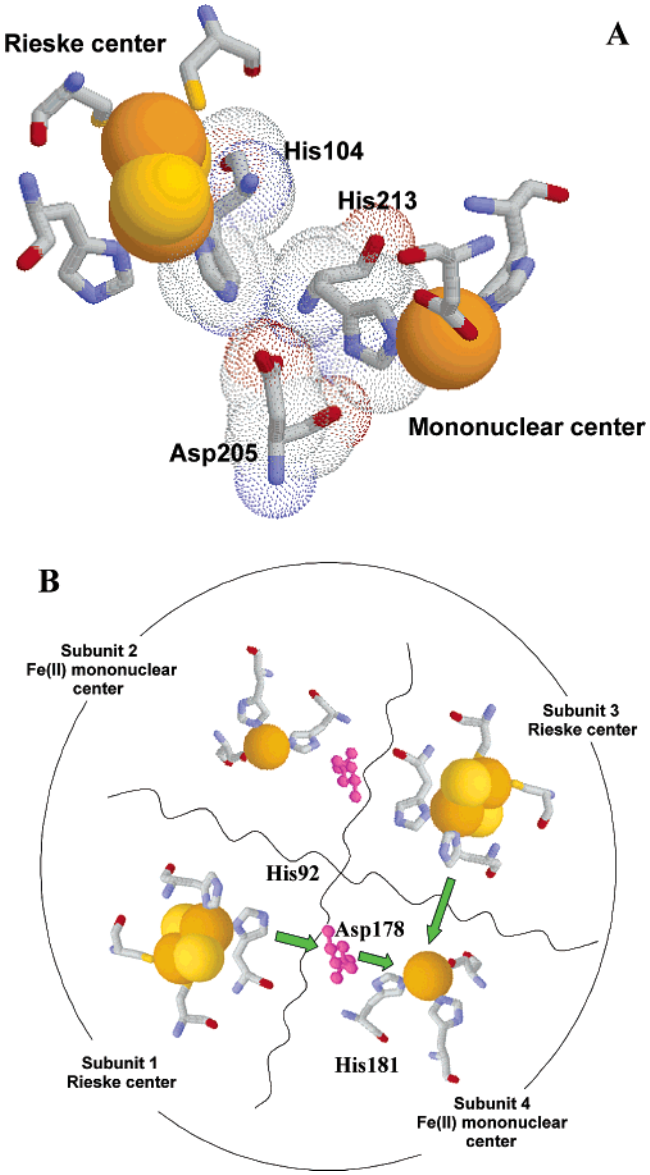


FIGURE 1: (A) Interaction of the Rieske cluster and the Fe mononuclear center on the neighboring subunits in NDO. (B) A representation of the active site of PDO based on biochemical and sequence data using the X-ray structure of NDO as a model (*13*, *14*). Green arrows in panel B indicate possible electron transfer pathways. Note that each Fe mononuclear center is positioned where it can receive electrons from two adjacent Rieske centers, one of which could be through the bridging Asp-178 (purple).

separated by more than 40 Å, which makes direct electron transfer between them unfavorable (*8*). However, because the mononuclear centers are located only ~12 Å from the Rieske centers on the adjacent subunits of the enzyme (Figure 1A), it is believed that normal catalysis involves intersubunit electron transfer. The ligating histidine of the Rieske center (His-104 in NDO, His-121 in BPDO, His-102 in NBDO, His-93 in CarDO, His-124 in CumDO, and His-108 in OMO) is in van der Waals contact with the ligating histidine of the iron mononuclear center (His-213 in NDO, His-224 in BPDO, His-211 in NBDO, His-183 in CarDO, His-234 in CumDO, and His-221 in OMO). The published structures show that these histidines are also in van der Waals contact with the so-called "bridging" aspartate (Asp-205 in NDO, Asp-221 in BPDO, Asp-203 in NBDO, Asp-180 in CarDO, Asp-231 in CumDO, and Asp-218 in OMO).

This aspartate residue is critically important for catalysis, as its substitutions in NDO (*9*), AntDO (*10*), and TDO (*11*) resulted in practically inactive enzymes (no turnover activity was detected in AntDO and TDO; activity was severely diminished in NDO), with the level of activity depending on the nature of the substitution. In some cases, much of this loss of activity can be attributed to poor iron binding by the mononuclear center (e.g., no iron is present in the mononuclear center in the D218E mutant of AntDO, and poor iron binding was assumed for the D219A mutant of TDO). However, in each of the NDO mutants (D205A, -E, -N, and -Q), as well as in the D218A and -N mutants of AntDO, the total iron content was similar to that in WT enzymes. In both NDO and AntDO, the Rieske centers could be reduced by their respective electron donor systems, but the rates of reduction of the Rieske centers in NDO and AntDO were not determined, probably because the electron donors in those systems are also Rieske proteins, so that there are only very small spectral changes to observe. It was proposed for NDO (*9*, *12*) and CarDO (*6*) that this bridging aspartate could be involved in electron transfer between the Rieske center of one subunit and the mononuclear center located on an adjacent subunit. The low activity of the Asp-205 variants in NDO was proposed to result from defective electron transfer from the Rieske to the mononuclear site. Slow electron transfer, however, was not the cause of inactive mutants of AntDO. Beharry et al. (*10*) showed that the oxidation of the Rieske center by oxygen in the mutant dioxygenase is similar to that in the WT enzyme. To account for the low oxygenase activity of the variants, they proposed that Asp-218 in AntDO affects the protonation state of the ligands of the Rieske center and/or steps involved in substrate hydroxylation. Thus, it is clear that the bridging aspartate is critical for proper activity in all of the Rieske oxygenases that were examined, but no definitive roles for this aspartate have been determined.

Although the overall degrees of homology are low between the sequences of PDO and the other Rieske oxygenases mentioned herein (which are quite similar to each other), there is considerable similarity in both the Rieske and mononuclear domains, suggesting that these regions are also structurally similar. Ligands for Fe both in the Rieske and in the mononuclear centers, as well as the bridging aspartate (D178 in PDO), are conserved and appear to be a signature of most Rieske oxygenases. Previously, we presented a model for the subunit arrangement in the PDO multimer (*13*) implying that in PDO this bridging aspartate also is a part of the electron transfer pathway from the Rieske center to the mononuclear site. Our results (*14*) indicated that structurally, two Rieske centers and two iron mononuclear sites on four different subunits must be located close to each other (Figure 1B). In that arrangement, electron transfer is possible from either of the two Rieske centers to either of the two adjacent Fe mononuclear sites; however, only some of these transfers would be facilitated by the bridging aspartate (Figure 1B). The work presented here provides insight into the diverse roles of the bridging aspartate in catalysis. We report the differences in electron transfer rates, overall reactivity, and substrate hydroxylation between D178A and D178N variants and WT PDO. Because in PDO the reductase is not a Rieske protein, we were able to monitor not only the oxidative but also the reductive part of the turnover. A

second manuscript addresses how Asp-178 substitutions in PDO mediate the physical interactions between the Rieske and mononuclear centers.

