SUPPORTING INFORMATION

Rate-determining Attack on Substrate Precedes Rieske Cluster Oxidation during *cis*-Dihydroxylation by Benzoate Dioxygenase

Brent S. Rivard,[†] Melanie S. Rogers,[†] Daniel J. Marell,[‡] Matthew B. Neibergall,[†] Sarmistha Chakrabarty,[†] Christopher J. Cramer,[‡] and John D. Lipscomb*[†]

[†]*Department of Biochemistry, Molecular Biology, and Biophysics and the Center for Metals in Biocatalysis, University of Minnesota, Minneapolis, Minnesota 55455*
[‡]*Department of Chemistry, Chemical Theory Center, and Supercomputing Institute, University of Minnesota, Minneapolis, Minnesota 55455*

Supplemental Experimental Procedures

Chemicals and Reagents. Water used in all experiments was purified with a Millipore Super-Q system. All commercial reagents were purchased from standard vendors and used without further purification. Gases were purchased from Matheson. Unless noted, all enzymatic reactions were conducted in a pH 6.9 buffered solution of MOPS (50 mM) and NaCl (100 mM).

Cloning, Heterologous Expression, and Purification of BZDO and BZDR. BZDO was cloned from genomic DNA of *Pseudomonas putida* mt-2 into the plasmid pET-29 using standard restriction enzyme-based methods. Heterologous expression utilized *E. coli* BL21(DE3) co-expressing a subset of proteins of the *Isc* pathway from the plasmid pACYC-*isc* that increased Rieske cluster loading in expressed protein.[1] The strain was grown in 2 liter flasks each containing 1 liter of LB media supplemented with kanamycin (50 μg/ml), chloramphenicol (34 μg/ml), 10 mg/ml ferrous ammonium sulfate at 30 °C and 150 RPM. When the optical density of the culture at 600 nm was 0.5, the temperature of the shaker was reduced to 20 °C. Culture growth continued while the cultures cooled to ~ 23 °C. When the OD of the culture at 600 nm reached 0.9, the protein expression was induced with IPTG (250 μM). The cells were harvested by centrifugation after 15 h of growth.

BZDO was purified using modifications to the previously reported procedure.[2] Cell paste (100 g) was lysed by sonication in 250 ml of a pH 7.5 buffered solution of HEPES (125 mM), glycerol (5%), DTT (1 mM) and 20 units DNase I (New England Biolabs) maintaining the slurry temperature < 10 °C. The lysate was centrifuged at 39,000 × g at 4 °C for 45 min to pellet the insoluble cellular debris. The cell -free extract containing soluble BZDO was loaded onto a 500 ml DEAE column (resin bed diameter ≈ 55 mm) equilibrated with pH 7.5 purification buffer consisting of HEPES (25 mM), glycerol (5%), and DTT (1 mM). Then, the column was washed with 1 liter of purification buffer supplemented with 100 mM NaCl. Protein fractionation and elution was achieved with a 5 liter linear gradient of NaCl from 100 to 250 mM in purification buffer at 15 ml/min. Coomassie stained SDS-PAGE gels were used to select pure fractions of BZDO and the resulting dilute pool was concentrated, frozen in liquid N_2, and stored at -80 °C.

The specific activity of the enzyme was assayed by monitoring O_2 consumption during steady state assays as described below. The specific activity of BZDO was ~ 12.5 units/mg (one unit of enzyme activity is equal to the amount of BZDO required to consumed 1 μmole O_2 per minute). This activity is higher than previously reported[2], however, the assay temperature (4 °C) and concentration of BZDR (15 μM) in the present assays was different from those previously reported.

The protein reductase required for catalytic turnover, BZDR, was cloned from genomic DNA of *Pseudomonas putida* mt-2 into the plasmid p11 (DNASU Plasmid Repository) using standard restriction enzyme-based methods resulting in an N-terminal His_6-tagged protein construct. Heterologous expression was similar to that described for BZDO, but the media was Terrific Broth containing ampicillin (50 μg/ml), chloramphenicol (34 μg/ml), 2.5 % glycerol and 10 mg/ml ferrous ammonium sulfate. The growth temperature was 35 °C.

To purify BZDR, 100 g of cell paste was lysed by sonication in 250 ml of a pH 8.0 buffered solution of sodium phosphate (50 mM) and NaCl (200 mM) containing 10 mM imidazole and 20 units DNase I (New England Biolabs) maintaining the slurry temperature below 10 °C. The lysate was centrifuged at 39,000 × g at 4 °C for 45 min to pellet the insoluble cellular debris. The cell free extract containing soluble expressed BZDR was loaded onto a 25 ml Ni-NTA column (Qiagen) equilibrated in the same buffer used during lysis. The column was washed with 250 ml of the phosphate/ NaCl buffer containing 20 mM imidazole and the expressed BZDR was eluted with the same buffer containing 250 mM imidazole. Coomassie-stained SDS-PAGE gels were used to select pure fractions of BZDR, and the resulting dilute pool was dialyzed in a pH 7.0 buffer of MOPS (25 mM), glycerol (5 %), and DTT (1 mM) and subsequently concentrated, frozen in liquid N_2, and stored at -80 °C.

