SUPPLEMENTAL MATERIAL

BTI1, an Azoreductase with pH Dependent Substrate Specificity

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Abbreviations: BHQ: Black Hole Quencher, BTI1: Biosearch Technologies, Inc. azoreductase 1, CHES: *N*-cyclohexyl-2-aminoethanesulfonic acid, FO: Flame Orange, MeR: Methyl Red, MeO: Methyl Orange, ESI: electrospray ionization, FAD: flavin adenine dinucleotide, FMN: flavin mononucleotide, HPLC: high pressure liquid chromatography, JGB: Janus Green B, LC: liquid chromatography, MES: 2-(*N*-morpholino)ethanesulfonic acid, MOPS: 3-(*N*-morpholino)propanesulfonic acid, MS: mass spectrometry, and Tris: tris(hydroxymethyl)aminomethane.

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  1 - ATGAAACTAGTCGTTATTAACGGTACACCAAGAAAATTAGGTAGAACTCGCGTTGTGGCA -  60
  1 - M  K  L  V  V  I  N  G  T  P  R  K  L  G  R  T  R  V  V  A  -  20
 61 - AAATATATTGCAGATCAATTTGAAGGGGAATTATACGATTTAGCAATAGAGGAATTGCCT - 120
 21 - K  Y  I  A  D  Q  F  E  G  E  L  Y  D  L  A  I  E  E  L  P  -  40
121 - TTATATAATGGCGAAGAATCGCAACGTGATTTAGAGGCAGTAAAAAAAATTAAAAGCGTTA - 180
 41 - L  Y  N  G  E  E  S  Q  R  D  L  E  A  V  K  K  L  K  A  L  -  60
181 - GTGAAAGCAGCAGACGGTGTAGTACTATGTACACCAGAATATCATAATGCAATGAGCGGA - 240
 61 - V  K  A  A  D  G  V  V  L  C  T  P  E  Y  H  N  A  M  S  G  -  80
241 - GCGCTGAAAAACTCGTTAGATTACTTAAGTAGTAGTGAGTTTATCCATAAACCTGTCGCA - 300
 81 - A  L  K  N  S  L  D  Y  L  S  S  S  E  F  I  H  K  P  V  A  - 100
301 - TTACTAGCTGTTGCGGGGGGCGGTAAAGGAGGAATTAACGCATTAAATAGTATGCGAACT - 360
101 - L  L  A  V  A  G  G  G  K  G  G  I  N  A  L  N  S  M  R  T  - 120
361 - GTTGCTAGAGGTGTTTACGCAAATGCAATCCCAAAACAAGTAGTACTTGATGGACTTCAC - 420
121 - V  A  R  G  V  Y  A  N  A  I  P  K  Q  V  V  L  D  G  L  H  - 140
421 - GTACAAGATGGTGAACTTGGAGAAGATGCAAAACCATTAATTCATGATGTAGTTAAAGAA - 480
141 - V  Q  D  G  E  L  G  E  D  A  K  P  L  I  H  D  V  V  K  E  - 160
481 - TTAAAAGCATATATGAGCGTATATAAAGAGGTGAAAAAACAACTAGGAGTGGAGTGA    - 537
161 - L  K  A  Y  M  S  V  Y  K  E  V  K  K  Q  L  G  V  E  *     - 178
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Figure S1. Gene and protein sequence of BTI1 (GenBank accession number EU664999). Underlined and in bold are Leucine 13 and Cysteine 70.

