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The metal- and substrate-dependences of 2,4'-dihydroxyacetophenone dioxygenase

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Abstract

While the enzyme, 2,4'-dihydroxyacetophenone dioxygenase (DAD), has been known for decades, very little has been characterized of the mechanism of the DAD-catalyzed oxidative cleavage of its reported substrate, 2,4'-dihydroxyacetophenone (DHA). The purpose of this study was to identify the active metal center and to characterize the substrate-dependence of the kinetics of the reaction to lay the foundation for deeper mechanistic investigation. To this, the DAD V1M mutant (*b*DAD) was overexpressed, purified, and reconstituted with various metal ions. Kinetic assays evaluating the activity of the reconstituted enzyme as well as the substrate- and product-dependences of the reaction kinetics were performed. The results from reconstitution of the apoprotein with a variety of metal ions support the requirement for an Fe³⁺ center for enzyme activity. Reaction rates showed simple saturation kinetics for DHA with values for k_{cat} and K_{DHA} of 2.4 s⁻¹ and 0.7 μ M, respectively, but no significant dependence on the concentration of O₂. A low-level inhibition ($K_I = 1100 \mu$ M) by the 4HB product was observed. The results support a minimal kinetic model wherein DHA binds to resting ferric enzyme followed by rapid addition of O₂ to yield an intermediate complex that irreversibly collapses to products.

Keywords

2,4'-dihydroxyacetophenone dioxygenase; Dioxygenase; Non-heme iron; Steady-state kinetics; Substrate-dependence

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Author contributions

Kenneth M. Roberts: Conceptualization, Supervision, Funding acquisition, Formal analysis, Investigation, Writing; **Gabrielle C. Connor:** Funding acquisition, Methodology, Investigation; **C. Haley Cave:** Funding acquisition, Investigation; **Gerard T. Rowe:** Conceptualization, Methodology; **Clinton A. Page:** Methodology.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.abb.2020.108441>.

ACCENSION CODES

*b*DAD F7J5X4

1. Introduction

Discovered in the 1980's for its unique activity [1], 2,4'-dihydroxyacetophenone dioxygenase (DAD) is a putative non-heme iron-dependent enzyme which catalyzes the oxidative cleavage of the α -hydroxyketone moiety of 2,4'-dihydroxyacetophenone (DHA) (Scheme 1) [1–4]. Sequence analyses of DAD [4,5], confirmed by structures solved using X-ray diffraction [5,6], show it is a member of the cupin-2 superfamily of enzymes, an immense family of enzymes characterized by a conserved metal-binding fold but no consensus activity [7,8]. While the National Center for Biotechnology Information currently lists over 2600 homologous enzymes classified as DAD [9], surprisingly little has been done to understand the enzyme or its chemistry beyond basic characterization of the two homologs from *Alcaligenes* sp. 4HAP (*a*DAD) [3] and *Burkholderia* sp. AZ11 (*b*DAD) [4] (81% identity). Both homologs are homotetramers comprised of ~20 kDa subunits, each purifying with one iron atom per subunit [3,4]. Interestingly, broad sequence homology analyses show that DAD shares essentially no sequence homology to previously-studied cupin-fold dioxygenases including acireductone dioxygenase (ARD') and the intradiol dioxygenases, indicating DAD represents a distinct class.

The oxidation of DHA by DAD results in cleavage of the C₁–C₂ bond to give 4-hydroxybenzoic acid (4HB) and formic acid (Scheme 1). The reaction shows superficial similarity to that of the intradiol dioxygenases [10–12] and the iron-dependent reaction of ARD' [13,14], each involving oxidative cleavage between two oxidized carbons (Scheme 1). An early mechanistic study of the DAD reaction in the presence of H₂¹⁸O or ¹⁸O₂ showed that the oxygen atoms incorporated into each of the two products originate strictly from O₂, supporting DAD as a true dioxygenase [2]. Since this study, however, no mechanistic investigation of DAD has been published. Surprisingly, while the oxidative cleavage reactions catalyzed by DAD and ARD' are strikingly similar, there has previously been no connection made between the mechanisms of these two reactions. Instead, disparate mechanisms for the DAD reaction have been proposed based on the reaction mechanisms of the intradiol and extradiol dioxygenases [4,5]. Studies using purified *b*DAD found the enzyme was active under aerobic conditions and did not require prior reduction [4], suggesting that catalysis was driven by an Fe³⁺ metal center, as Fe²⁺ is prone to oxidize in the presence of O₂. This observation led the authors to propose a mechanism derived from the intradiol dioxygenases (Scheme 2A) [4,10]. Simply, coordination of DHA to DAD-Fe³⁺, followed by loss of a proton, results in an enediol analogous to the catecholic 1,2-diol substrates of the intradiol dioxygenases. This intermediate delocalizes an electron to the Fe³⁺ center generating Fe²⁺ and a benzylic radical. Dioxygen (O₂) can ligate to the reduced iron and attack the intermediate resulting in insertion of an O atom into the C₁–C₂ bond. This mechanism is problematic in that intradiol dioxygenases do not pass through a formal Fe²⁺ state for O₂-activation but instead formation of the peroxy bridge at the substrate and Fe³⁺-center is concerted [10,15]. Support for an intradiol dioxygenase-like mechanism, however, has come from biomimetic studies, with the distinction that such reactions begin with an Fe²⁺ center [16,17]. An alternative mechanism has been proposed from colorimetric observation of refractive crystals of *a*DAD [5]. Specifically, as extradiol dioxygenase enzymes are known to be colorless and intradiol dioxygenases are characteristically red-

colored [15], the colorless *a*DAD crystals were used as evidence for a mechanism requiring an Fe²⁺ center as seen for the extradiol dioxygenases (Scheme 2B). That many iron-dependent enzymes are colorless in the oxidized Fe³⁺ state was not considered. In the proposed mechanism, the enzyme begins as DAD-Fe²⁺ which, already being in the reduced state, binds O₂ directly. The deprotonation of the DAD·DHA·O₂ ternary complex results in a delocalized benzylic radical which recombines to yield products. However, that the enzyme does not require reduction for initiation of activity was not addressed. That DHA cleavage occurs *between* the hydroxyl groups may provide evidence against the mechanism of Scheme 2B as extradiol dioxygenases show near absolute specificity for cleavage *adjacent* to the 1,2-diol [15]. Intradiol-cleaving mutants of extradiol dioxygenases have been characterized, however [18,19], suggesting that the site of cleavage is likely due to bond proximity as opposed to a requisite behavior of the reaction.

