Supporting Information for

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Isotope Effects of Enzymatic Dioxygenation of Nitrobenzene and 2-Nitrotoluene by Nitrobenzene Dioxygenase

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S1 Chemicals

Stock solutions of nitrobenzene (99%), 2-nitrotoluene (99%), catechol (99%), 3-methylcatechol (98%), and 2-nitrobenzyl alcohol (97%) - all purchased from Sigma-Aldrich (Buchs, Switzerland) - were prepared in methanol (99.99%). Buffer solutions were prepared in nanopure water (18.2 $M\Omega \cdot cm$, Barnstead NANOpure Diamond Water Purification System) and the pH was adjusted with sodium hydroxide (98%) and hydrochloric acid (32%). The following chemicals were purchased from various manufacturers and used without further treatment: potassium phosphate monobasic (KH_2PO_4, 99.5%), potassium phosphate dibasic anhydrous (K_2HPO_4, 99%), bis-tris (99%), potassium chloride (99.5%), ammonium sulfate (99%), dithiothreitol (99%), ethanol (99.9%), gylcerol (99%), kanamycin monosulfate from *Streptomyces kanamyceticus* (75%), ampicillin sodium salt (95%), isopropyl β-D-1-thiogalactopyranoside (99%), β-nicotinamide adenine dinucleotide reduced disodium salt (93%), ammonium ferrous sulfate hexahydrate (99%), sodium nitrite (99%), sulfanilamide (99%), *N*-(1-naphthyl)ethylenediamine (99%), sodium chloride (99.5%), sodium persulfate (99%), and phosphoric acid (85%). The carrier and reference gases for GC/IRMS and LC/IRMS were helium (99.999%), N_2 (99.9999%), CO_2 (99.999%), and H_2 (99.999%) from Carbagas (Rümlang).

S2 Purification of Nitrobenzene Dioxygenase

S2.1 Bacterial strains and growth conditions

Escherichia coli VJS415(pK19::927),[2] which carries the *nbzAaAbAcAd* genes from *Comamonas* sp. strain JS765[1], was used for purification of the terminal oxygenase component of nitrobenzene dioxygenase. *E. coli* DH5α(pJPK13::Fd)[2] only carries the *nbzAb* gene and was used for purification of the ferredoxin component. Both strains were grown at 37 ℃ with shaking in LB (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 5 g L^{-1} NaCl) containing 100 μg mL^{-1} kanamycin sulfate to late exponential phase (OD_{600} 3-4). *E. coli* BL21(DE3)(pDTG871) which carries the *nbzAa* gene, was used for purification of the reductase components and grown at 30 ℃ in TB (24 g L^{-1} yeast extract, 12 g L^{-1} tryptone, 4 g L^{-1} glycerol, 2.3 g L^{-1} KH_2PO_4, 16.4 g L^{-1} K_2HPO_4) containing 200 μg mL^{-1} ampicillin. When the cultures reached early exponential phase (OD_{600} 0.5-1.0), 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) were added and cells were incubated for additional three hours.

All cells were harvested by centrifugation (12,000 x g at 4 ℃ for 10 min) and stored at -20 ℃. The purification procedures were adapted from Parales et al.[3] and are described below for each of the three enzyme components. An automated fast protein liquid chromatography system (Bio-Rad Laboratories) was used with columns and resins from GE Healthcare. Purified protein was stored at -80 ℃.