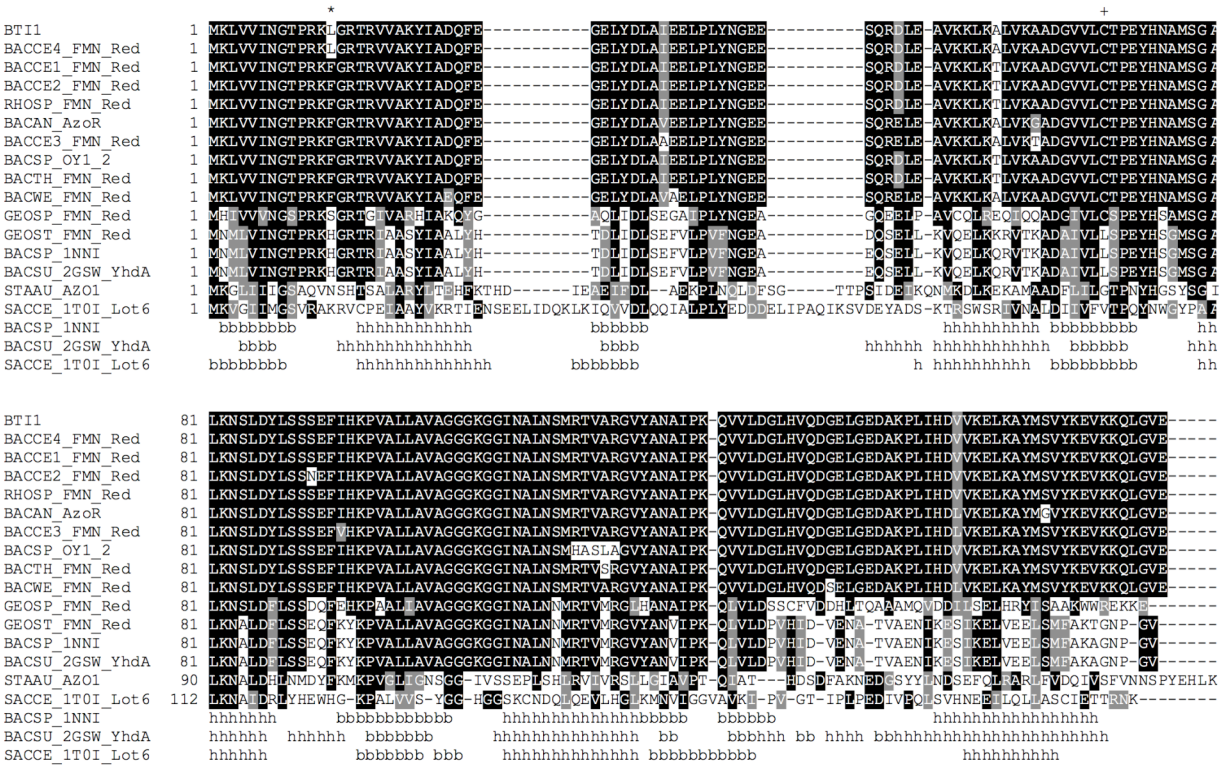

Figure S2. Alignment of BTI1 and related protein sequences. Sequences used for the alignment: BTI1: uncultured *Bacillus* sp., NADPH-dependent azoreductase, ACI12881 (this study); BACAN_AzoR: *Bacillus anthracis* str. A0488, azoreductase, ZP_02215196 (unpubl.); BACCE1_FMN_Red: *B. cereus* ATCC 14579, azoreductase, NP_831994 (10); BACCE2_FMN_Red: *B. cereus* G9241, azoreductase, ZP_00236672 (9); BACCE3_FMN_Red: *B. cereus* R309803, NADPH azoreductase, ZP_04289233 (unpubl.); BACCE4_FMN_Red: *B. cereus* Rock4-18, NADPH azoreductase, ZP_04207938 (unpubl.); BACSP_OY1_2: Bacillus sp. OY1-2, azoreductase, BAB13746 (21); BACSP_1NNI_AzBz_Red: *B. subtilis* azobenzene reductase, 1NNI (unpubl.); BACSU_2GSW_YhdA: *B. subtilis*, put. NADPH-dependent azobenzene FMN-reductase YhdA, 2GSW (unpubl.); BACTH_FMN_Red: *B. thuringiensis* Bt407, NADPH azoreductase, ZP_04139267 (unpubl.); BACWE_FMN_Red: *B. weihenstephanensis* KBAB4, azobenzene reductase, YP_001644949 (16); GEOSP_FMN_Red: *Geobacillus* sp. WCH70, NADPH-dependent FMN reductase, YP_002948705 (unpubl.); GEOST_FMN_Red: *G. stearothermophilus*, azoreductase, BAB85975 (20); RHOSP_FMN_Red: *Rhodobacter sphaeroides*, azoreductase, AAN17400 (24); STAAU_AZO1: *Staphylococcus aureus* NADPH-dependent aerobic

flavo-azoreductase, AAT29034 (5); and SACCE_1T0I_Lot6: *Saccharomyces cerevisiae* NA(D)PH-dependent FMN reductase Ylr011wp, 1T0I (13). The definition FMN_Red is used as provided by GenBank. Residues corresponding to BTI1 Leucine 13 and Cysteine 70 are indicated by "*". The secondary structure features (b: β-sheet, h: α-helix) for 1nni, 2gsw, and 1t0i are indicated below the alignment.