MATERIALS AND METHODS

Construction of the PDO Expression Plasmid. Genomic DNA was isolated from *B. cepacia* with the DNeasy tissue kit from Qiagen. The gene encoding PDO was amplified by PCR using DyNAzyme DNA polymerase from Finnzymes. Primers used for the amplification were 5′-GGGGAATTC-CATATGCTGACCCACCAAGAAAACGAATTGC-3′ (forward primer; underlined bases indicate an NdeI site) and 5′-CGCGGATCCGTTATTGCTTGACTTGATAGTCCGTTGCGAG-3′ (reverse primer; underlined bases indicate the BamHI site). BamHI and NdeI restriction enzymes were obtained from Invitrogen and used according to the manufacturer's recommendations. PCR products and the pET11a vector (Novagen) digested by the restriction enzymes were further purified by QIAquick gel extraction (Qiagen). PCR fragments were inserted into the pET11a vector (Novagen) using a DNA ligation kit from Novagen, transformed into competent HMS174 *Escherichia coli* cells (Novagen), and purified using QIAprep spin miniprep kits from Qiagen. The correct sequence was verified by sequencing the entire PDO gene. Sequencing was carried out at the University of Michigan Core Facility.

Construction of D178A and D178N Variants. Substitution of the aspartate 178 codon with that of alanine (D178A) or asparagine (D178N) was carried out using the QuickChange site-directed mutagenesis kit (Stratagene) following the recommended protocol and using the GenElute HP plasmid midiprep kit in a vacuum format (Sigma) for the isolation of plasmid DNA. Plasmid pET11a containing the nucleotide sequence encoding PDO from *B. cepacia* DB01 was used as a template with the complementary oligonucleotide primers 5′-GAAGGCGCGATCGCTTCCGCGCACAGCTCG-3′ and 5′-CGAGCTGTGCGCGGAAGCGATCGCGCCTTC-3′ for D178A and 5′-CTCGAAGGCGCGATCAATTC-CGCGCACAGCTCG-3′ and 5′-CGAGCTGTGCGCGGA-ATTGATCGCGCCTTCGAG-3′ for D178N, with the underlined sequences indicating mutant codons. Primers were purchased from IDT. Successful mutation of the codons was verified by nucleotide sequencing of the entire PDO gene.

Expression and Purification of Recombinant Enzymes. Plasmids containing unmodified WT or the mutated PDO gene were transformed by heat shock into competent *E. coli* strain C41(DE3) (Avidis) (*15*). Colonies were selected on Luria-Bertani (LB) agar plates supplemented with 100 mg/L carbenicillin. A single colony was inoculated into 100 mL of LB liquid medium supplemented with 100 mg/L carbenicillin in a 0.5 L Erlenmeyer flask and incubated for 16−18 h (overnight), at 37 °C with shaking at 250 rpm. On the following day, 10 mL of the overnight culture was used to inoculate 1 L of Terrific Broth (TB) medium supplemented with 100 mg/L carbenicillin and 2 mM phthalate in a 2.7 L Fernbach flask. The culture was further supplemented with 1 mM cysteine, 0.1 g/L ferrous sulfate, and 0.1 g/L ferric citrate. These additional supplements resulted in a more than 10-fold increase in the yield of PDO. Cultures were grown in an INNOVA orbital shaker at 30 °C and 200 rpm until the optical density of the culture at 600 nm reached 5−6. At this time, the expression of PDO was induced by adding 0.7 mM IPTG (final), and the cultures were grown for an additional 40 h. Aliquots were withdrawn periodically for determination of optical density (at 600 nm), pH, and protein concentrations. The PDO yield in cell cultures was assessed using the EasyLyse Bacterial Protein Extraction Protocol (Epicenter) in conjunction with SDS−PAGE and reached up to 1 g/L of cell culture. Cells were harvested by centrifugation. Approximately 130 g of cells was collected from 6 L of *E. coli* culture. Pellets were resuspended in the lysis buffer [35 mM KP_i (pH 7.0), 5 mM phthalate, 100 μM PMSF, and 4 mg/L DNAse I], with ∼40 mL of lysis buffer used per 15−20 g of wet cell paste, sonicated on ice for ∼4 min to ensure full resuspension, and lysed by being passed twice through a French pressure cell (at 2000 psi). Cell debris was precipitated by ultracentrifugation for 1 h at 100000*g*. The supernatant contained the PDO (as verified by SDS−PAGE), which was further purified as described previously (*16*), excluding the separation of the PDO−PDR complex.

Spectrophotometry, Assays, and Kinetics. Concentrations of enzymes were determined spectrophotometrically using a $\Delta\epsilon_{575}$ of 2.38 mM^{-1} cm^{-1} and a $\Delta\epsilon_{466}$ of 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 due to consumption of NADH. Assay mixtures contained 0.2 μM PDO, 5 mM phthalate, and 100−200 μM NADH in 0.1 M HEPES (pH 7.8). For activity measurements in the presence of iron, the solution containing PDO was preincubated with $Fe(NH_4)_2(SO_4)_2$ (FAS) (prepared freshly as an anaerobic solution with a known concentration) for 3−5 min to allow the absorbance to stabilize prior to the addition of 0.2 μM PDR. Note that measured activity does not represent maximum PDO activity, but rather the activity under the conditions described above at a PDO:PDR stoichiometry of 1:1. PDO WT-APO that lacks iron in the mononuclear center (≤2.08 Fe atoms/monomer) and a less stringently depleted preparation (PDO WT-APO[20]) that contained ∼2.2 Fe atoms/monomer were prepared as previously described (*13*). Iron and sulfur content in PDO was determined by inductively coupled plasma high-resolution mass spectrometry (ICP) using a Finnegan Element Instrument from Thermo Finnegan Co. (at University of Michigan Geological Services). Iron content was also verified by the ferrozine assay (*17*). The binding of PDR to PDO was evaluated by determining the turnover numbers, as described above, at varying levels of PDO, keeping the PDR concentration constant (0.2 μM). The extent of phthalate binding was estimated by monitoring the activity of PDO in steady-state assays in the presence of varying amounts of phthalate. KaleidaGraph, version 3.6 (Synergy Software), was used to fit the raw data to the binding equation $y = C_0 + C_1x/(C_2 + x)$. Parameters indicating Fe(II) binding were determined on the basis of the changes in the steady-state activity of PDO observed during the titration of WT, WT-APO, D178A, and D178N samples with an anaerobically prepared FAS solution. The data were fit to the Hill equation [$y = C_0 + C_1x^n/(C_2 + x^n)$, where n is the Hill coefficient] using KaleidaGraph.