S2.2 Purification of Ferredoxin

Frozen cell pellet (50 g) was mixed with 50 mL BTGED buffer (50 mM Bis-Tris, 5% glycerol, 5% ethanol, 1 mM sodium dithiothreitol, pH 6.8) and thawed on ice. The cell suspension was passed twice through a chilled French pressure cell and centrifuged at 10,000 x g for 30 min at 4 ℃. The supernatant was dialyzed in fresh BTGED buffer and loaded onto a Q-Sepharose fast flow ion exchange column (approximate bed volume 170 mL) equilibrated with 600 mL BTGED. Unbound protein was removed with 170 mL BTGED at 2 mL min^{-1}. Bound protein was eluted with a linear gradient from 0 to 500 mM KCl in BTGED (total volume 690 mL at 2 mL min^{-1}). Fractions containing ferredoxin were combined and concentrated by ultrafiltration (10 kDa membrane, 1 bar nitrogen gas). Afterwards, 1.5 M ammonium sulfate was added and the solution was loaded onto an octyl-sepharose 4 fast flow column (22 mL bed volume) equilibrated with 100 mL of 1.5 M $(NH_4)_2SO_4$ in BTGED. Unbound protein was removed with 100 mL of 1.5 M $(NH_4)_2SO_4$ in BTGED at 1 mL min^{-1}. Ferredoxin eluted with unbound proteins. Fractions containing ferredoxin were combined and loaded onto a phenyl-sepharose 6 fast flow (high sub) column (19 mL bed volume) equilibrated with 100 mL 1.5 M $(NH_4)_2SO_4$ in BTGED. Unbound protein, including ferredoxin, was removed with 200 mL of 1.5 M $(NH_4)_2SO_4$ in BTGED at 1 mL min^{-1}. Fractions containing ferredoxin were combined, concentrated by ultrafiltration (10 kDa membrane, 1 bar nitrogen gas), and dialyzed in 40 mM phosphate buffer (pH 7.0).

S2.3 Purification of Reductase

Frozen cell pellet (60 g) was mixed with 60 mL BTGED buffer and thawed on ice. The cell suspension was mixed with 1 mg mL^{-1} DNase I, passed twice through a chilled French pressure cell, and centrifuged at 10,000 x g for 30 min at 4 ℃. The supernatant was dialyzed in fresh BTGED buffer and loaded onto a Q-Sepharose fast flow ion exchange column (approximate bed volume 170 mL) equilibrated with 600 mL BTGED. Unbound protein was removed with an isocratic flow of 340 mL BTGED at 2 mL min^{-1}. Bound protein was eluted with a linear gradient from 0 to 500 mM KCl in BTGED (total volume 660 mL at 2 mL min^{-1}). Fractions containing reductase were combined and concentrated by ultrafiltration (10 kDa membrane, 1 bar nitrogen gas). Afterwards, 1.0 M ammonium sulfate was added and the solution was loaded onto a phenyl-sepharose 6 fast flow (high sub) column (19 mL bed volume) equilibrated with 100 mL 1.0 M $(NH_4)_2SO_4$ in BTGED. Unbound protein was removed with 50 mL of 1.0 M $(NH_4)_2SO_4$ in BTGED at 1 mL min^{-1}. Bound protein was eluted with a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$ in BTGED (total volume 120 mL at 1 mL min^{-1}). Fractions containing reductase were combined and concentrated by ultrafiltration (10 kDa membrane, 1 bar nitrogen gas). Again, 1.0 M ammonium sulfate was added to the solution which was loaded onto a phenyl-sepharose fast flow (low sub) column (approximate bed volume 30 mL) equilibrated with 150 mL 1.0 M $(NH_4)_2SO_4$ in BTGED. Unbound protein was removed with 75 mL of 1.0 M $(NH_4)_2SO_4$ in BTGED at 1 mL min^{-1}. Bound protein was eluted with a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$ in BTGED (total volume 180 mL at 1 mL min^{-1}). Fractions containing