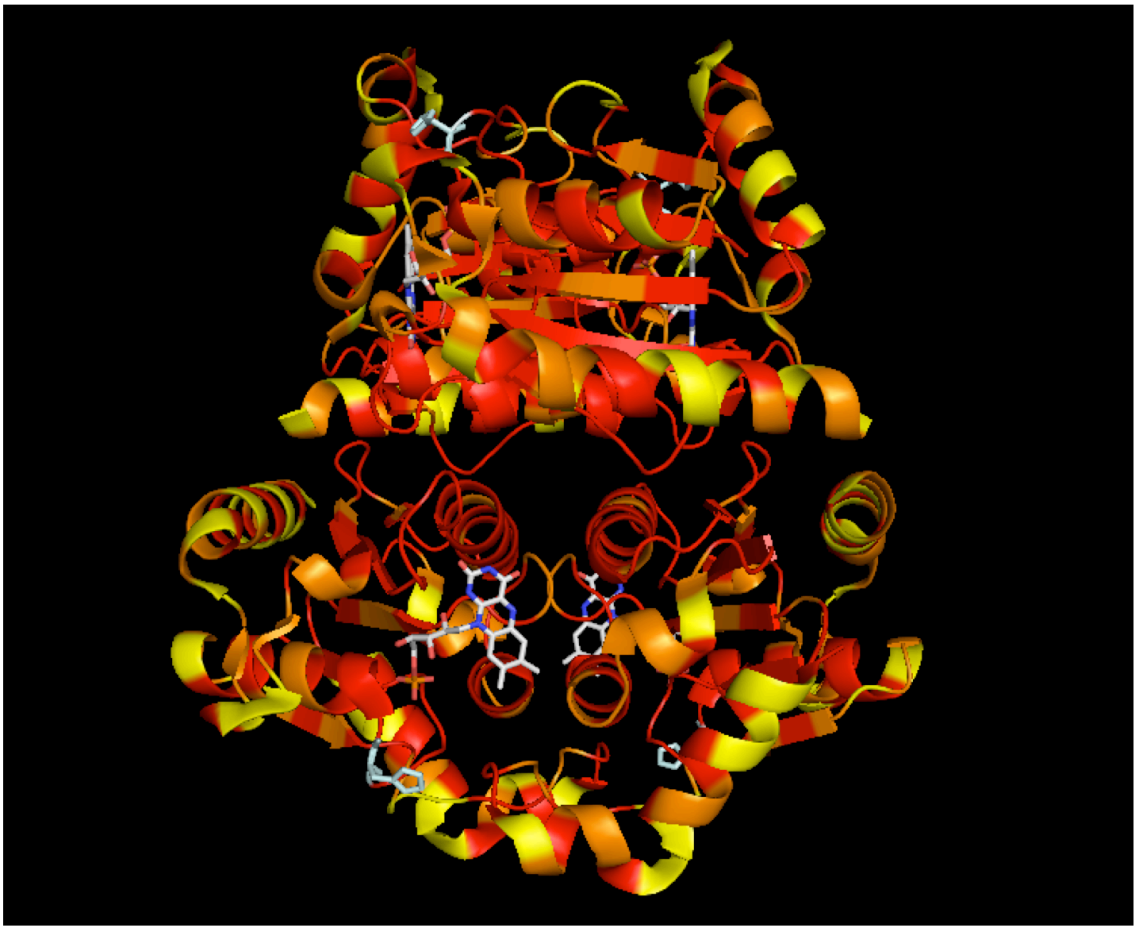

Figure S3. BTI1 tetramer core residues are conserved in YhdA. Overall cartoon α-helix and β-sheet homo-tetramer structure of *Bacillus* sp. YhdA (PDB ID: 2GSW). Conservation between BTI1 and YhdA is indicated in red (identical residues), orange (similar residues), and yellow (non-conserved residues). Stick models of the FMN co-factors and the variable residue 13 (His in YhdA) show the location the four active sites.