Product Analysis. Reduction of PDO (80−300 μM before mixing) was achieved by exposing an anaerobic enzyme complemented with 0.5−1 μM 5-deazariboflavin and 1−5

mM glycine in an ice bath to a 1000 W halogen lamp (*18*). When required, reduced enzyme was supplemented with the anaerobically prepared FAS solution. Reactions were quenched by filtering the enzyme from the solution with Microcon-YM30 concentrators (Amicon, Co.) or by heating the samples for 3 min at 95 °C. Precipitates from denatured samples were removed by spinning them for 2 min in a tabletop centrifuge. Samples were frozen at 77 K until product analysis was carried out. Control experiments using previously extracted DHD verified that no DHD deterioration occurs during the freezing and thawing and/or heating of samples up to 95 °C for up to 5 min. Product analysis was performed using a Waters HPLC system equipped with a Waters 441 detector ($\lambda = 250$ nm). An Aminex HPX-87H column running isocratically in 9 mM H_2SO_4 at 65 °C with a flow rate of 0.9 mL/min was used for product separation. Data were recorded and analyzed with Millenium32 software (Waters).

Rapid Reaction Kinetics. PDO and PDR samples for stopped-flow studies (20−40 μM before mixing) were made anaerobic by ≥10 cycles of vacuum/gas exchange with purified argon and finally overlaid with ~2 psi of purified argon. Enzymes were reduced (if required) by titrating the samples anaerobically with a sodium dithionite solution. The oxidation states of enzymes were monitored using a Shimadzu UV 2051PC spectrophotometer. Experiments were performed in 0.1 M HEPES (pH 7.8) at 22 °C in the presence of 3 mM phthalate (if required). Kinetic data were acquired using a Kinetic Instruments, Inc., stopped-flow spectrophotometer in single-wavelength mode. Reactions were observed at 575 nm, a wavelength that corresponds to a shoulder in the Rieske center visible absorption spectrum of PDO_{ox}, and at 640 nm, a wavelength where the absorbance of the semiquinone form of PDR is greater than the absorbance change due to the reduction of the Rieske center. Quantification of semiquinone formation was based on a $\Delta\epsilon_{640}$ of 2.010 mM^{-1} cm^{-1} for $PDR_{2e^-;\text{semiquinone FMN}}$ minus $PDR_{3e^-;\text{fully reduced}}$, a $\Delta\epsilon_{640}$ of 2.468 mM^{-1} cm^{-1} for $PDR_{1e^-;\text{oxidized FMN}}$ minus $PDR_{2e^-;\text{semiquinone FMN}}$, and a $\Delta\epsilon_{640}$ of 0.497 mM^{-1} cm^{-1} for $PDO_{reduced}$ minus $PDO_{oxidized}$. Data collection and analysis were performed using program A, which employs the Marquardt−Levenberg algorithm (*19*) and was developed in our laboratory by C.-J. Chiu, R. Chang, J. Dinverno, and D. P. Ballou (University of Michigan). Rates and amplitudes were obtained from curve fitting (mean values from at least three independent experiments were within 15% of each other unless specified otherwise). All chemicals were analytical grade and used without further purification.

RESULTS

Binding of Phthalate to PDO. The affinity of both WT and mutant PDO for phthalate was estimated by monitoring the activity of PDO in the presence of varying amounts of phthalate (see Materials and Methods). Activity was also found to be dependent on the iron content of the mononuclear center. When Fe(II) is present in the mononuclear center of PDO (WT PDO), K_m values for phthalate are in the micromolar range (K_{m1} in Table 1), whereas with no Fe(II) present (PDO WT-APO), the K_m is increased by ~3 orders of magnitude (millimolar values of K_{m2} in Table 1). Consistent with the low iron content of the D178A and D178N variants [less than 20% of the mononuclear centers contain Fe(II)], activity of the mutant PDOs exhibited a biphasic dependence on the phthalate concentration (Table 1). One subpopulation of the mutant PDO exhibited affinity for phthalate that was more similar to that of WT PDO with Fe(II) ion present in the mononuclear center (5.8 and 9.3 μM), while the other D178A and D178N subpopulation reacts with phthalate in a manner similar to that of the WT APO enzyme (millimolar K_m value). Overall, it can be concluded that D178A and D178N substitutions did not greatly affect the affinity of phthalate for the enzyme.

Table 1: K_m Values for Phthalate in the PDOS

	K_{m1} (μM) mononuclear center contains Fe(II)	K_{m2} (mM) mononuclear center lacks Fe(II)
WT	0.2	
WT APO		1.6
D178N	5.8	1.1
D178A	9.3	0.9

Table 2: K_m Values for PDR in the PDOS

PDO sample	mononuclear centers containing Fe(II) (%)	K_m (μM)
WT-Fe(II)	100	7.9 ± 0.6
WT-APO[a]	~100	8.1 ± 1.5
D178A	21[b]	18 ± 2
D178N	20[b]	16 ± 4
WT-APO[20 c]	20	16 ± 2
WT-APO[d]	>8	37 ± 2

[a] PDO WT-APO was supplemented with FAS (20 μM FAS) in excess of the PDO concentration to reconstitute the mononuclear centers. Precise determination of the Fe content after the reconstitution was not possible. [b] Assuming that iron detected by ICP analysis is the sum of the Fe in the Rieske and mononuclear centers only. [c] PDO WT-APO prepared according to a less stringent procedure (see Materials and Methods). [d] EDTA (1 mM) was added to the PDO WT-APO sample that was not supplemented with Fe(II) to prevent iron contamination.

PDO−PDR Interaction. We determined the K_m values for PDR in the steady-state kinetics of the PDOS. Fitting the data for WT PDO with the Michaelis−Menten equation gives a K_m value of 7.9 ± 0.6 μM for PDR (Table 2). PDO that lacks iron at the mononuclear center (WT APO in Table 2) had a K_m of 37 ± 2 μM, ~5-fold greater than that when the mononuclear center is fully populated. PDO (both WT and mutant) with intermediate Fe content (~20%) exhibited intermediate K_m values of ~17 μM for PDR (Table 2). Thus, D178A and D178N substitutions did not result in large changes in the interaction of PDR with PDO. WT APO that was reconstituted with Fe(II) behaved essentially like WT PDO.

