Supporting Information

Detection and Kinetic Characterization of a Highly Reactive Heme–Thiolate Peroxygenase Compound I

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Materials

Wild-type extracellular aromatic peroxygenase of *A. aegerita* (isoform II, pI 5.6, 46 kDa) was produced in stirred-tank bioreactors with a soybean-flour suspension as growth substrate, and purified as described previously.[1] The enzyme preparation was homogeneous by SDS/PAGE, and exhibited an $A_{418\ nm}/A_{280\ nm}$ ratio of 1.7. The specific activity of the peroxygenase was 59 U/mg, where 1 U represents the oxidation of l μmol of 3,4-dimethoxybenzyl alcohol to 3,4-dimethoxybenzaldehyde in 1 min at room temperature. *m*CPBA (3-chloroperoxybenzoic acid) was obtained from Aldrich and purified by stirring in 100 mM pH 7.4 phosphate buffer for 1 h followed by filtering and washing thoroughly with water. *p*-Isopropylbenzoic acid, *p*-ethylbenzoic acid, *p*-toluic acid, THF, THF-d_8, cycloheptanecarboxylic acid, cyclopentanecarboxylic acid, cyclohexanecarboxylic acid, 1,4-dioxane, 2,2-dimethylbutyric acid were obtained from Aldrich and purified by recrystallization or distillation under vacuum. D_2O was obtained from Cambridge Isotope Laboratories, Inc. Water used in all experiments was de-ionized (Millipore, Milli-Q). Buffer solutions were prepared by mixing sodium citrate and citric acid in water and adjusted with NaOH. Phosphate-citrate buffer was prepared by mixing 0.2 M dibasic potassium phosphate with 0.1 M citric acid and the pH was adjusted with KOH.

Instrumentation

UV-vis spectral measurements were made with a Hewlett Packed 8453 diode array spectrophotometer at room temperature. Stopped-flow experiments were performed with a Hi-Tech SF-61 DX2 double mixing instrument with a 1 cm path length equipped with an ISOTEMP 3013 D thermostat bath. NMR spectra were recorded on a 500 MHz Bruker Avance II spectrometer. GC-MS analyses were run using an Agilent 7890A GC coupled to a 5975 Inert MSD with a Rtx-5Sil MS column.

Reaction Kinetics

Kinetic data were collected at 4 ^{o}C in 100 mM buffer, citrate buffer for pH 5.0 and phosphate-citrate buffer for pH 3.0-7.0. Each experiment was repeated two or three times. Concentrations presented are the final concentrations after mixing. Kinetic data for substrate hydroxylation were obtained in double-mixing mode using either diode array detection or single wavelength mode at 417 nm. Values of k_1 were obtained by the fitting of initial rates with a series of *m*CPBA concentrations. Values of k_{obs} were processed by fitting the kinetic

profile to a single exponential equation using Kinetic Studio from Hi-Tech. Values of k_2 were obtained from the slope of a k_{obs} vs. the [substrate] plot. SVD and global analyses were analyzed by ReactLab^{TM} Kinetics from Jplus Consulting.

Figure S1. Absorbance traces at 417 nm upon mixing of ferric enzyme with 2 equiv of *m*CPBA over a range of pH.

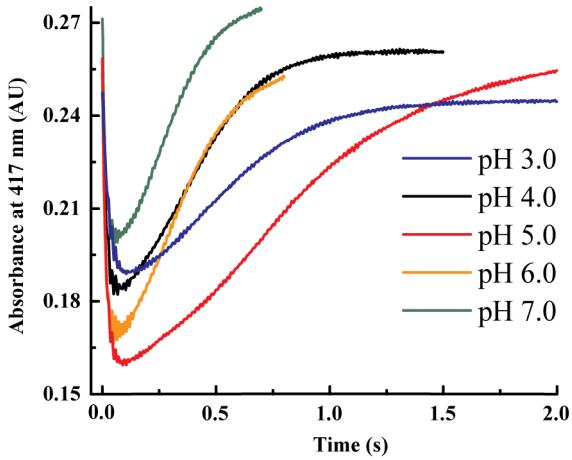

Figure S2. Water suppressed ^{1}H-NMR of the reaction mixture of oxidation of *p*-ethylbenzoic acid by 0.22 µM of *Aae*APO and H_2O_2. Labeled resonances correspond to the product (*R*)-4-(1-hydroxyethyl)benzoic acid at 7.748 ppm (2H, dd), 7,330 ppm (2H, dd) and 1.368 ppm (3H, d). The benzylic proton resonance was obscured by the suppressed water peak.