As DAD has only been superficially characterized, the proposed mechanisms for the oxidative cleavage of DHA by DAD are purely speculative. The study presented here begins the process of elucidating the formal mechanism by investigating the identity of the chemically-active metal center and the steady-state kinetics of the reaction. Specifically, the dependences of the steady-state reaction on both substrates and the products are evaluated and a minimal kinetic model is proposed in the context of the previously proposed chemical mechanisms.

2. Materials and methods

2.1. Materials

The pUDP1.3 m vector containing the *dad c.1G>A* gene was a generous gift from Dr. Kiyofumi Maruyama at Gifu University, Gifu, Japan. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). *Escherichia coli* BL21(DE3) pLysS competent cells were purchased from Agilent (Santa Clara, CA). *E. coli* BL21(DE3) competent cells, NdeI and BamHI restriction enzymes, and T4 ligase were purchased from New England Biolabs (Ipswich, MA). Ampicillin, chloramphenicol, and isopropyl β-D-1-thiogalactopyranoside were purchased from GoldBio (St. Louis, MO). Q-Sepharose Fast-Flow chromatography resin was purchased from GE Healthcare (Chicago, IL). Iron(III) chloride, 4-hydroxybenzoic acid, trisodium nitrilotriacetic acid hydrate, and ethanol (200 proof) were purchased from Acros Organics, a subsidiary of ThermoFisher Scientific (Waltham, MA). Sodium dithionite (85%) was purchased from Alfa Aesar (Tewksbury, MA). Zinc nitrate tetrahydrate was purchased from EMD, now part of MilliporeSigma (Burlington, MA). 2-bromo-4'-hydroxyacetophenone was purchased from TCI Chemicals (Portland, OR). Mono- and dibasic potassium phosphate and ammonium sulfate were purchased from VWR (Radnor, PA). The pET20b(+) vector was purchased from ThermoFisher Scientific. All other reagents were purchased from Fisher Scientific, a subsidiary of ThermoFisher Scientific. All commercial reagents were used as received without further purification.

UV–visible absorbance assays were performed using an Agilent Cary 8454 UV–visible spectrophotometer fitted with a Quantum Northwest (Liberty Lake, WA) t2 Sport Peltier-driven temperature-controlled cuvette holder and a Semrock (Rochester, NY) BrightLine

267 nm blocking edge long-pass filter. Solution O₂ concentrations were determined and consumption assays were performed using a Hansatech (King's Lynn, Norfolk, England) Oxygraph + system.

2.2. Synthesis of 2,4'-dihydroxyacetophenone (DHA)

The DHA substrate was synthesized from 2-bromo-4'-hydroxyacetophenone modifying the acylation method of Robertson and Robinson [20] and the deacylation method of Zhang et al. [21] into a one-pot reaction. To a 100-mL flask containing 1.00 g (12.2 mmol) sodium acetate and 250 μ L (4.4 mmol) acetic acid in 25.0 mL ethanol, 1.00 g (4.7 mmol) 2-bromo-4'-hydroxyacetophenone was added while stirring. The mixture was refluxed for 75 min with complete consumption of 2-bromo-4'-hydroxyacetophenone indicated by TLC. The mixture was allowed to cool to room temperature. While stirring, 30 mL 0.5 M NaOH was added to the mixture and the reaction stirred at room temperature for 45 min. The reaction was neutralized with 5 mL 2 M hydrochloric acid and the product extracted with ethyl acetate. The extract was washed with brine, dried with magnesium sulfate, and the solvent removed by rotary evaporation. The crude product was recrystallized with water to yield a pale yellow solid (0.447 g, 2.94 mmol, 63%). ¹H NMR (60 MHz, D₂O): δ 7.85 (d, *J* = 9.2 Hz, 2H), δ 6.93 (d, *J* = 8.8 Hz, 2H), δ 4.91 (s, 2H).

2.3. Protein expression and purification

In our hands, we found that the expression of DAD from pUDP1.3 m was constitutive and not enhanced in the presence of IPTG. As the cloning site of pUC18 follows a *lac* promoter, it was concluded that the *dad c.1G > A* was too distal in the cloned cDNA to be induced by IPTG and instead was downstream of an alternative promoter that permitted constitutive expression. To support better control of the expression of the *dad c.1G > A* gene and to increase expression yields, the gene was subcloned from pUDP1.3 m into pET20b(+). Specifically, *dad c.1G > A* (which codes for DAD V1M from *Burkholderia* sp. AZ11) [4] was amplified from pUDP1.3 m by polymerase chain reaction using oligonucleotide primers incorporating an upstream NdeI restriction site (forward: 5'-ATACATATGGTCGACAAAGCCGTATCCG) and a downstream BamHI (reverse: 5'-ATAGGATCCTCAGCGGAACAGCTTCTCGA) restriction site. Positioning of the NdeI site resulted in placement of the ATC start codon 7-bp downstream of the pET20b(+) vector. Both the amplified gene and pET20b(+) were double-digested with Nde and BamHI and subsequently ligated using T4 ligase to yield the pDAD construct. The *dad c.1G > A* gene of pDAD was verified by sequencing and the vector transformed into *Escherichia coli* BL21(DE3) cells either containing or lacking the pLysS plasmid.