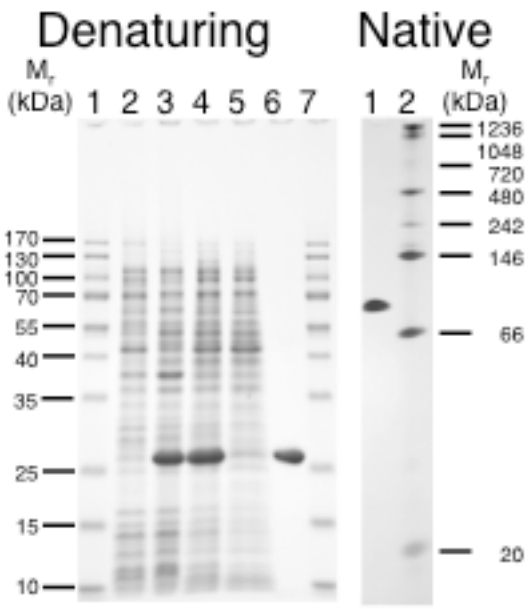

Figure S4. *Left panel*: Expression in *E. coli* BL21(DE3) pLysS and protein purification of BTI10 azoreductase. Lanes 1 and 7: PAGE-Ruler size markers. Relative molecular weight indicated on the left. Lane 2, uninduced culture. Lane 3, induced culture. Lane 4, S10 extract. Lane 5: Nickel column flow through. Lane 6, eluted BTI10. *Right panel*: BTI10 azoreductase is a tetramer. Purified BTI10 (lane 1) was separated by native 14 % PAGE. Lane 2: Invitrogen's NativeMark size marker. Relative molecular weights indicated to right.

Component	Abs signature (nm)	HPLC Elution time (min)	Calculated mass ($g \cdot mol^{-1}$)	Observed mass
Riboflavin	268, 369, 441	0.9	376.36	377.08
FMN	268, 369, 445	3.2	456.34	**457.06**
FAD	266, 369, 446	4.2	785.55	786.00
BTI10 bound	274, 369, 446 (native) 268, 369, 447 (denat.)	8.6 3.2	N/A	**457.10**
BTI10 polypeptide	N/A	N/A	22,209.5	22,210.8

Table SI. HPLC and MS analyses of the BTI1 polypeptide and bound co-factor. Left panel: Flavin absorption signatures and HPLC elution times. Right Panel: Calculated and observed mass of flavins, BTI10 bound co-factor and polypeptide. For experimental details see supplemental methods.

SUPPLEMENTAL MATERIALS AND METHODS

Reagents, media and bacterial strains. All oligonucleotides were synthesized in house. Platinum *Taq*, Accuprime *Pfx*, pBAD/gIII, pSecTag2a, His-bond Ni-affinity media, polyacrylamide gels, NativeMark protein size marker, and *E. coli* LMG 194 were from Invitrogen (Carlsbad, CA). PAGE-Ruler denaturing protein size marker was from Fermentas (Glen Burnie, MD). BioSafe Coomassie protein stain was from Bio-Rad, (Hercules, CA). Restriction enzymes, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA ligase, T4 polynucleotide kinase, bovine alkaline and Antarctic phosphatases as well as *E. coli* Turbo cells were from New England BioLabs (Ipswich, MA). His-bind resin and buffer kit, pET28a, benzonase, *E. coli* TOP 10, and *E. coli* BL21(DE3) pLysS were from Novagen / EMD Biosciences (Madison, WI). Bicinchoninic acid (BCA)-assay kit was from Pierce (Rockford, IL). Plasmid preparation and DNA cleaning kits were from Qiagen (Valencia, CA). All other reagents and buffers were from Sigma-Aldrich (St. Louis, MO).

Isolation and sub-cloning of BTI1 azoreductase. DNA was isolated from a 1 g soil sample collected from just below the surface of a grassy field in Novato, CA. It was amplified with PCR primers based on previous work (21) and extended with terminal NcoI and HindIII restriction sites: ESBTI3 5' - CGAGCCATGGTGATGAAACTAGTCGTTATTAACGGTACA - 3' and ESBTI5 5' - GTCTGAAAGCTTTCACTCCACTCCTAGTTGTTTTT - 3'. DNA amplification was achieved with Accuprime Pfx and thermocycling (95 °C 2 min, [95 °C 20 s, 60 °C 30 s, 68 °C 60 s] x 35) on an ABI9700 (Applied Biosystems, Foster City, CA). Amplification products were digested with NcoI and HindIII and ligated into the same sites of pBAD/gIII. Positive clones were identified by colony PCR with plasmid-specific and insert-spanning primers by

using the same program and Platinum Taq. The nucleotide sequence reported in this paper was deposited in the GenBank database under accession number EU664999.