PDO Iron Content and Steady-State Catalytic Activity. Recombinant WT PDO as purified contained 2.85 ± 0.15 Fe atoms/monomer and had a steady-state turnover activity of 4.1 s^{-1} (Table 3), corresponding to ~70% of the activity observed previously under the same conditions with PDO purified from *B. cepacia* (*13*). The iron content (2.1 ± 0.1 Fe atoms/monomer) and activity (0.16 s^{-1}) of the recombinant WT APO are similar to those of PDO WT-APO described previously (*13*). The iron content in the variants as purified (~2.2 Fe atoms/monomer; Table 3) was only a fraction of that in the WT enzyme, suggesting weaker binding of iron in the mononuclear center (two Fe ions are bound at the Rieske center).[3] Incubation of the D178A and D178N

Table 3: Steady-State Turnover Activity and Coupling of DHD Formation for WT, D178A, and D178N PDO Variants

		steady-state turnover				oxidative half-reaction	
		no extra Fe(II)		with extra Fe(II)[a]		coupling (%)	
	no. of Fe atoms per monomer (±5%)	TN (s^{-1})	coupling (%)	TN (s^{-1})	coupling (%)	no extra Fe(II)	with extra Fe(II)[a]
WT	2.85	4.1	100	4.1	100	95	95
WT-APO	2.08	0.16	82	3.2	96	<2	95
D178N	2.20	0.037	3.7	0.30	3.7	8	94
D178A	2.21[c]	0.040	16.7	0.29[b]	16.9	<2	7

[a] The concentration of anaerobically added FAS was 20 μM for the WT enzyme and 0.5−1 mM for the D178A and D178N variants. Thus, TN in the presence of extra Fe(II) represents the activity of the enzyme under the assay conditions in a saturating Fe(II) environment. [b] An estimate. See the text. [c] Iron content of D178A variant samples used in this work. Some preparations exhibited an iron content as low as 2.05 Fe atoms/monomer.

variants with EDTA as described in Materials and Methods for the preparation of the PDO WT-APO enzyme resulted in the proteins containing ~2.1 Fe atoms/monomer. Given the apparently weak binding of Fe(II), it is surprising that any iron, beyond that of the Rieske centers, was found after this procedure. This might indicate that at least part of the non-Rieske iron is bound at sites other than the mononuclear center. Small amounts of such adventitious iron were previously detected in WT PDO after its reconstitution with Fe(II) (*13*).

The steady-state activity of the mutants was >100-fold lower than that of the recombinant WT enzyme. This activity is so low that the direct reaction of reduced PDR with oxygen, which is slow (0.01−0.03 s^{-1}), interferes with the assays by contributing to the overall NADH consumption. This has negligible effects on the determination of the rates in the steady-state WT PDO catalysis. Turnover numbers presented in Table 3 were calculated under the assumption that PDR autoxidation does not contribute to the consumption of NADH; therefore, true turnover rates could be somewhat lower than those reported. If, for example, half of the oxidation of NADH were due to the autoxidation of PDR, the actual catalytic steady-state turnover rates would be 1.5−3-fold lower than those presented in Table 3.

Addition of iron to the reaction mixture did not affect the activity of WT PDO, and the catalytic activity of the WT APO enzyme that lacks iron in the mononuclear center could be recovered by adding a nearly stoichiometric amount of Fe(II). Stoichiometric additions of Fe(II) effected no change in the observed turnover rates of D178N and D178A. However, some activity was recovered via addition of much higher concentrations of Fe(II), but even at millimolar concentrations of iron, the activity of the mutants was less than 10% of that of WT PDO (Table 3). In contrast to that of D178N, activation of D178A in the presence of excess Fe(II) appeared to be transient, with turnover rates in most cases dropping back to 0.04 s^{-1} soon after the addition of Fe(II). Presumably, the iron in this variant becomes oxidized and dissociates from the PDO. The observed activity of the mutants in the presence of high concentrations of iron did not depend on the method used for the addition of iron (direct addition to the media, preincubation under aerobic or anaerobic conditions, or preincubation with the anaerobic

[3] The assumption behind the iron content measurements is that the extinction coefficients of the Rieske centers of the mutants remain similar to that of the WT enzyme. As we did not determine the extinction coefficients independently, the measured iron contents in D178N and D178A are only approximations of the actual values. However, extinction coefficients for most Rieske proteins are similar.

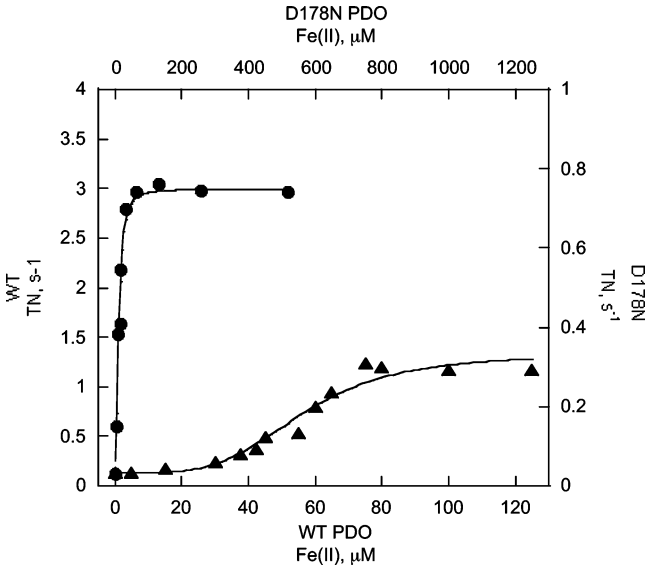


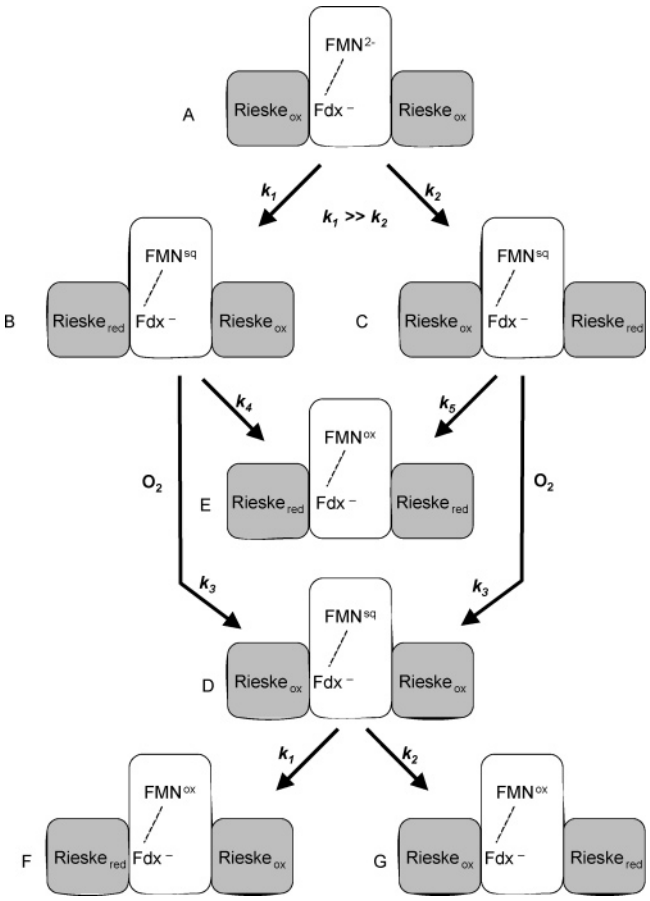
FIGURE 2: Steady-state turnover activity of PDO WT-Fe(II) and mutant PDO in the presence of extra Fe(II): PDO WT-Fe(II) (●) and D178N (▲). Conditions were as described in Materials and Methods. Data were fitted with the Hill equation (see Materials and Methods).

reduced enzyme). Addition of catalase and SOD (up to 250 units/mL each) did not result in any change in the observed turnover rates, indicating that H_2O_2 and $O_2^{-\bullet}$ were not impeding the reconstitution and the steady-state turnover.