BZDO Steady State Activity Assays. The k_{cat} was estimated by monitoring the rate of O_2 consumption during catalytic turnover using a Hansatech Oxytherm oxygen electrode at 4 °C, 50 mM MOPS buffer, pH 6.8 plus 100 mM NaCl with the concentration of BZDR optimized and the substrates saturated. The reactions contained benzoate or a fluorobenzoate (10 mM), NADH (0.6 mM), BZDR (15 μM), O_2 (250 μM) and BZDO at 0.18 μM, 1.2 μM, 0.6 μM, or 4.8 μM when assaying with benzoate, 4-FB, 3,5-FB, or 3,4,5-FB, respectively. Reactions were initiated by addition of NADH.

Anaerobic Technique and Chemical Reduction of BZDO. Solutions of BZDO were made anaerobic by purging the headspace of a sealed vial while stirring on ice with high purity argon gas dispensed through an additional O_2 scrubbing column (Agilent). Reduction of the mononuclear Fe and Rieske cluster for single-turnover experiments was conducted in an anaerobic glove bag (Coy). After adding methyl viologen (20 μM), sodium dithionite was added from a

concentrated stock until the blue color of reduced methyl viologen persisted for ≥ 20 min indicating complete reduction of BZDOs metal centers. The methyl viologen and dithionite were removed with a PD-10 desalting column (GE Healthcare) in the anaerobic chamber.

Preparation of Nitric Oxide Adducts. Reduced BZDO was prepared as above and substrate was anaerobically added to the concentration indicated for each experiment in the Figure legends. NO solutions were made by first removing O_2 from a sealed vial of reaction buffer by sparging with high purity argon gas dispensed through an additional O_2 scrubbing column (Agilent). The deoxygenated buffer was then sparged with NO gas (pretreated by passing through 6 N NaOH) in a fume hood until saturated. Gas-tight syringes were used to dilute the NO solution to the required concentration, and this solution was either directly added to reduced BZDO or loaded onto a deoxygenated stopped-flow instrument and rapidly mixed with reduced BZDO. It is important to note that two cautions be exercised when working with NO gas. First, it should only be handled in a fume hood as it poses a significant health risk if inhaled. Second, strict anaerobicity must be maintained in experimental solutions as exposure to O_2 in aqueous solution results in formation of nitrous acid.

Fitting Procedures for Reaction Time Courses and Substrate Concentration Dependencies. Nonlinear regression fitting of the kinetic traces of the Rieske oxidation reactions was performed using the Applied Photophysics Pro-Data Software Suite (version 4.2.12). For multistep reactions, the time course is expected to be described an equation with the same number of exponential terms as reaction steps. The observed absorbance ($A_{t,obs}$) is given by: [3]

$$A_{t,obs} = A_{\infty} + \sum_{i=1}^{n} a_i e^{(-\frac{t}{\tau i})}$$

where $1/\tau_i$ is the reciprocal relaxation time of the phase in s^{-1}, a_i is the observed amplitude of phase i (of n) in absorbance units, t is time (s) and A_{∞} is the final absorbance.

For a two-step reaction where the first step is fast reversible binding, a hyperbolic dependence of the $1/\tau_{obs}$ on substrate or O_2 concentration may apply if the observable step that follows is comparatively slow: [4]

$$\text{E} + \text{ligand} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{E·ligand} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{E-ligand'}$$

Plots of $1/\tau_{obs}$ versus either substrate, or oxygen, concentration were fit to the hyperbolic function below using Origin:

$$\frac{1}{\tau_{obs}} = \frac{k_2[\text{ligand}]}{(\frac{k_{-1}}{k_1}) + [\text{ligand}]} + k_{-2}$$

The parameters of the equation yield the apparent K_d for the binding reaction (k_{-1}/k_1) and forward and reverse rate constants for observable conversion which follows binding. The accuracy of the K_d value depends upon how closely the binding reaction approaches rapid equilibrium, and specifically, whether k_{-1} is >> than k_2.

Spectroscopy. Electronic absorption spectra were recorded on either a Hewlett-Packard 8453 diode array spectrophotometer or an Agilent Technologies Cary 60 scanning spectrophotometer. X-band EPR spectra were recorded with a Bruker ELEXSYS E-500 system equipped with an Oxford ESR 910 liquid helium cryostat.