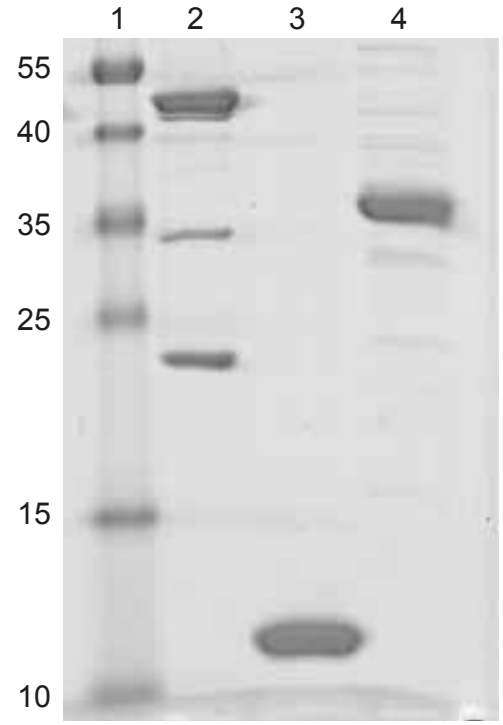

Figure S1 SDS-PAGE analysis of the purified NBDO components oxygenase (lane 2), ferredoxin (lane 3), and reductase (lane 4). The scale on the left refers to molecular masses in kDa according to the standard bands in lane 1. Molecular masses have previously been determined for alpha and beta subunits of oxygenase (50 and 23 kDa), for ferredoxin (11 kDa), and for reductase (35 kDa).[3]

reductase were combined, concentrated by ultrafiltration (10 kDa membrane, 1 bar nitrogen gas), and dialyzed in 40 mM phosphate buffer (pH 7.0).

S2.4 Purification of Oxygenase

Frozen cell pellet (65 g) was mixed with 65 mL BTGED buffer and thawed on ice. The cell suspension was mixed with 1 mg mL^{-1} DNase I, passed twice through a chilled French pressure cell, and centrifuged at 10,000 x g for 30 min at 4 ℃. The supernatant was dialyzed in fresh BTGED buffer and loaded onto a Q-Sepharose fast flow ion exchange column (approximate bed volume 170 mL) equilibrated with 720 mL BTGED. Unbound protein was removed with 215 mL BTGED at 2 mL min^{-1}. Bound protein was eluted with a linear gradient from 0 to 500 mM KCl in BTGED (total volume 680 mL at 2 mL min^{-1}). Fractions containing oxygenase were combined and concentrated by ultrafiltration (100 kDa membrane, 1 bar nitrogen gas). Afterwards, 1.0 M ammonium sulfate was added and the solution was loaded onto a phenyl-sepharose 6 fast flow (high sub) column (19 mL bed volume) equilibrated with 100 mL 1.0 M $(NH_4)_2SO_4$ in BTGED. Unbound protein was removed with 100 mL of 1.0 M $(NH_4)_2SO_4$ in BTGED at 1 mL min^{-1}. Bound protein was eluted with a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$ in BTGED (total volume 260 mL at 1 mL min^{-1}). Fractions containing oxygenase were combined and concentrated by ultrafiltration (100 kDa membrane, 1 bar nitrogen gas).

S3 Input data for isotopomer model

Table S1 Labeling position of nitrobenzene isotopomers included in the numerical model[4] and probabilities of these isotopomers to react with the light (k_{12}) or heavy (k_{13}) reaction rate given as γ^{12C} and γ^{13C}, respectively, for the two considered reaction mechanisms.

Isotopomer	Position of isotopic substitution						step-wise [a]		concerted [b]	
	C1	C2	C3	C4	C5	C6	γ^{12}C	γ^{13}C	γ^{12}C	γ^{13}C
il	^{12}C	^{12}C	^{12}C	^{12}C	^{12}C	^{12}C	1	0	1	0
i$h1$	^{13}**C**	^{12}C	^{12}C	^{12}C	^{12}C	^{12}C	1	0	0	1
i$h2$	^{12}C	^{13}**C**	^{12}C	^{12}C	^{12}C	^{12}C	1/2	1/2	1/2	1/2
i$h3$	^{12}C	^{12}C	^{13}**C**	^{12}C	^{12}C	^{12}C	1	0	1	0
i$h4$	^{12}C	^{12}C	^{12}C	^{13}**C**	^{12}C	^{12}C	1	0	1	0
i$h5$	^{12}C	^{12}C	^{12}C	^{12}C	^{13}**C**	^{12}C	1	0	1	0
i$h6$	^{12}C	^{12}C	^{12}C	^{12}C	^{12}C	^{13}**C**	1/2	1/2	1/2	1/2

[a] A step-wise mechanism involves attack of oxygen at C2 or C6.