The activity of the enzymes at different levels of added iron is presented in Figure 2. The response for added iron was simple for PDO WT-APO but was distinctly cooperative for the D178N variant with an apparent Hill coefficient (n) of 5.5 ± 1.5; the apparent dissociation constants, K_d, were 1 and 550 μM for WT and D178N, respectively (as shown by the sigmoidal curve; see Figure 2). The affinity of Fe(II) for D178A was not determined, because the changes in the steady-state turnover activity upon addition of FAS were not reliable (see above).

Reaction of Reduced PDR with Oxidized Forms of PDO. The reduction of the Rieske [2Fe-2S] center in PDO by fully reduced PDR (three electrons) results in the formation of the flavin semiquinone form of PDR (transition from form A to forms B and C in Scheme 2). As the electron is delivered from the [2Fe-2S] center of PDR to the Rieske center, the $FMNH^-$ in PDR very rapidly regenerates the reduced [2Fe-2S] center, resulting in the FMN semiquinone. In WT PDO, the steady-state turnover rate[4] at a PDO (Rieske):PDR stoichiometry of 1:1 was ~4 s^{-1}, defining a lower limit for the requirement of PDR to deliver electrons

Scheme 2: Interaction of PDR (white) with Rieske Centers of Two Adjacent PDO Monomers (gray)[a]



[a] In this model, only one reduced PDR binds per two Rieske centers and, thus, can only deliver electrons efficiently to one of the Rieske centers. In normal catalysis, NADH is present so that reduced PDR can be regenerated as the Rieske center delivers electrons to the mononuclear center. During the rate-limiting steps involved in oxygenation of phthalate, PDR might reorient to reduce the second Rieske center more efficiently.

to PDO. Thus, in anaerobic reactions of equimolar concentrations of reduced PDR with oxidized PDO, only the kinetic phases of semiquinone formation with rates of ≥ 4 s^{-1} are likely to be relevant for normal catalysis. The reduction of WT PDO by reduced PDR exhibits two kinetic phases corresponding to PDR FMN semiquinone formation (and, as a consequence, to PDO Rieske center reduction) (Figure 3) that are relevant for catalysis: one with a kinetic rate of $\sim$200 s^{-1} and a second with a kinetic rate of $\sim$3 s^{-1} (k_1 and k_2 in Table 4 and Scheme 2).

However, only $\sim$50% of all available PDR molecules formed an FMN semiquinone in the two fast kinetic phases, indicating that at a PDO:PDR stoichiometry of 1:1 not all PDR molecules are involved in rapid electron transfer to the Rieske centers of PDO. In our previous studies (*13*), we established that two Rieske centers are capable of electron transfer to any given iron mononuclear center and proposed a cooperative assembly of PDO subunits resulting in an arrangement of two Rieske and two mononuclear centers as shown in Figure 1B. One model satisfying such an arrangement is a head-to-tail bundle of four monomers that makes up the PDO tetramer [α_4 multimer, consistent with the initial gel filtration results (*20*) and described in detail in refs *13* and *14*)]. However, a similar arrangement can be achieved for a hexameric (α_6) PDO structure (the hexamer is consistent with our most recent results; see footnote 2), including one with two trimer head-to-tail rings, each similar to the ring of α-subunits of NDO, sitting one atop the other (Scheme 3). The results of product analysis in single-turnover experiments with PDO, as well as the fact that iron remains in a ferrous state at the end of catalysis (*13*), are inconsistent with either the trimeric (α_3) or hexameric ($\alpha_3\beta_3$) cyclic structures typical for other Rieske dioxygenases, where only one Rieske center is located near each mononuclear Fe(II) [e.g., NDO (*2*)]. The model shown in Figure 1B postulates two Rieske centers being close, so it is possible that only one PDR at a time can effectively transfer electrons to Rieske centers (as represented in Scheme 2 by a single PDR molecule located within efficient electron transfer distance of the Rieske centers on two adjacent PDO monomers). If so, only half of the reduced PDR molecules would be capable of reducing PDO at catalytically relevant rates. In normal steady-state catalysis with both NADH and O_2 present, a single PDR might serve to reduce a pair of nearby Rieske centers, and

[4] As mentioned previously, the steady-state activity assay does not determine the maximum rate of turnover, but the rate under specific, reproducible conditions (see ref *13*).

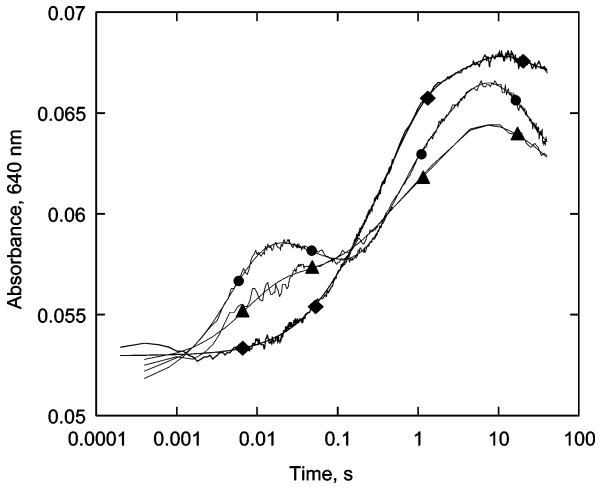


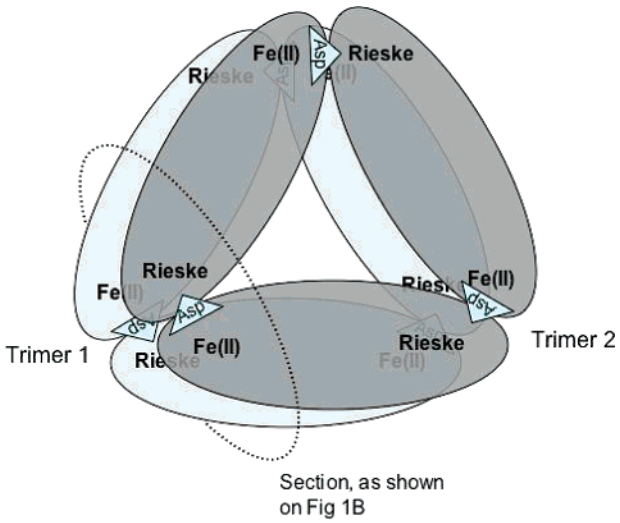
FIGURE 3: Reaction of oxidized PDO with a stoichiometric amount of reduced PDR ([PDO Rieske]:[PDR] = 1:1) in 0.1 M HEPES (pH 7.8) in the presence of 3 mM phthalate at 22 °C: 38 μM PDO WT-Fe(II) (●), 38 μM D178N (◆), and 24 μM (normalized to 38 μM) PDO WT-APO (▲). All concentrations given are those before mixing in the stopped-flow spectrophotometer. PDO WT-Fe(II) and D178N traces are averages from four measurements, and the PDO WT-APO trace is the average from seven measurements. Smooth lines are fits obtained from the parallel reaction model.