The overexpression of DAD V1M (hereafter "*bDAD*") was adapted from the procedure of Enya et al. [4] LB-Miller media (100 mL) supplemented with 100 μ g mL⁻¹ ampicillin (and 50 μ g mL⁻¹ chloramphenicol if pLysS was present) was inoculated with a single colony of *E. coli* BL21(DE3) pDAD cells and the culture grown overnight at 37 °C, 250 RPM. Culture flasks (1.0 L) containing 400 mL LB-Miller supplemented with 4.0 mM magnesium sulfate 1.0 mM iron(III) chloride, 34 μ g mL⁻¹ chloramphenicol (if necessary), and 100 μ g mL⁻¹ ampicillin were each inoculated with 4.0 mL of overnight culture and grown at 37 °C, 250 RPM. At OD₆₀₀ 0.7–0.9, expression of *bDAD* was induced with 500 μ M IPTG and the

cultures grown for a further 5.0 h at 37 °C, 250 RPM. Cells were harvested by centrifuging the cultures at 4700g for 30 min. The supernatant was decanted and the cell pellet frozen overnight at -80 °C. Expression of the *dad c.1G > A* gene from the pDAD vector in *E. coli* BL21(DE3) cells showed specific response to IPTG and gave high-yield overexpression of bDAD with the enzyme being as much as 50% of the cell lysate protein by SDS-polyacrylamide electrophoresis (Fig. S1).

All purification steps were performed at room temperature unless indicated. The pellets were thawed and resuspended in 50 mM potassium phosphate, pH 7.0 (Buffer A; 8 mL per gram cell pellet) and the resuspension homogenized by passing twice through an 18G needle then stirred for 30 min. The cell mixture; were lysed by sonification using a Branson Ultrasonics (Danbury CT) Sonifier S-450A (six rounds of 45 s at 50% duty cycle, 4.5 power). Mixtures were incubated on ice for 5 min between sonication rounds. Lysed mixtures were centrifuged at 14,400g for 30 min at 4 °C to remove the insoluble fraction. A large, unrecoverable portion (~50%) of the expressed DAD is lost to the insoluble fraction likely due to the instability of apoprotein resulting from insufficient loading of the metal cofactor. The lysis supernatants were combined, aliquoted into 15-mL disposable centrifuge tubes, and incubated in a 65 °C bath for 8 min. The tubes were inverted ten times to mix, then incubated a further 10 min at 65 °C. Denatured protein was removed by centrifugation (14,400g, 30 min, 4 °C) and the supernatants combined. Ammonium sulfate was added to the supernatants to 40% saturation and the mixture stirred at 4 °C for 40 min. The precipitated protein was harvested by centrifugation (14,400g, 30 min, 4 °C). The resulting pellet was resuspended in Buffer A (an equivalent volume as was used for resuspension) and dialyzed against Buffer A to remove salts. The dialyzed protein was loaded onto Q Sepharose Fast-Flow (bDAD appears greyish blue when bound) and washed with five column volumes of Buffer A followed by three column volumes of Buffer A + 150 mM sodium chloride. The bDAD protein was eluted with a gradient of 150–400 mM sodium chloride over 10 column volumes. Fractions containing bDAD were verified by SDS-PAGE with those showing greater than 95% DAD combined and dialyzed against Buffer A. In our hands, bDAD typically shows as two bands by SDS-PAGE (Fig. S1), similar to that seen with aDAD [3], which can be resolved into a single homogeneous band upon incubation in SDS at 95 °C for greater than 1 h (Addition of β -mercaptoethanol did not affect the double-banding.) bDAD purified as described was found to be stable for several months at 4 °C. It is notable that concentrated stocks of purified bDAD show a strong gray color. Due to increased instability of the enzyme below pH 7.0, all bDAD stocks and samples were kept at pH 7.0 prior to use in activity assays. The concentration of purified bDAD was determined using a “per monomer” molar absorptivity of 48,200 M⁻¹ cm⁻¹ based on the equation from Pace et al. [22] When determined by a bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL), the molar absorptivity was consistently found to be within 10% of the value calculated from Pace et al.

2.4. DAD-metal ion reconstitution

For the metal-replacement study, the native metal cofactor was removed by dialyzing the purified bDAD against 20 mM ethylenediaminetetraacetic acid (EDTA), 20 mM nitrilotriacetic acid (NTA), 20 mM sodium dithionite, 50 mM potassium phosphate, pH 7.0

(four changes over 48 h) at 4 °C. Such treatment resulted in the loss of the gray coloring of the protein. Following metal removal, the apoprotein (apoDAD) was dialyzed against four changes of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 7.0 (four changes) at 4 °C to remove the chelators and reducing agent and then centrifuged at 22,800g, 4 °C for 30 min to remove any aggregated solids. The supernatant was collected and stored at 4 °C.

Reconstitution of DAD with various metal ions was performed by incubating solutions of apoDAD in the presence of five equivalents of a metal salt (one of: calcium chloride dihydrate; copper(II) nitrate hemipentahydrate; magnesium sulfate; manganese(II) chloride tetrahydrate; nickel(II) sulfate hexahydrate; or zinc nitrate tetrahydrate). Specifically, reconstitution mixtures were prepared by adding 15 µL of 10 mM metal salt (150 nmol) in 2 mM HCl to a solution of 20 µM apoDAD (30 nmol) in 50 mM MES, pH 7.0 (1.50 mL final volume). Mixtures were incubated at room temperature and enzyme activity tested after 2 h and after overnight incubation (minimum of 12 h).