[b] A concerted mechanism involves attack of oxygen(s) at C1 and C2 or at C1 and C6.

S4 Illustrations of kinetics and isotope fractionations

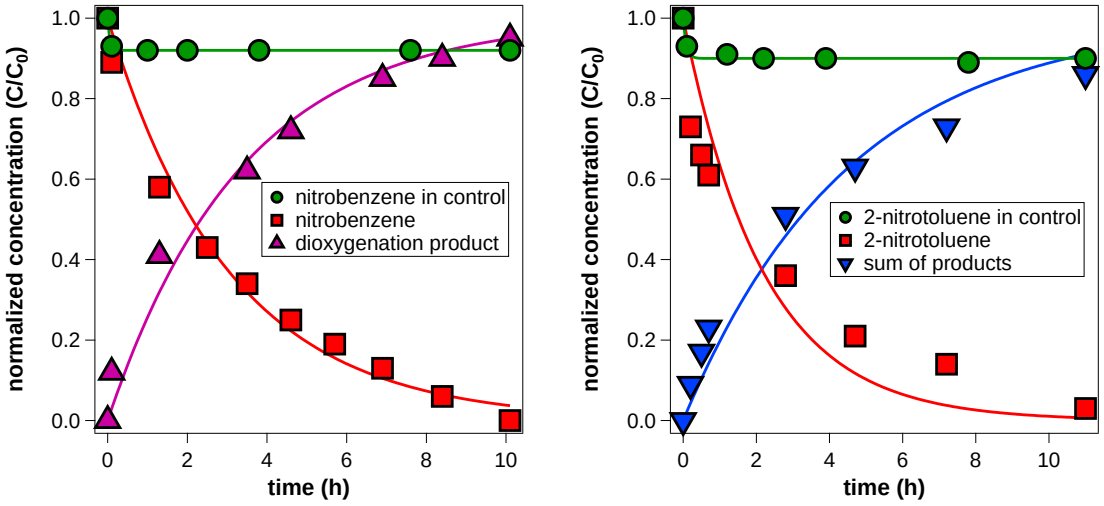

Figure S2 Left: kinetics of nitrobenzene in a control experiment with autoclaved *E. coli* clones (green circles), nitrobenzene in an experiment with *E. coli* clones (red squares), and the dioxygenation product nitrite (purple triangles). Right: kinetics of 2-nitrotoluene in a control experiment with autoclaved *E. coli* clones (green circles), 2-nitrotoluene in an experiment with *E. coli* clones (red squares), and the sum of dioxygenation and methyl group oxidation products (purple triangles).