Table 4: Reduction of the Rieske Centers in PDO by Reduced PDR[a]

	k_1 (s^{-1}) [*A* (%)]	k_2 (s^{-1}) [*A* (%)]	k_3 (s^{-1}) [*A* (%)]
WT-Fe(II)	201 (25)	2.7 (30)	0.4 (15)
WT-APO	156 (20)	3.0 (25)	0.5
D178A	−	3.1 (50)	0.8
D178N	−	2.2 (40)	0.3

[a] k_1, k_2, and k_3 are kinetic rates of semiquinone formation (one-electron loss at the FMN in PDR due to reduction of the Rieske center in PDO). *A* is the fraction of PDR that forms the FMN semiquinone state at the end of the phase. A small (<10−15%) contribution of a faster phase, with a k_1 of $\sim$10−50 s^{-1}, was observed in some samples of D178A and D178N variants. Conditions as described in the legend of Figure 3.

Scheme 3: Possible Arrangement of Subunits in the PDO Hexamer[a]



[a] Two head-to-tail trimers (α_3) are stacked one atop the other. Asp designates the bridging aspartate (Asp-178 in PDO).

the continuous supply of electrons from NADH might in that case enable a PDR−PDO complex with a stoichiometry of 1:2 to turn over at catalytically relevant rates. A full understanding of this interaction and the transfer of electrons from PDR to PDO will require extensive investigation.

In addition to two fast rates of semiquinone formation, a slower increase in semiquinone concentration with an apparent kinetic rate of ~0.4 s^{-1} was observed for all samples. This rate might be related to reorganization of PDR molecules binding to PDO, with the formation of FMN^{sq} in the PDR FMN^{2-} molecules that replace PDR FMN^{sq} at the binding site. As this process is slow, it is not relevant for normal catalysis.

The presence of Fe(II) at the mononuclear center did not significantly affect the course of the reaction of PDO_{ox} with PDR_{red}. All three phases (two fast and one slow) of semiquinone formation were observed, not only in native WT PDO but also in PDO WT-APO (Table 4).

A kinetic phase with a rate of ~10 s^{-1} (between ~20 and 100 ms) that results in the apparent decrease in absorbance at 640 nm was due to the contribution of the oxidation of the Rieske center of PDO in its reaction with a trace of contaminating oxygen (Scheme 2; k_3 for the transition from forms B and C of the PDO−PDR complex to form D in Scheme 2).[5] This phase was not detectable in PDO WT-APO, D178A, or D178N because the oxidation of the Rieske center is much slower in these species than in WT PDO. The decrease in absorbance observed ~20 s after the mixing was not related to residual oxygen in the system and was associated with a second electron transfer from PDR to PDO. Such transfers resulted in the loss of PDR semiquinone due to the formation of fully oxidized FMN (transitions to forms E−G with the rates k_4 and k_5 in Scheme 2).

The reaction of reduced PDR with oxidized D178A and D178N PDO variants results in semiquinone formation at a rate of ~2−3 s^{-1}, similar to a slower semiquinone formation kinetic phase (k_2) in WT PDO (Table 4). No fast phase of FMN semiquinone formation (k_1) was observed. The lack of fast phases in the variants is not likely due to the lack of iron at the mononuclear site because WT APO exhibits a fast phase of reduction.

Reaction of Reduced Forms of PDO with O_2. When the mononuclear center contains iron and is fully competent for catalysis, the reactions of oxygen with reduced PDO occur in three kinetic phases (*13*) (Table 5 and Figure 4). Rieske centers in recombinant WT PDO-Fe(II) exhibit the same general pattern of oxidation, with the majority of the centers (~65%) oxidized in the fast phase at a rate of ~45 s^{-1}. A fraction of the Rieske centers in the recombinant enzyme reacted with O_2 very slowly (~3×10^{-4} s^{-1}), as shown by the increase in absorbance at times >1000 s (Figure 4). This phase, which contributes ~25−30% of the absorbance change, clearly belongs to a fraction of less-functional enzyme, and it correlates with the lower turnover number as measured in steady-state catalysis of the recombinant WT PDO-Fe(II) as compared to that of the WT enzyme isolated from *B. cepacia* (4 and 6 s^{-1}, respectively).

Both reduced D178N and D178A in the presence of the substrate reacted with oxygen slowly, with ~90% of the Rieske centers being oxidized at rates of $1-2 \times 10^{-3}$ s^{-1}, similar to that observed in PDO WT-APO (Table 5 and Figure 4). This result is consistent with the low iron content in the mononuclear centers of the mutant enzymes. Addition of high levels of FAS to the reaction mixture [10 min anaerobic preincubation of the reduced PDO (40 μM) with 500 μM FAS] brought about a marked (~100-fold) increase in the rates of the reaction of oxygen with the D178A and D178N variants (Table 5 and Figure 4). However, these rates still were significantly lower than those in WT PDO. Addition of catalase and SOD (up to 8000 units/mL each) had no effect.

Substrate Hydroxylation and Coupling. The conversion of phthalate into its dihydrodiol by WT PDO is a tightly coupled reaction in which NADH, phthalate, and oxygen are consumed in 1:1:1 stoichiometry to provide one molecule of product (*14*). Nearly 100% coupling[6] was also observed in the steady-state reaction of WT APO reconstituted with 20 μM excess Fe(II) (Table 3). In PDO WT-APO, steady-state catalysis was also tightly coupled (with coupling approaching 90%, Table 3), even though with the iron-depleted mononuclear centers NADH consumption was 10−200-fold slower than in Fe(II)-reconstituted PDO (Table 2). This indicates that Fe(II) present in a small fraction of the mononuclear centers is responsible for the observed residual activity, as proposed previously (*13*).

In single-turnover experiments, when the electron supply is limited to that available from the reduced Rieske centers, DHD formation is tightly coupled to the oxidation of the Rieske centers for both WT and WT APO reconstituted with Fe(II) (coupling of >95%), but no product was detected for WT APO protein without extra Fe(II) added (Table 3).

Small amounts of DHD were detected in a steady-state turnover reaction with "as purified" Asp-178 variants.