Reconstitution of DAD with iron was performed anaerobically using a glass tonometer. For each mixture, 20 µM apoDAD in 50 mM MES, pH 7.0 (5 mL) was placed in the body of a tonometer with a sidearm port. The sidearm was charged with an appropriate volume of 10 or 20 mM ferrous ammonium sulfate hexahydrate in 2 mM HCl and the sidearm was then attached to the tonometer body and sealed. Beyond the stopcock on one end, the tonometer was capped with a rubber septum. The contents were made anaerobic through 10 vacuum/N₂ cycles with the tonometer gently rocked such that the contents in the sidearm were kept isolated. After the vacuum/N₂ cycles, the contents were mixed by inverting into and out of the side arm 10 times, collecting the bulk of the final mixture into the body of the tonometer. Two additional vacuum/N₂ cycles were performed. The anaerobic solution was sampled from the tonometer by inserting the needle of a 25 µL glass syringe with Teflon plunger through the septum and stopcock at the one end of the tonometer and collecting 15 µL of mixture. Anaerobic samples were immediately added to reaction mixtures.

2.5. DHA-consumption assays

The oxidative cleavage of DHA by *b*DAD was monitored by UV absorbance spectroscopy. The conversion of DHA to 4HB results in a decrease in absorbance above 260 nm with maximum at 277 nm ($\epsilon_{277} = 11,400 \text{ M}^{-1} \text{ cm}^{-1}$) corresponding to the λ_{max} for DHA. Due to our observation that DHA undergoes photobleaching at wavelengths below 270 nm, a long-pass filter with a 267 nm cut-off was used. DHA consumption assays were performed by adding enzyme to preincubated solutions of increasing concentrations of DHA and monitoring the decrease in absorbance at 277 nm at 0.5 s intervals. In a typical assay, 3.0 mL of a solution of DHA in 50 mM MES, 100 mM sodium chloride, pH 6.0 (Buffer B) was added to a 1.0 × 1.0 cm quartz cuvette (Agilent) and preincubated with stirring in the cuvette holder at 20.0 °C for at least 1.5 min. The reaction was initiated with 2.5 µL of 5.0 µM (4.2 nM final) *b*DAD in Buffer A and the absorbance monitored at 0.5 s intervals. Inhibition assays were performed by adding 20 µL of DHA stock to a cuvette containing 3. mL of a solution of either 4HB or formic acid in Buffer B. Reactions were similarly initiated with 2.5 µL of 5.0 µM *b*DAD. For the metal reconstitution assays, reactions were initiated with 15 µL of reconstitution mixture.

For assays performed at non-ambient O₂ concentrations (1% and 100%), samples were prepared by bubbling appropriate N₂/O₂ mixtures into a sealable cuvette containing solutions of DHA. Specifically, 1% or 100% O₂ mixtures were prepared using a Maxtec (Salt Lake City, UT) MaxBlend 2 oxygen blender. The absolute concentration of output O₂ was determined by bubbling the N₂/O₂ mixture into an O₂ probe cell containing 2.0 mL of Buffer B at 20.0 °C for 10 min and measuring the concentration of dissolved O₂. A screw-top sealed quartz cuvette (1.0 × 1.0 cm) equipped with a vent needle was charged with 3.0 mL of DHA in Buffer B and loaded into the temperature-controlled cuvette holder at 20.0 °C. The measured O₂ mixture was then bubbled through a needle into the DHA solution while stirring for 5 min. After bubbling, the needle was removed and the reaction immediately initiated by addition of *b*DAD using a Hamilton (Reno, NV) Gastight syringe, then monitoring the consumption of DHA at 277 nm.

Time-dependent traces of the consumption of DHA by *b*DAD were complicated by a first-order decrease in the absorbance over the first 30 s of the reaction. Reaction samples across various concentrations of DHA and *b*DAD showing clear linearity after 90 s were fit with equation (1) yielding an invariant value for the rate constant (k_{decay}) of $0.039 \pm 0.008 \text{ s}^{-1}$ (Fig. S2). As the value for k_{decay} is less than 2% of the value for the rate constant for turnover (k_{cat} ; described below), it is most likely that this feature is a complication of the assay and not a component of the enzyme reaction. The decay feature is significantly reduced but not eliminated by initiating the reaction with enzyme. To this, initial rates of DHA consumption were determined using KaleidaGraph 4.5 (Synergy Software, Reading, PA) from fits of the A₂₇₇ traces over the first 30–90s with equation (1) applying a fixed value of 0.039 s^{-1} for k_{decay} and a value for ϵ_{277} of $11,400 \text{ M}^{-1} \text{ cm}^{-1}$. Reported values were calculated as the average slope of at least three independent samples. Analyses of the concentration-dependence of initial rates were also performed using KaleidaGraph.

$$Abs = Ae^{-k_{\text{decay}}t} + mt + b \quad (1)$$

2.6. O₂-consumption assays

The consumption of O₂ in the oxidation of DHA by DAD was monitored using a Hansatech Oxygraph + system. Reactions were performed by adding 2.0 mL of air-saturated 25 μM DHA in Buffer B to a jacket-cooled probe cell (20.0 °C) calibrated at air-saturation. The mixture was preincubated at 20.0 °C for at least 5 min and the reaction initiated with 20 μL of 5.0 μM *b*DAD. The time-dependent concentration of O₂ was monitored until reaction completion. The rate of O₂ consumption was determined as the slope of the trace over the first 60 s using KaleidaGraph with the reported value calculated as the average slope of five independent samples.