Supplemental Results

Authentic Standards of Dearomatized, 1,2-*cis*-Diol Products. The 1,2 *cis*-diol products of benzoate and all fluorobenzoates in Table 1 were produced using *Ralstonia eutrophus* strain B9, a mutant that excretes the *cis*-diols into the media, and purified according to previously

reported procedures. [5] [6] [7] Benzoate, 4-FB, and 3,5-FB *cis*-diols were obtained in high purity and characterized by ^{1}H- and, when applicable, ^{19}F-NMR. Residual proteated solvent (δ 3.31) was the reference compound for ^{1}H-NMR. These products were characterized and found to have the NMR spectral characteristics listed below. Using the methods referenced above, the 3,4,5-FB *cis*-diol was obtained in moderate purity. Based on comparison with the other *cis*-diols, we assigned one observed multiplet (δ_H 5.37) to 3,4,5-FB *cis*-diol (500 MHz, CD_3OD). The amount of 3,4,5-FB *cis*-diol in the mixture was quantified by comparison to the ^{1}H-NMR integration values of a known amount of mesitylene.

(1*S*,6*R*)-1,6-*cis*-dihydroxycyclohexa-2,4-diene-1-carboxy acid (benzoate *cis*-diol). Off white solid: ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.11 (1H, dd, J_1 = 9Hz, J_2 = 5Hz), 5.95-5.91 (1H, m), 5.80 (2H, br m), 4.85 (1H, s).

(1*S*,6*R*)-4-fluoro-1,6-*cis*-dihydroxycyclohexa-2,4-diene-1-carboxyic acid (4-FB *cis*-diol). Pink solid: ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.08-6.04 (1H, m), 5.95 (1H, dd, J_1 = 10Hz, J_2 = 6Hz), 5.27 (1H, dt, , J_1 = 12 Hz, J_2 = 3 Hz), 4.83 (1H, q, J = 3 Hz). ^{19}F NMR (470 MHz, CD_3OD): δ_F -118.65 (1F, sextet, J_1 = 6 Hz).

(1*S*,6*S*)-3,5-difluoro-1,6-*cis*-dihydroxycyclohexa-2,4-diene-1-carboxyic acid (3,5-FB *cis*-diol). Off white solid: ^{1}H NMR (500 MHz, CD_3OD): δ_H 5.61(1H, t, J = 6 Hz), 5.20 (1H, d, J = 12 Hz), 4.93 (1H, s). ^{19}F NMR (470 MHz, CD_3OD): δ_F -111.17 (1F, t, J = 11 Hz), -111.28 (1F, m).