The isolated BTI1 (Biosearch Technologies, Inc. azoreductase #1) gene sequence was codon-optimized for expression in *E. coli* and mammalian cells. *De novo* gene synthesis in house and by Gene Oracle (Mountain View, CA) used in-house synthesized oligonucleotides and published protocols (11). The non-conserved Cys70 residue (Fig. S2) was replaced with a Val to minimize the possibility of oxidative damage in the absence of reducing agents and to enable future single site cysteine mutagenesis. Wild type and C70V BTI azoreductase genes were inserted into the SfiI and ApaI sites of pSecTag2a and in frame with the C-terminal Myc- and His-peptides to generate pBTI3 and pBTI4. The BTI1 wt and C70V containing NcoI-PmeI fragments from pBTI3 and pBTI4 were inserted into the NcoI and blunt-ended HindIII (filled in with Klenow fragment and dNTPs) of pET28a to create pBTI9 and pBTI10. The C-terminally tagged wt and C70V BTI proteins are referred to as BTI9 and BTI10. The C-terminal peptide sequence extension (in single letter amino acid code) is:

GSFGPEQKLISEEDLNSAVDSCAAALEHHHHHH -COOH

Correct insertion was confirmed by restriction digestion and bidirectional sequencing with insert-specific or plasmid-specific primers (pBAD: pBAD forward and reverse; pSecTag2a: T7 promoter and bGH polyA reverse; pET28: T7 promoter and T7 terminator) (Sequetech, Mountain View, CA and ELIM Bio, Hayward, CA).

Enzyme expression in *E. coli* and purification. BTI9 and BTI10 were expressed from pBTI9 and pBTI10 in *E. coli* BL21(DE3)pLysS by using Novagen's Studier-based T7 expression system (19) as recommended, but with a with 0.8 mM IPTG to induce expression that proceeded

for 2 h. Samples removed before induction and at harvest were used to assess the level of expression. Treatment with 100 U Benzonase and 25 U hen egg white lysozyme for 20 min at 20 °C ensured complete lysis and DNA-removal prior to denaturation and electrophoretic separation of proteins by Tris-glycine 14 % SDS-PAGE. Protein in gels was stained with BioSafe Coomassie stain (Bio-Rad) and visualized in an Alpha Imager.

Lysis and purification proceeded as recommended by Novagen, except that Benzonase (20 U / ml) and hen egg white lysozyme (5 U / ml) was added to complete lysis and DNA digestion. Ni-affinity column chromatography was as recommended, except that the column was washed with 20 and 100 mM imidazole in buffer (20 mM Tris-Cl pH 7.9, 500 mM NaCl) and eluted with 400 mM imidazole (in the same buffer) to yield > 95 % pure proteins as determined by SDS-PAGE. The buffer was exchanged of pooled fractions enzyme to 20 mM Tris-Cl pH 7.9 or deionized H_2O by Sephadex G50 size-exclusion chromatography prior to determination of protein concentration, spectrophotometric assessment of FMN content, and enzymatic activity measurements. Three standards were employed to determine the concentration of azoreductase protein by the BCA-assay: Bovine serum albumin, Bovine gamma globulin, and azoreductase itself freeze-dried in pre-weighed Eppendorf tubes, weighed, and reconstituted in H_2O. The ratio of concentrations obtained was 0.82:0.47:1, respectively. Hence the azoreductase itself was used as a standard for all preparations of the BTI enzyme used for activity measurements. Native protein was separated by non-denaturing gel-electrophoresis essentially as before (6).

Bioinformatics. Plasmid sequence analysis and in silico manipulation were performed by using ApE (A Plasmid Editor) (M. Wayne Davis, www.biology.utah.edu/jorgensen/wayned/ape/) and tools hosted at JustBio (www.justbio.com). Protein sequence alignment of group II azoreductases

was created with CLUSTAL_X 2.0.7 (12) and conserved residues shaded with BOXSHADE 3.2.1 at www.ch.embnet.org/software/BOX_form.html using 50 % cutoff for conservation. 3D structures were analyzed by using MACPYMOL 0.99 (www.pymol.org). The ProtParam tool at ExPASy (us.expasy.org/tools/protparam.html) was employed to predict the mass of the BTI azoreductase variants. Identification of conserved domains was performed at NCBI (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (14).