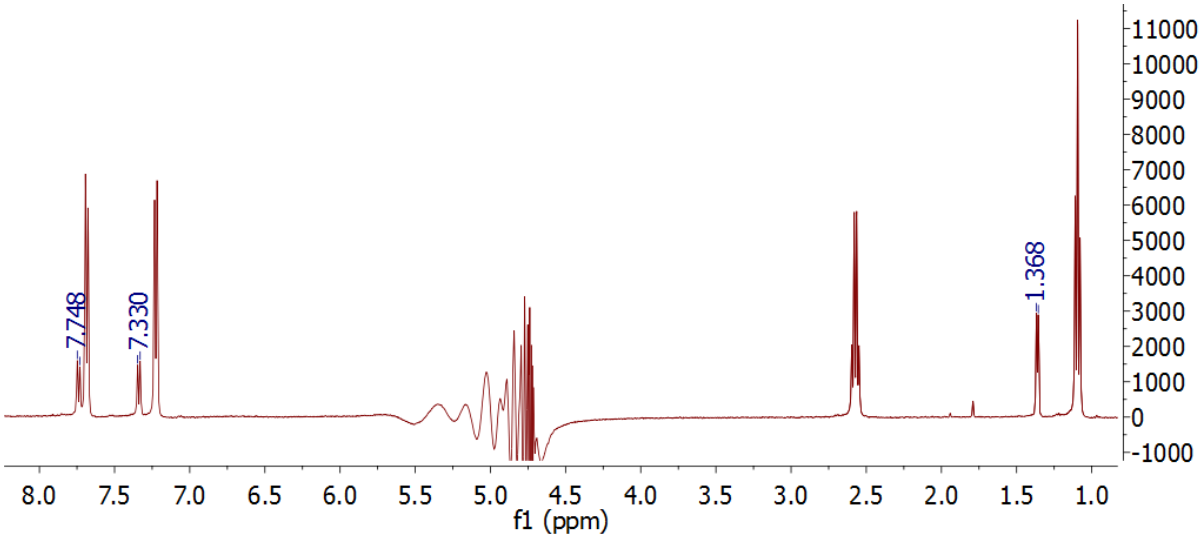

Figure S3. Rate constants for the hydrogen abstraction by MnO_4^-, ROO•, RO• and HO• vs. the strength of the O-H bonds formed for toluene (solid circles) and *p*-ethylbenzene (open diamonds).[2] Plotting rate constants for hydrogen abstraction by *Aae*APO-I with *p*-toluic acid and *p*-ethylbenzoic acid on the curves gives an Fe^{IV}O-H bond strength of about 103 kcal/mol.

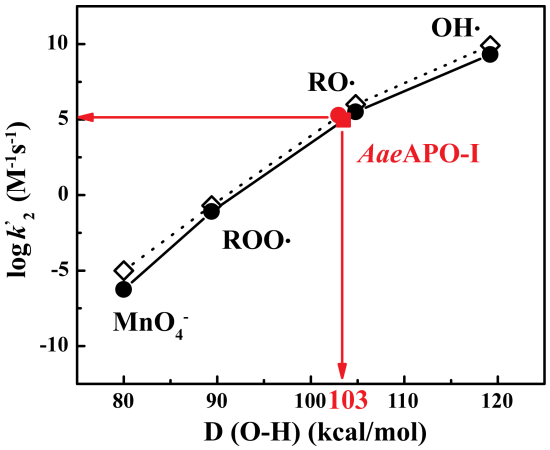

Table S1. Summary of all substrates, equivalent C-H bonds, BDE, k_2, k_2' and logk_2'.

Number	Substrates	Equivalent C-H Bonds*	BDE (kcal/mol)	k_2 ($M^{-1}s^{-1}$)	k_2'** ($M^{-1}s^{-1}$)	logk_2' ($M^{-1}s^{-1}$)
1	*p*-Isopropylbenzoic acid	1	83	2.0 (±0.09)×10^5	2.0×10^5	5.3
2	*p*-Ethylbenzoic acid	2	85.5	3.9 (±0.2)×10^5	1.9×10^5	5.3
3	*p*-Toluic acid	3	90	2.8 (±0.2)×10^5	9.3×10^4	5.0
4	THF	4	92	1.7 (±0.05)×10^4	4.3×10^3	3.6
4'	THF-d_8	4		4.0 (±0.1)×10^3		
5	Cycloheptane Carboxylic acid	4	94	3.0 (±0.1)×10^4	7.5×10^3	3.9
6	Cyclopentane Carboxylic acid	8	95.6	1.0 (±0.1)×10^4	1.3×10^3	3.1
7	Cyclohexane Carboxylic acid	6	99	1.0 (±0.2)×10^4	1.7×10^3	3.2
8	1,4-Dioxane	8	96	3.5 (±0.02)×10^2	4.4×10^1	1.6
9	2,2-Dimethylbutyric acid	9	100	6.0 (±0.05)×10^1	6.7	0.8

*The numbers of equivalent C-H bonds were based on the numbers of sites being hydroxylated. **Second-order rate constants k_2' were adjusted based on the number of equivalent C-H bonds in the substrates.

References:

(1) (a) Ullrich, R.; Nüske, J.; Scheibner, K.; Spantzel, J.; Hofrichter, M. *Appl. Environ. Microbiol.* **2004**, *70*, 4575. (b) Ullrich, R.; Liers, C.; Schimpke, S.; Hofrichter, M. *Biotechnol. J.* **2009**, *4*, 1619.

(2) (a) Gardner, K. A.; Kuehnert, L. L.; Mayer, J. M. *Inorg. Chem.* **1997**, *36*, 2069. (b) Mayer, J. M. *Acc. Chem. Res.* **1998**, *31*, 441.