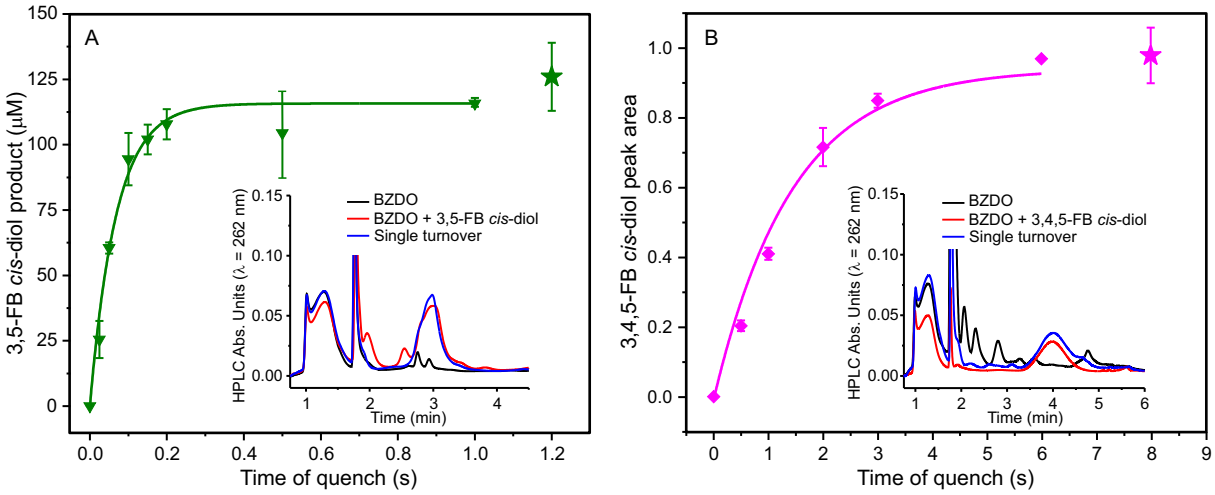

Figure S1. Product analysis of single turnover reactions shows a correlation with the fast phase of Rieske cluster oxidation. Reduced BZDO (400 μM) was rapidly mixed 1:1 with O_2 saturated reaction buffer containing (A) 3,5-FB (10 mM) or (B) 3,4,5-FB (10 mM) and chemically quenched as described in the Experimental Procedures. The yield at the end of the reaction (= 4 min) is shown by a star. The product formation time course can be fit to a single exponential equation for each substrate (solid lines), yielding the k_{obs}. In each case, a single new HPLC peak consistent with an authentic standard of the *cis*-diol product is observed (insets). For replicated points, $n = 3$ and the errors bars represent 1 standard deviation of the mean.

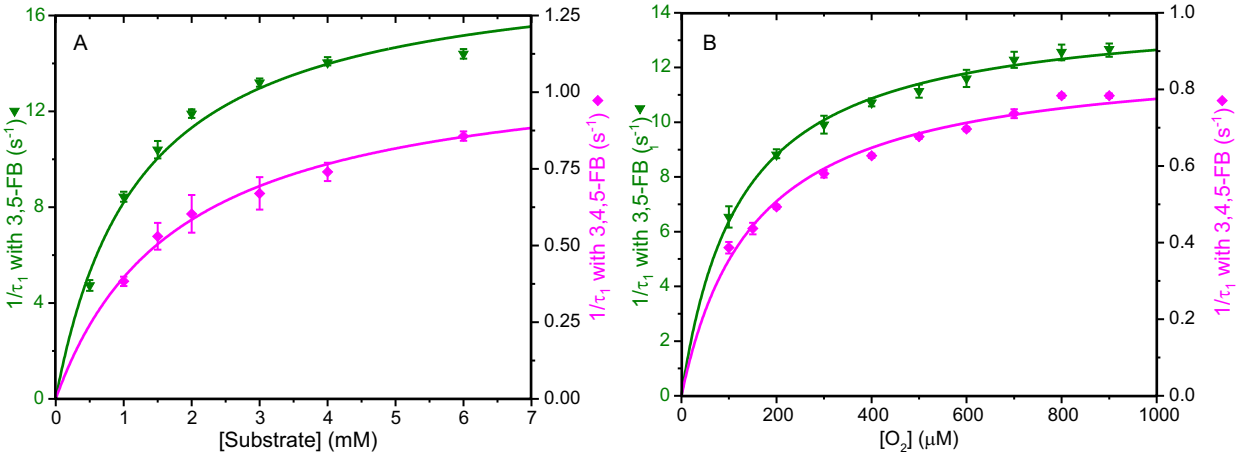

Figure S2. Substrate and O_2 concentration dependence of RRT-1 reveals a common subsequent slow step. Reduced BZDO (60 μM) was mixed 1:1 in a stopped-flow spectrophotometer with reaction buffer containing either (A) O_2 (saturated solution at 4 °C = 1.8 mM) and varied concentrations of 3,5-FB or 3,4,5-FB, or (B) varied concentrations of O_2 and 3,5-FB (20 mM) or 3,4,5-FB (20 mM). RRT-1 from multi-exponential fitting of stopped-flow traces (n = 6 and 4 for the reaction of 3,5-FB and 3,4,5-FB, respectively) was plotted vs (A) substrate or (B) O_2 concentration. Reported error of each point is one standard deviation of the mean. Apparent K_d and $k_{formation}$ values were determined by fitting the data to a hyperbolic function (solid curve) and are reported in Table 2.

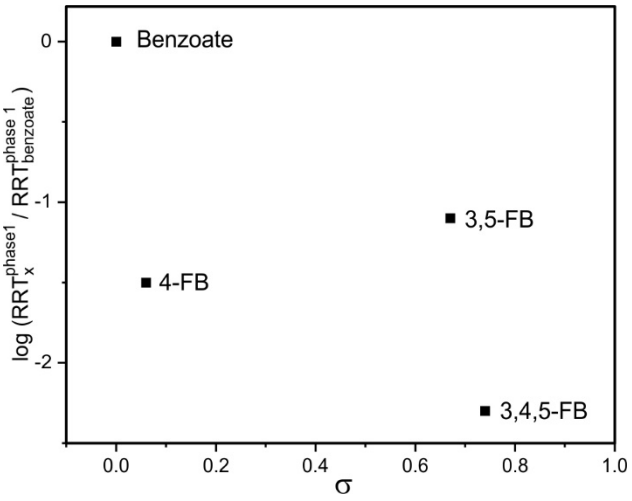

Figure S3. The Hammett plot for the reaction using the substrates shows no clear trend.

Supplemental References

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