Substrates. Alizarin Yellow R (5-[4-Nitrophenylazo]salicylic acid), Am-azo 1 ({3-(4-[2-hydroxy-1-phenylcarbamoyl-propenylazo]-2-methyl-phenylazo)-phenyl}-trimethyl-ammonium Chloride), Basic Blue 41 (2-[[4-[ethyl(2-hydroxyethyl)amino]phenyl]azo]-6-methoxy-3-methyl-benzothiazolium, methyl sulfate salt, C.I. 11105), Disperse Orange 3 (4-[4-Nitrophenylazo]aniline), Janus Green B (JGB; 3-diethylamino-7-(4-dimethylaminophenylazo)-5-phenylphenazinium chloride, C.I. 11050), Methyl Orange (MeO; 4-[4-(Dimethylamino)phenylazo]benzenesulfonic acid sodium salt, C.I 13025), *ortho*-Methyl Red (*o*-MeR; 2-(4-Dimethylaminophenylazo)benzoic acid, C.I. 13020), *para*-Methyl Red (*p*-MeR; 4-(4-Dimethylaminophenylazo)benzoic acid), Nitro-naphthyl-DMA (*N*,*N*-dimethyl-4-(4-nitro-1-naphthylazo)aniline), Orange G (1-Phenylazo-2-naphthol-6,8-disulfonic acid disodium salt, C.I. 16230), Orange I (4-(4-Hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt, C.I. 14600), Orange II (4-(2-Hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt, C.I. 15510), and Ponceau BS (2-((2-hydroxy-1-naphthalenyl)azo)-5-((4-sulfophenyl)azo)-benzenesulfonic acid, disodium salt, C.I. 26905) were all from Sigma-Aldrich (St. Louis MS). Citrus yellow (1-methyl-4-((methylphenylhydrazono)methyl)-pyridinium methylsulfate, Basic Yellow 87), Flame Orange (FO; 2-[(4-aminophenyl)azo]-1,3-dimethyl-1H-imidazolium chloride, Basic Orange 31), and

Ruby Red (2[[4-(dimethylamino)phenyl]azo]-1,3-dimethylimidazolium chloride, Basic Red 51) were gifts from Ciba Corp. (Tarrytown, NY).

Synthesis of compounds. Synthesis and characterization of Black Hole Quencher 10 (BHQ-10; 4-(Methyl-{4-[2-sulfo-4-(4-sulfo-phenylazo)-phenylazo]-phenyl}-amino)-butyric acid disodium salt) proceeded essentially as published (7). All reagents and solvents were either ACS reagent grade or HPLC/spectroscopy grade and were obtained from Sigma-Aldrich, Acros, TCI or Burdick & Jackson unless otherwise noted. MALDI mass spectrometry was performed on a Bruker Biflex III MALDI-TOF instrument. Electrospray mass spectrometry was performed on a Finnigan LC Q and LC-mass spectrometry was performed on a Micromass LCT ESI mass spectrometer coupled with a Waters 2790 HPLC and a 996 PDA detector. NMR analysis (^{1}H: 400 MHz) was performed by Acorn NMR (Livermore, CA).

4-(Methyl-phenyl-amino)-butyric acid. N-Methylaniline (32.1 g, 0.30 mol) and ethyl-4-bromobutyrate (61.7 g, 0.32 mol) were added to a 250 mL flask, and the mixture was heated at 115 °C for 20 h. After cooling, the mixture was dissolved in ethyl acetate (500 mL). The solution was washed with 4.0 M sodium carbonate solution (2 x 300 mL), dried ($MgSO_4$), filtered, and evaporated under vacuum to leave an oil that was distilled under vacuum to afford as a colorless oil (46.2 g, 70 %; b.p.115 °C at 0.37 mm Hg). The oil was added to a 1 L flask containing 1 M potassium hydroxide (500 mL) and the mixture was heated at reflux for 4 h. After cooling, the mixture was washed with ether (2 x 200 mL). The organic layer was dried ($MgSO_4$), filtered, and evaporated under vacuum to recover the unreacted starting material. The aqueous layer was treated with concentrated hydrochloric acid to adjust the pH to the range 7-8. The concentration

of the product in the aqueous solution was calculated by subtracting the recovered starting material from the initial reaction quantity. Water was added to adjust the concentration of the 4-(methyl-phenyl-amino)-butyric acid solution to 0.5 M. This solution was used without further purification.