3. Results and discussion

3.1. The metal-dependence of DAD

The requirement for a non-heme iron in the DAD-catalyzed oxidation of DHA was investigated through metal-replacement. The metal ion of recombinantly-expressed *b*DAD

was removed by chelation and the resulting apoprotein (apoDAD) was reconstituted with various exogenous metal ions. Direct removal of the metal ion by dialyzing *b*DAD against 20 mM EDTA and 20 mM NTA at pH 7.0 as chelators for a full week resulted in only 10% activity loss indicating that the metal ion is held very tightly by the enzyme. Successful removal of the metal ion was accomplished by dialyzing the enzyme with 20 mM EDTA and 20 mM NTA in the presence of 20 mM dithionite as a reducing agent (at pH 7.0). After 24 h of dialysis in the reducing buffer, the enzyme showed a loss of ~85% activity. An additional 24 h of dialysis resulted in apoprotein with 2% activity (Table 1). The loss of activity coincided with the disappearance of a shoulder in the absorbance spectrum at 360 nm (Fig. 1) as well as loss of the distinct gray color of the enzyme.

In attempts to recover enzyme activity, the apoDAD was incubated with five equivalents of various divalent metal ions in 50 mM MES, pH 7.0 under air exposure. The activity of the reconstitution mixtures was determined by adding samples of each to air-saturated solutions of 83 μ M DHA in 50 mM MES, 100 mM NaCl, pH 6.0 in a UV–visible spectrophotometer, monitoring the consumption of DHA as a decrease in absorbance at 277 nm. Incubation of apoDAD with Ca^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , or Zn^{2+} resulted in no effect on the background activity after either 2 h or overnight (Table 1). In contrast, incubation with Cu^{2+} resulted in a further decrease in the low-level activity of apoDAD, indicating that the ion may be inhibitory to the enzyme.

As the Fe^{2+} ion is generally unstable in air at neutral pH, apoDAD in 50 mM MES, pH 7.0 was reconstituted with Fe^{2+} under anaerobic conditions at several apoDAD:Fe ratios (1:0, 1:1, 1:2, 1:5, and 1:10) using a glass tonometer. The reconstitution mixtures were then either directly assayed or exposed to air prior to addition to the reaction mixture. When the anaerobic reconstitution mixtures were assayed directly, the initial rates indicated an increase of as much as three-fold in activity over apoDAD alone (Table 1), but still amounting to less than 10% of the activity of purified *b*DAD. Interestingly, in all cases, the rate of DHA consumption increased over time (Fig. 2 inset, red dashes). When assayed directly, the time-dependent traces for the anaerobic reconstitution mixtures were identical regardless of the time of incubation under anaerobic conditions (Fig. 2 inset). In contrast, exposing the anaerobic reconstitution mixtures to air for 2 h resulted in significant increases in activity with maximal activity achieved after overnight exposure (Table 1; Fig. 2 inset). The rates of DHA consumption by apoDAD:Fe reconstitutions exposed to air were linear and demonstrated a significant dependence on the apoDAD:Fe ratio, with rates increasing up to a ratio of 1:10 (Table 1; Fig. 2). After overnight exposure to air, the 1:5 and 1:10 apoDAD:Fe reconstitutions achieved 80 and 85% of the activity seen with *b*DAD alone, respectively. UV–visible absorbance spectra of the reconstituted mixtures after overnight exposure to air showed a shoulder at 360 nm similar to that seen in the as-isolated *b*DAD (Fig. 1). This feature increased in intensity with the apoDAD:Fe ratio (Fig. 1), coinciding with the observed increase in activity. The intensity of the shoulder maximized in the 1:10 reconstitution, being similar in intensity to that of the purified *b*DAD. While the anaerobic reconstitution of apoDAD with Fe^{2+} resulted in a broad spectrum increase in absorbance, no absorbance feature was observed at 360 nm prior to incubation in air (Fig. 1 inset) indicating that this feature is specific to an oxidized DAD- Fe^{3+} complex. Further, that *b*DAD shows this feature supports that the enzyme is isolated in the oxidized Fe^{3+} state.

The near-complete recovery of activity with apoDAD:Fe reconstitution mixtures (in conjunction with the absence of activity from other tested metal ions) strongly supports a non-heme iron as the active site metal cofactor. Due to the high insolubility of the Fe^{3+} ion in neutral solutions, it is not possible to reconstitute apoDAD directly with Fe^{3+} . However, the low activity seen from the anaerobic apoDAD: Fe^{2+} mixtures followed by large increases in activity upon oxidation signifies that the active state of the non-heme iron is, in fact, the oxidized Fe^{3+} . This explains the earlier observations that *b*DAD does not require reduction prior to initiation of the reaction [4]. The small but significant increase in the activity of the anaerobic apoDAD- Fe^{2+} mixtures is likely due to oxidation of the Fe^{2+} ion by trace O_2 in the anaerobic preparations. The requirement for a significant excess of Fe^{2+} to achieve maximal activity suggests that Fe^{2+} does not bind tightly to the apoDAD. Incubating apoDAD with an excess of Fe^{2+} establishes an equilibrium of bound and unbound Fe^{2+} . Oxidation of the unbound Fe^{2+} results in insoluble Fe^{3+} precipitate (which is removable by centrifugation). In contrast, oxidation of the DAD-bound Fe^{2+} results in DAD- Fe^{3+} which is robust and chemically active. This is supported by the observation that *b*DAD must be reduced prior to removing the metal center to yield apoDAD. The requirement for an Fe^{3+} center supports an intradiol dioxygenase-like mechanism such as that in Scheme 2A in contrast to the Fe^{2+} -dependent mechanism of the extradiol dioxygenases (Scheme 2B). Specifically, DHA binds to the free DAD- Fe^{3+} to form a binary complex that reacts with O_2 to promote the oxidative cleavage of the DHA substrate. Whether the DAD-DHA binary complex results in reduction of the metal in preparation for O_2 binding (Scheme 2A) or instead binds O_2 directly to form the peroxy bridge (as seen with intradiol dioxygenases) is unclear at this time.