[5] When larger amounts of O_2 were introduced into the system, the magnitude of this phase increased and the rate obtained from the fits approached 40 s^{-1}, the rate of oxidation of the Rieske center at an atmospheric oxygen concentration.

[6] In steady-state reactions, coupling is defined as the amount of DHD formed per NADH oxidized. In single-turnover reactions, coupling is defined as the amount of DHD formed per two Rieske centers oxidized.

Table 5: Reaction of Reduced PDO with Oxygen

	k_1 (s^{-1}) [*A* (%)]	k_2 (s^{-1}) [*A* (%)]	k_3 (s^{-1}) [*A* (%)]	k_4 (s^{-1}) [*A* (%)]	k_5 (s^{-1}) [*A* (%)]
WT-Fe(II) (*B. cepacia*)[a]	41 (70)	1.8 (20)	0.1 (10)		4×10^{-3} [b] (<10)
recombinant WT-Fe(II)	45 (65)	0.6 (5)			
D178N				0.025 (10)	2.1×10^{-3} (90)
D178A				0.025 (10)	1.0×10^{-3} (90)
WT-APO[a]					1.8×10^{-3} (100)
D178N-APO					2.3×10^{-3} (100)
WT-APO with 20 μM Fe(II)	35 (30)		0.3 (70)		
D178N with 500 μM Fe(II)	7 ± 3 (20 ± 5)		0.35 (30)	0.045 (50)	
D178A with 500 μM Fe(II)			0.85 (32)	0.10 (68)	

[a] Data from ref *12*. [b] Phase present in some preparations, probably due to incomplete reconstitution of the mononuclear center of PDO. *A* is the relative contribution of the kinetic phase to the total absorbance change. Conditions as described in the legend of Figure 4.

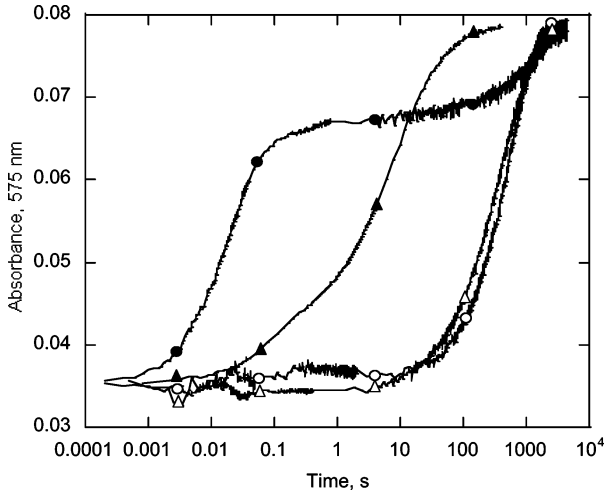


FIGURE 4: Oxidation of reduced PDO by oxygen. PDO (36 μM) was mixed with 250 μM O_2 in 0.1 M HEPES (pH 7.8) in the presence of 3 mM phthalate at 22 °C: PDO WT-Fe(II) (●), PDO WT-APO (○), and D178N before (△) and after (▲) anaerobic preincubation with 500 μM FAS for 10 min prior to mixing. All concentrations (except for the FAS) are those before mixing in the stopped-flow spectrophotometer.

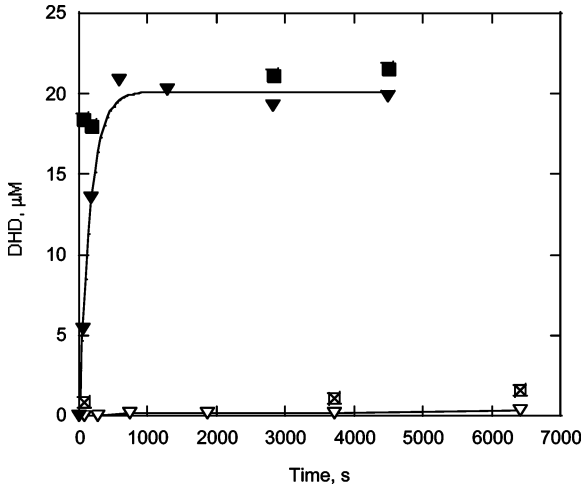


FIGURE 5: Product formation in the reaction of 43 μM (after mixing) reduced PDO D178N with oxygen in the presence of 3 mM phthalate. The reaction was initiated at time zero by the addition of the oxygenated buffer (625 μM after mixing) containing 3 mM phthalate. DHD formation was assessed after we quenched the reaction by filtering out the enzyme (triangles) and by heat denaturation (squares) in the absence of any additional Fe(II) (▽ and crossed box) and after anaerobic preincubation for 10 min with 500 μM FAS (▼ and ■). DHD release in the presence of extra Fe(II) was fit with a single-exponential function with a rate constant (k) of 6.2×10^{-3} s^{-1}.

Substitution with asparagine and alanine resulted in enzymes that exhibited ~4 and ~16% coupling of DHD formation, respectively, which could be a low estimate if PDR is also reacting with oxygen directly. Addition of excess Fe(II), although increasing the turnover rates of the variants, did not significantly improve the coupling of phthalate hydroxylation, and SOD and catalase had no effect. The reaction of reduced forms of D178N and D178A variants as purified with oxygen resulted in 8 and <2% coupling, respectively (Table 3). Anaerobic preincubation of the D178N variant in the presence of excess Fe(II) (500 μM) resulted in a dramatic increase in the coupling (to ~95%) to DHD formation (Table 3 and Figure 5), but for the D178A variant, addition of similar amounts of FAS failed to significantly increase the coupling of DHD formation (up to 7% only, Table 3). As in the WT enzyme (*14*), in both D178N and D178A variants product formed rapidly, presumably, on par with the observed oxidation of the Rieske centers, but the release of DHD from PDO was slow [$(6.2 \pm 0.7) \times 10^{-3}$ s^{-1}] (Figure 5).

DISCUSSION

The X-ray structures of NDO (*2*), BPDO (*3*), NBDO (*4*), CarDO (*6*), CumDO (*5*), and OMO (*7*) predict that the Rieske and Fe mononuclear centers interact across the $\alpha-\alpha$ interfaces of the $\alpha_3\beta_3$ multimers and that this interaction is facilitated by a bridging aspartate (e.g., Asp-205 in NDO) (Figure 1). We had previously proposed a somewhat similar arrangement of subunits in PDO (*13*, *14*) with the bridging Asp-178 facilitating one of the two possible electron transfer pathways from the Rieske center to the Fe mononuclear site (see the modeled structure in Figure 1B). For most of the above-mentioned oxygenases, substitutions of the bridging aspartate resulted in a drastic decrease of the steady-state rates of catalysis, but the reasons for that effect were unclear, as discussed above.