Black Hole Quencher-10 (BHQ-10), 4-(Methyl-{4-[2-sulfo-4-(4-sulfo-phenylazo)-phenylazo]-phenyl}-amino)-butyric acid. Concentrated sulfuric acid (2 mL) was added dropwise to a solution of 4-amino-1,1'-azobenzene-3,4'-disulfonic acid sodium salt (1.56 g, 4.0 mmol) in water (30 ml) in a 100 ml flask. The resulting suspension was cooled in an ice bath. A solution of sodium nitrite (0.32 g, 4.6 mmol) in water (30 mL) was added dropwise over 15 min, causing formation of a yellow-brown solution. The mixture was stirred for 1 h. To a 250 mL flask were added saturated aqueous sodium bicarbonate (50 mL) and the solution of 4-(methyl-phenyl-amino)-butyric acid solution (10 mL, 5 mmol), and the mixture was cooled in an ice bath. The cold acidic solution was added to the basic mixture portionwise over 15 min. Saturated aqueous sodium bicarbonate was added to raise the pH to 7.5 and then the mixture was stirred overnight. The reaction mixture was added directly to a column of macroporous polystyrene (13 x15 cm) in water and the column was eluted with water. Fractions of the deep purple band were collected and were checked for purity using C18 impregnated silica TLC plates (Analtech). Pure fractions were combined and then concentrated to 100 ml by rotary evaporation. The solution was filtered and then evaporated to afford a dark brown solid (1.62 g, 67%). TLC rf 0.27 (C18 impregnated silica plate 15% methanol in water); λ_{max} 516 nm (ε 30,400) in carbonate-bicarbonate buffer pH 8.5, 516 nm (ε 28,700) in PBS pH 7.0; ^{1}H NMR (D_2O + 2% (v/v) 30% NaOD) 1.78 (quintet, J = 7.4 Hz, 2H), 2.19 (t, J = 7.5 Hz, 2H), 2.82 (s, 3H), 3.25 (t, J = 7.3 Hz, 2H), 6.59 (d, J = 9.2 Hz,

2H), 7.20 (d, J = 8.6 Hz, 2H), 7.61-7.68 (m, 2H), 7.81 (d, J = 8.6 Hz, 2H), 7.96 (d, J = 8.6 Hz, 2H) and 8.37 (d, J = 2.2 Hz, 1H); 13C NMR 26.1, 37.5, 40.8, 54.4, 114.4, 120.8, 126.0, 126.3, 128.9, 129.5, 129.6, 142.2, 145.8, 147.6, 153.1, 153.6, 155.4, 156.0, 185.6; ESI-MS observed (-) 560.1 (M-H)-, calculated 561.01 (M = $C_{23}H_{23}N_5O_8S_2$).

Co-factor determination and peptide mass verification. Absorption spectra of purified enzyme were recorded on a HP 8435 diode array UV-VIS spectrophotometer in Tris-Cl pH 7.9, 100 mM NaCl.

Anion exchange high pressure liquid chromatography (AX-HPLC) in 38 mM Tris-Cl pH 8.0 with a NaBr gradient (progressive 0-300 mM over 8 min then linear up to 1 M at 10 min) provided clearer separation between native BTI azoreductase and candidate flavins; riboflavin, FMN and FAD than achieved by an earlier method (3).

Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) was carried out on a Micromass LCT (Manchester, UK) using a Z-spray mk2 source, interfaced with a Waters Acquity ultra high pressure LC (UPLC) (Milford, MA).

BTI10 and flavin reference samples were examined similarly to a published protocol (1). Samples were separated by UPLC on a BEH300 C18 column (Waters, Milford, MA) in a linear gradient of denaturing 100% 50 mM ammonium formate, pH 5.9 to 100% acetonitrile over 6 min at a flow rate of 400 µl / min. For ES ionization, positive ions were generated at 2.8 kV at the capillary, at 40 V at the cone, and at 4 V at the extraction cone, with a desolvation gas flow rate of 750 L/hr, a cone gas flow rate of 50 L/hr, a capillary temperature of 350 °C, and a source temperature of 120 °C. Masses were acquired in TOF full scan mode (m/z: 200:40000) at two scans per second. Horse heart myoglobin (16,951.49 Da) was used as an internal standard for

the determination of the full mass of the BTI azoreductase peptide. Multicharged proteins were deconvoluted by the MaxEnt-1 program, as part of the MassLynx 4.1 software (Waters, Milford, MA).

Enzymatic reduction of azo dyes at different pH. Ammonia-MES, -MOPS, -HEPES, and -CHES buffers that also promote β-NADPH stability (15, 17, 23) were used at 20 mM final concentrations.