Anaerobic reconstitution of the purified *b*DAD with five equivalents of Fe^{2+} resulted in an increase in activity of 7% which increased to 13% upon overnight incubation in air (Table 1; Fig. 2). As this concentration of Fe^{2+} was sufficient to almost completely reconstitute apoDAD activity, it is expected that the additional activity in *b*DAD reconstitutions is due to the presence of apoDAD in the purified *b*DAD. The observed 10% increase in activity upon reconstitution supports that, under the methods described here, purified *b*DAD has an iron occupancy of 90%.

3.2. Substrate stoichiometry

To determine the substrate stoichiometry of the reaction, *b*DAD was added to an air-saturated solution of DHA in an O_2 probe cell at 20.0°C and the concentration of O_2 was monitored over 60 s. The value for k_{cat} calculated from the consumption of O_2 is in good agreement with the value from the consumption of DHA (see below) giving an O_2 :DHA consumption ratio of 0.96:1 (Table 2). This value supports a 1:1 stoichiometry for DHA and O_2 in the reaction. Together with the $^{18}\text{O}_2$ and H_2^{18}O studies by Hopper [2], the 1:1 substrate stoichiometry completes the definition of DAD as a true dioxygenase. As both 4HB and formic acid each receive a single O atom originating from O_2 , the 1:1 stoichiometry observed in the current study strongly supports a mechanism in which a single O_2 substrate molecule is subsequently cleaved with one atom going to each of the DHA cleavage products.

3.3. Substrate-dependence of the steady-state kinetics

The contributions of both DHA and O₂ to the kinetics of the oxidation of DHA by *b*DAD were evaluated from the dependence of the turnover rate on the concentration of DHA. To this, solutions of DHA (0.2–25 μM) were prepared at either air-saturation (260 μM dissolved O₂) or bubbled with 1 or 100% O₂ (12 or 1200 μM dissolved O₂) in a UV–visible spectrophotometer at 20.0 °C. Reactions were initiated with 4.2 nM *b*DAD and the rate of DHA consumption was monitored at 277 nm. Time-dependent traces for samples with limiting O₂ concentrations (*e.g.* 25 μM DHA, 12 μM O₂) showed incomplete consumption of DHA, as expected. The observed rate constants at each DHA–O₂ concentration pair were plotted against the concentration of DHA (Fig. 3). The dependence of the rate on the concentration of DHA demonstrated simple saturation kinetics at each concentration of O₂. Consequently, the complete data set was globally fit with a two-substrate model (equation (2)) with the apparent kinetic parameters given in Table 2. The value for k_{cat} is in good agreement with the value of 2.4 s^{−1} reported by Enya et al. for *b*DAD [4] and more than twice that of the approximately 1 s^{−1} seen with the *a*DAD homolog [3]. In contrast, we report a lower value for K_{DHA} than the 1.6 μM previously reported [4]. That the dependence of the observed rate constant on the concentration of DHA is well-described by a simple saturation model supports that the formation of the DAD–DHA complex is not complicated by other kinetic factors including substrate inhibition or cooperative substrate binding. The potential for substrate inhibition was investigated by performing reactions with concentrations of DHA up to 125 μM at air saturation. Under these conditions, no significant inhibition of activity was observed (not shown). Further, while *b*DAD is reported to be tetrameric in solution [4], the lack of any notable sigmoidal behavior in the DHA-dependence supports the absence of cooperativity in DHA binding.

$$k_{\text{obs}} = \frac{k_{\text{cat}}[\text{DHA}][\text{O}_2]}{K_{\text{O}_2}[\text{DHA}] + K_{\text{DHA}}[\text{O}_2] + [\text{DHA}][\text{O}_2]} \quad (2)$$

Surprisingly, the apparent K_{O_2} was calculated to be indistinguishable from zero (Table 2) which can easily be seen from the near identity of the DHA-dependence at all three concentrations of O₂ (Fig. 3). The apparent independence of the rate of the reaction on the concentration of O₂ limits the kinetic mechanism to a few, select mechanisms. First, it is possible that the value for the apparent K_{O_2} reflects the value of the intrinsic K_{O_2} . Such a value for K_{O_2} is highly unlikely as this would put DAD in direct competition with important aerobic enzymes including ferritin (K_{O_2} : ~1 μM) [23] and cytochrome *c* oxidase (K_{O_2} : ~1 μM) [24,25]. Alternatively, the O₂-dependence could be masked by a subsequent, irreversible step as can be seen in a rapid-equilibrium ordered mechanism: [26–28]. In such a model, this irreversible step would follow the rapid-equilibrium binding of O₂. Specifically, DAD and O₂ would be in rapid equilibrium with the DAD·O₂ complex, with formation of the complex undergoing irreversible conversion to an intermediate. The concentration-dependence on O₂ is masked as the binary complex is forming and deforming so rapidly that it appears as if saturated with O₂. This pseudo-saturated binary complex is then trapped by the subsequent binding of DHA into the active DAD·O₂·DHA ternary complex (Scheme 3). A rapid-equilibrium mechanism does not require O₂ to bind first,

however, as long as the subsequent step is irreversible. In such a case, DHA could bind reversibly to form the DAD·DHA binary complex followed by the equilibrium binding of O₂ to form the DAD·DHA·O₂ complex. Irreversible conversion to intermediate or product would mask the dependence of the rate on the O₂ concentration. A similar effect would be observed if the DAD·DHA binary complex reacted immediately and irreversibly upon collision with O₂ (Scheme 4). As long as the rate of such a step was very rapid in comparison to the subsequent step, any concentration-dependence on O₂ would be masked.