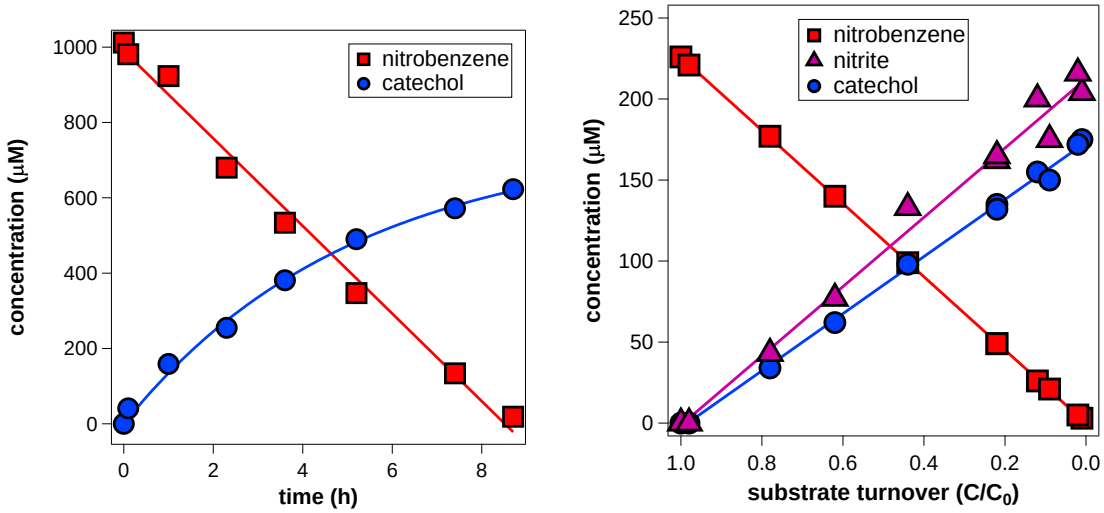

Figure S3 Left: kinetics of nitrobenzene (red squares) and catechol (blue circles) during transformation in crude cell extract. Right: concentrations of nitrobenzene (red squares), nitrite (purple triangles), and catechol (blue circles) during transformation by purified NBDO.

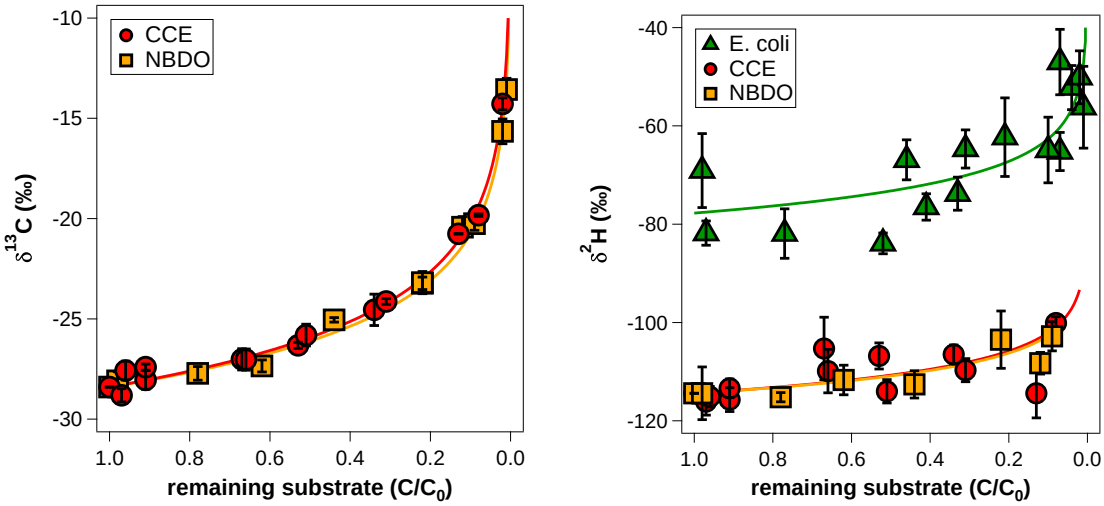

Figure S4 Left: carbon isotope signatures of nitrobenzene versus remaining substrate concentration for experiments with crude cell extract (red circles) and purified NBDO (yellow squares). Right: hydrogen isotope signatures of nitrobenzene versus remaining substrate concentration for experiments with *E. coli* clones (green triangles), crude cell extract (red circles) and purified NBDO (yellow squares).

S5 References

[1] Lessner, D. J., Johnson, G. R., Parales, R. E., Spain, J. C., and Gibson, D. T. (2002). Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. *Appl. Environ. Microbiol.*, 68(2):634–641.

[2] Mahan, K. M. (2014). *Structure function analysis of nitrobenzene 1,2-dioxygenase.* PhD thesis, University of California Davis.

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[4] Wijker, R. S., Adamczyk, P., Bolotin, J., Paneth, P., and Hofstetter, T. B. (2013). Isotopic analysis of oxidative pollutant degradation pathways exhibiting large H isotope fractionation. *Environ. Sci. Technol.*, 47(23):13459–13468.