The results presented in this paper show that Asp-178 substitutions in PDO bring about significant changes in the properties of the mononuclear centers. Not only is the dissociation constant for Fe(II) increased by more than 2 orders of magnitude, but also Fe(II) binding in the variants appears to be cooperative, unlike that in the WT enzyme. These results suggest that the effects of the mutations are not limited to the immediate vicinity of the Fe mononuclear center but probably also affect the subunit interactions within the PDO multimer. Because the bridging aspartates link the two subunits, this behavior might be expected.

Like the results reported for other Rieske oxygenases (*9*, *10*), D178 PDO variants could be reduced by their reductive

partner, PDR. While the K_m values of the PDO−PDR interaction were not significantly affected by the D178 substitutions, the rates of the catalytically relevant electron transfers were substantially lower than in the WT enzyme, an effect that cannot be solely attributed to the loss of iron at the mononuclear center. Reduced Rieske centers of PDO D178A and D178N were oxidized by oxygen much more slowly than in WT PDO. As discussed above, this effect was only partially due to the low Fe(II) content of the mononuclear centers. Taken together, the increase in Fe(II) dissociation constants, the decreases in the rate of Rieske center reduction by PDR, and the slower rates of the reaction of reduced PDO with oxygen (Tables 4 and 5) can easily account for the ~100-fold decrease in the overall steady-state turnover rates observed for D178A and D178N variants.

Phthalate Dihydroxylation in the Steady-State and Oxidative Half-Reaction. Overall, the coupling of DHD formation to electron delivery was found to depend on two main factors: (i) the fraction of Rieske center that is oxidized via the mononuclear Fe(II) center and is responsible for catalysis versus the fraction that involves direct reaction of the Rieske center with oxygen [which is a function of the electron transfer efficiency to the Fe(II)] and (ii) the fraction of the active, iron-containing, mononuclear centers. In WT PDO, phthalate dihydroxylation to DHD is tightly coupled to NADH oxidation (Table 3). Steady-state catalysis remains similarly tightly coupled, even in PDO WT-APO, the enzyme that has only a small fraction of the mononuclear centers containing Fe(II).

Like that in NDO, the Rieske center in PDO does not carry out catalysis with the mononuclear center of the same subunit (see the discussion in ref *13*). In the reaction of oxygen with reduced NDO, the stoichiometry of product formation is one molecule of product per each Rieske center that is oxidized (*21*). The second electron, essential for substrate dihydroxylation, is provided by the ferrous mononuclear ion so that it was found in the ferric state at the end of the reaction. In contrast, in analogous reactions with PDO, upon completion of the single-turnover catalytic reaction, the mononuclear iron was found to be in the ferrous state (*14*). Thus, the Rieske center from a second subunit of PDO must provide the second electron for catalysis. The resultant stoichiometry is 1 DHD molecule produced per 2 Rieske centers oxidized (*14*). It is unknown whether this mode of oxidation (with two different Rieske centers supplying electrons to a single mononuclear center) is relevant during steady-state turnover where one Rieske center could, in principle, consecutively provide both electrons for the catalysis via a continuous supply of electrons from PDR and NADH.

Very little DHD formed in the reaction of reduced WT APO with oxygen because only those Rieske centers that are adjacent to mononuclear sites that contain iron can contribute to phthalate hydroxylation. It can be concluded that in PDO WT-APO, oxidation of the Rieske center proceeds predominantly through a noncatalytic pathway, presumably through a direct reaction of the Rieske centers with oxygen, with no DHD formation. Addition of FAS to PDO WT-APO restored the coupling in single-turnover experiments (Table 3). It is likely that only half of the mononuclear centers need to be reconstituted to generate the maximum possible amount of product (*14*).

In D178A and D178N PDO variants as purified, the amount of non-Rieske iron observed by ICP was ~0.2 Fe atom/monomer. As shown previously (*14*), to produce one DHD molecule, two reduced Rieske centers must cooperate in providing one electron each for the catalysis. Thus, in oxidative half-reactions, PDO with 20% of the mononuclear sites containing Fe(II) would be able to generate up to 40% of the total amount of product possible,[7] which would correspond to 40% coupling. The low coupling of DHD formation observed in single-turnover experiments with D178A and D178N variants (Table 3) indicates that either some mononuclear sites in the mutants are not active or most of this non-Rieske iron is actually bound elsewhere and thus is not relevant for catalysis. The latter correlates with the significantly weaker binding of iron at the mononuclear centers of D178 variants and with the previously found adventitious iron in Fe-reconstituted samples of WT PDO (*13*). That would also explain the marked similarity of the rates of oxidation of the Rieske centers in the variants and in PDO WT-APO (see Table 5), as a faster Fe-dependent phase would be expected to be present in the enzyme with 20% of the mononuclear sites populated with Fe(II). On the basis of the amount of product observed in single-turnover experiments, less than 1% for D178A and ~4% for D178N of the mononuclear centers in variants in as purified actually contained Fe(II).

In D178A and D178N variants, the rates of the catalytically relevant oxidation of the Rieske center (i.e., observed under Fe saturation conditions) are much smaller than in WT PDO. Moreover, in steady-state reactions, the coupling of DHD formation is low. Even addition of high concentrations of iron does not significantly improve the coupling, as the amount of product generated is determined predominantly by the difference between the catalytic and noncatalytic Rieske center oxidation rates. In the oxidative half-reaction, the amount of DHD formed depends not on the rates of electron transfer from the Rieske center but on the amounts of available mononuclear centers that contain Fe(II). Thus, addition of extra Fe(II) to the D178N variant, which can be reconstituted with iron, resulted in a marked increase in the level of DHD formation with the coupling improving from ~8 to nearly 100% (Table 3).[8] Similar addition to the as purified Dl78A variant, which cannot be readily reconstituted, did not increase the level of DHD formation (Table 3).

Overall, substitution of the bridging aspartate with either alanine or asparagine resulted in impaired enzymes with significantly similar properties. The observed differences between these variants, as described above, are largely explained by the inability of D178A to retain Fe(II) at the mononuclear center.

In this work, we have demonstrated that the observed low rates of catalytic activity of the D178A and D178N PDO variants can be ascribed to the cumulative effect of the low intrinsic iron content of the mutants and the combination of

[7] Assuming that for every two Rieske centers in PDO only one mononuclear center contains Fe(II).

[8] Considering that only one active Fe-containing mononuclear center per two reduced Rieske center is necessary for the hydroxylation of all phthalate bound to PDO, ~100% coupling for D178N reconstituted with Fe(II) indicates that at least 50% of the mononuclear centers had been reconstituted with Fe(II).

the decreased rates of reduction and oxidation of the Rieske center. Hydroxylation is still possible in the variants; however, the reaction appears uncoupled due to the slow rates of electron transfer between the Rieske and mononuclear centers and the small fraction of variant enzymes that contain iron at the mononuclear centers. The effect of substitutions in the Asp-178 position were not limited to those described above. In a following paper, we describe how these substitutions affect the state of the Rieske center and its ligands and how they influence subunit interactions that play important roles in the regulation of catalysis.

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