Typically, distinguishing the substrate binding order with one substrate showing rapid-equilibrium binding is done through the use of product inhibition or substrate analogs as dead-end inhibitors and determining their modes of inhibition relative to each substrate [27,28]. However, as the ability of O₂ to bind DAD is dependent on the redox state of the iron center [29–31], the redox state of the active enzyme clarifies the binding order. As the reaction begins with DAD in the oxidized Fe³⁺ state, O₂ cannot bind until formation of the DAD·DHA binary complex wherein O₂ can either react directly with the complex (as seen for intradiol dioxygenases) or to a reduced metal intermediate as that proposed by Enya et al. (Scheme 2A). Addition of O₂ in this manner results in irreversible product formation (Scheme 4). It is possible that O₂ does not initially bind the metal at all but instead directly attacks the bound substrate, similar to early proposals for ARD' [32]. However, such a mechanism would still require prior formation of the DAD·DHA binary complex.

3.4. Product inhibition of DAD activity

A common method for distinguishing a rapid-equilibrium ordered mechanism is by evaluating any product inhibition of the steady-state reaction. To this, reaction assays were performed by adding DAD to preincubated mixtures containing DHA and either formic acid or 4HB in a UV absorbance spectrometer at 20.0 °C. The pH of the assay mixtures was 6.0, and the relevant species of inhibitor in solution can be assumed to be the deprotonated form (formate or 4-hydroxybenzoate, respectively). Reaction rates were determined from the consumption of DHA at 277 nm. In assays with formate, no apparent inhibition of enzyme activity was observed in the presence of as high as 1.25 mM formate. In contrast, initial screening of enzyme activity in the presence of 1.25 mM 4HB suggested product inhibition may be a factor in the steady-state kinetics. To further investigate the potential for inhibition by 4HB, reactions were performed by adding DAD to DHA-4HB mixtures across a range of concentrations: 0.45–25 μM DHA; 0–1.25 μM 4HB. Each DHA-4HB concentration pair was plotted against the concentration of DHA (Fig. 4). The concentration-dependent data was globally fit to a competitive inhibition model (equation (3)) with the best-fit values for the apparent kinetic parameters reported in Table 2. A low-level competitive product inhibition by 4HB was observed with a value for K_I of ~1 mM. Fitting the data with a mixed inhibition model (not shown) did not significantly improve the quality of the fit and suggested that any uncompetitive mode of inhibition must be no stronger than 10 mM in magnitude. The large value of K_I for 4HB and the lack of any inhibition by formate suggests that product inhibition does not play a significant factor in the steady-state kinetics of DAD and that product release is effectively irreversible. Further, as the background absorbance of 4HB is too large to perform assays above 1.25 mM, the inhibition by 4HB was too weak to assist in kinetic confirmation of the substrate binding order.

$$k_{\text{obs}} = \frac{k_{\text{cat}}[\text{DHA}]}{K_{\text{DHA}} \left(1 + \frac{[\text{4HB}]}{K_I} \right) + [\text{DHA}]} \quad (3)$$

4. Conclusions

Intact *b*DAD was successfully reconstituted from apoDAD upon anaerobic incubation with iron. Full activity was achieved after aerobic incubation of the reconstituted enzyme providing strong support for an Fe³⁺ as the active metal center. Further, kinetic analyses support a mechanism in which DHA binds DAD to form a complex which reacts rapidly with O₂ to facilitate product formation (Scheme 4). This is in contrast to mechanisms in which O₂ binds the enzyme first, requiring a reduced Fe²⁺ in the resting state. Product inhibition of the reaction is weak and does not present a significant contribution to the kinetics of the reaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4HB	4-hydroxybenzoic acid
apoDAD	2,4'-dihydroxyacetophenone dioxygenase apoprotein
ARD'	iron-dependent acireductone dioxygenase
DAD	2,4'-dihydroxyacetophenone dioxygenase
<i>a</i>DAD	<i>Alcaligenes</i> sp. 4HAP 2,4'-dihydroxyacetophenone dioxygenase
<i>b</i>DAD	<i>Burkholderia</i> sp. AZ11 2,4'-dihydroxyacetophenone dioxygenase
DHA	2,4'-dihydroxyacetophenone

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Fig. 1.

UV-visible absorbance spectra for DAD-Fe reconstitutions. Samples consisted of the anaerobic mixing of 20 μM DAD in 50 mM MES, pH 7.0 with varying equivalents of Fe^{2+} in 2mM HCl followed by overnight incubation in air. Spectra were determined from 10-fold dilutions of the reconstitution mixtures with 50 mM MES, pH 7.0. DAD-Fe mixtures: apoDAD (red crosses); apoDAD:Fe 1:1 (orange exes); apoDAD:Fe 1:2 (yellow diamonds); apoDAD:Fe 1:5 (green triangles); apoDAD:Fe 1:10 (blue circles); and bDAD (gray dashes). Inset: Reconstitution of apoDAD-Fe 1:10. Mixtures: apoDAD (red crosses), anaerobically-prepared apoDAD-Fe 1:10 (purple squares), and apoDAD-Fe 1:10 incubated in air overnight (blue circles). Spectra were determined without dilution. Each spectrum represents the

average of at least four replicates. Spectra are offset for clarity. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2.

The consumption of DHA by DAD reconstituted with Fe^{2+} . Reaction samples consisted of adding aerobically-incubated DAD-Fe reconstitutions to 83 μM DHA in 50 mM MES, 100 mM NaCl, pH 6.0 at 20.0 $^{\circ}\text{C}$. The final enzyme concentration in each assay was 100 nM. DAD-Fe mixtures: apoDAD (red crosses); apoDAD:Fe 1:1 (orange exes); apoDAD:Fe 1:2 (yellow diamonds); apoDAD:Fe 1:5 (green triangles); apoDAD:Fe 1:10 (blue circles); bDAD (gray dashes), bDAD:Fe 1:5 (black solid line). Inset: The effect of incubation time on DHA consumption by apoDAD:Fe 1:5 reconstitutions incubated aerobically or anaerobically

prior to assay. Incubation time: none (red dashes), 2 h anaerobic (black circles), 2 h aerobic (green dotted line), overnight aerobic (blue solid line). Each trace represents the average of four to five replicate traces. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3.

The dependence of the observed rate constant in the oxidation of DHA by *b*DAD on the concentrations of the substrates. Reactions samples consisted of mixtures containing varying concentrations of DHA and 12, 260, or 1200 μM O₂ in 50 mM MES, 100 mM NaCl, pH 6.0 at 20.0°C. Reactions were initiated with *b*DAD (4.2, 8.3, 17, 25, 33, or 41 nM). Each point represents the average of three to four replicates. The lines indicate the global fit of the concentration-dependent data with equation (2) with best-fit values for the kinetic parameters given in Table 2.

Fig. 4.

Product inhibition by 4HB in the oxidation of DHA by *b*DAD. Reaction samples consisted of 4.2 nM *b*DAD added to varying concentrations of DHA and 0 (red circles), 300 (orange squares), 600 (yellow diamonds), 900 (green triangles), or 1250 μM 4HB (blue exes) in 50 mM MES, 100 mM NaCl, pH 6.0 at 20.0 °C. Each point represents the average of three to six replicates. The dashed curves indicate the global fit of the concentration-dependent data with equation (3). Best-fit values for the kinetic parameters are given in Table 2. Inset: Double-reciprocal plot of the concentration-dependent data with the dashed lines

representing the globally fit curves. Some points omitted for clarity. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Scheme 1.

The oxidative cleavage reactions catalyzed by DAD, ARD' , and the intradiol dioxygenases.

Scheme 2.

Proposed mechanisms for the DAD-catalyzed oxidative cleavage of DHA. (A) Adapted from Enya et al. [4] (B) Adapted from Keegan et al. [5].

Scheme 3.

Rapid-equilibrium ordered binding model with equilibrium formation of a DAD·O₂ complex followed by DHA binding.

Scheme 4.

Minimal kinetic model for the addition of O₂ to a DAD·DHA binary complex resulting in irreversible product formation.

Table 1

Observed V/E values for DHA consumption by various DAD-Metal reconstitutions.

Enzyme-Metal	DAD: Metal Ratio	Anaerobic (s ⁻¹)	Time in Air	
			2 h (s ⁻¹)	Overnight (s ⁻¹)
hDAD-Fe ²⁺	1:0			1.99 ± 0.02 ^c
		1.92 ± 0.02 ^a	2.01 ± 0.02	2.02 ± 0.01
		2.06 ± 0.05 ^a	2.24 ± 0.03	2.29 ± 0.02
apoDAD-Fe ²⁺	1:0			0.04 ± 0.01 ^c
		0.05 ± 0.01 ^a	0.11 ± 0.01	0.12 ± 0.01
		0.13 ± 0.04 ^a	0.68 ± 0.01	0.75 ± 0.01
		0.07 ± 0.03 ^a	0.81 ± 0.03	1.41 ± 0.03
		0.14 ± 0.02 ^a	1.11 ± 0.04	1.61 ± 0.01
apoDAD-Ca ²⁺	1:10	0.16 ± 0.02 ^a	1.09 ± 0.03	1.72 ± 0.04
		<i>nd</i>	0.06 ± 0.01 ^b	0.06 ± 0.01
		<i>nd</i>	0.01 ± 0.01 ^b	0.01 ± 0.01
		<i>nd</i>	0.05 ± 0.01 ^b	0.04 ± 0.01
		<i>nd</i>	0.04 ± 0.01 ^b	0.04 ± 0.01
apoDAD-Mg ²⁺	1:5	<i>nd</i>	0.04 ± 0.01 ^b	0.04 ± 0.01
		<i>nd</i>	0.04 ± 0.01 ^b	0.04 ± 0.01
		<i>nd</i>	0.04 ± 0.01 ^b	0.04 ± 0.01
		<i>nd</i>	0.04 ± 0.01 ^b	0.04 ± 0.01
		<i>nd</i>	0.03 ± 0.01 ^b	0.03 ± 0.03

^a Determined from anaerobic reconstitution.

^b Determined from aerobic reconstitution.

^c Determined using aerobic stocks of hDAD or apoDAD; *nd*: not determined.

Table 2

Substrate-dependent steady-state kinetic parameters.

Consumption Assay	k_{cat} (s^{-1})	K_{DHA} (μM)	k_{cat}/K_{DHA} ($\times 10^6 M^{-1} s^{-1}$)	K_{O_2} (μM)	k_{cat}/K_{O_2} ($\times 10^6 M^{-1} s^{-1}$)	4HB K_I (μM)
DHA ^a	2.42 ± 0.03	0.63 ± 0.03	3.8 ± 0.2	0.09 ± 0.26	28 ± 82	
DHA ^b	1.92 ± 0.04	0.74 ± 0.10	2.6 ± 0.3	<i>nd</i>	<i>nd</i>	1100 ± 400
O ₂	2.32 ± 0.16					

^aDetermined from fits of the data in Fig. 3 to equation (2).

^bDetermined from fits of the data in Fig. 4 to equation (3); *nd*, not determined.