SUPPLEMENTAL RESULTS

The BTI azoreductase. The BTI1 (Biosearch Technologies, Inc. azoreductase #1) azoreductase gene isolated by PCR amplification of DNA from a locally obtained soil sample encodes a protein of 178 amino acids (Figs. S1 and S2) that is only 96 % identical to the OY1-2 azoreductase on which the primers were based, but identical in sequence to a putative FMN reductase from *B. cereus* Rock 4-18. An alignment of *Bacillaceae* protein sequences (Fig. S2) including two structure-determined group II azobenzene reductases (PDB ID: 1nni and 2gsw; unpublished), as well as the *Saccharomyces cerevisiae* oxidoreductase Lot6p (Ylr011wp) (PDB ID: 1t0i) (13), reveal certain variable residues; a surface exposed patch within the last alpha helix, one residue within the enzyme hydrophobic core (Cys 70), and one at the edge of the active site (Leu 13). Strong conservation between BTI1 and YhdA of residues of the FMN harboring active site (27/28 identical or conserved residues) enabled 3D Modeling of BTI1 based on the three structures 1nni, 2gsw, and 1t0i (not shown). The model confirmed a flavodoxin core with conserved flavin binding sites at the dimer subunit interface and accessible N- and C-termini (**cf. Fig. S3**).

A functionalized form of BTI1, BTI10, with C-terminal His_6 and Myc peptides for ease of purification and identification by western analysis, as well as the non-conserved Cys 70 mutated to Val to enable site-directed insertion of single cysteine residues was constructed and expressed in *E. coli* (see supplementary methods for details). Neither the added peptides, nor the Cys70Val mutation affected enzymatic activity (not shown), similarly to related enzymes (22). Large quantities (>10 mg / 100 ml culture) of concentrated (> 4 mg / ml) enzyme that remained soluble and active over several months at + 4 °C were easily obtained.

Verification of expressed protein. Purified BTI10 migrated as a ~27 kDa single band in SDS-PAGE (Fig. S4, left panel), slightly slower than predicted. The identity of BTI10 was verified by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). The observed mass of 22,210.8 Da for the free polypeptide (Tab. SI, right panel) agreed well with the calculated mass of the polypeptide having undergone post-translational removal of the N-terminal fMet residue (22,209.5 Da).

The oligomeric state of the BTI azoreductase as assessed by native PAGE revealed that purified BTI10 migrated as a single band with a Mr of 90 ± 10 kDa (Fig. S4, right panel), consistent with a homo-tetrameric configuration (Mcalc: 90.7 kDa). Two further variants of BTI1(C70V) in which the C-terminal tag was either left attached, or removed by proteases, also migrated as homo-tetramers. Oligomerization of the C-terminally trimmed proteins only occurred at concentrations over 10 mg • ml^{-1} (not shown).

The BTI1 co-factor is FMN. The yellow color of the BTI azoreductase and its relatedness to known FMN-dependent azoreductases suggested a firmly bound flavin co-factor. The type of flavin bound to an enzyme is known to influence its reductive potential (4, 8, 18) and this prompted us to characterize the BTI10-associated flavin(s). The flavin absorption signature

revealed by UV-vis spectroscopy was slightly blue-shifted in the native enzyme as compared to the denatured enzyme and free flavins (not shown). The absorption spectrum of BTI10 (with maxima at 275, 374, and *ca.* 450 nm) changed upon reduction with NADPH into one predominant peak at 275 nm consistent with flavin reduction.

Additional anion exchange HPLC (AX-HPLC) provided clear separation between the candidate flavins and native BTI10 protein. Reference samples of pure RF, FMN and FAD eluted at 1.0, 3.2, and at 4.2 min, respectively (Tab. SI, left panel). Native protein retained the flavin as evidenced by significantly delayed elution (8.6 min) of a peak with a flavin absorption signature. The absolute majority of the flavin released by denaturation in 2 % SDS eluted at 3.2 min and only a trace amount at 4.2 min. Results from co-injection of denatured BTI10 and each flavin, or with a mixture of the three, clearly confirmed co-elution of the BTI10-bound flavin with FMN. Thermal denaturation of BTI10 also released the bound flavin, but it eluted as several peaks correlating with FMN and riboflavin suggesting partial heat-induced de-phosphorylation of FMN. These data are consistent with FMN as a non-covalently bound cofactor, and association of trace amounts of FAD.

The co-factor identity was verified when purified BTI10 was subjected to LC-ESI-MS; the LC time of migration and the absorption spectrum of the flavin released from the azoreductase were consistent with that of FMN and more importantly, the observed molecular mass of the released flavin (457.10 Da) also was consistent with that of free FMN (457.06 Da) (Tab. SI, right panel). Close scrutiny of the MS data in the mass range of FAD revealed a mass of 786.1, barely above background, again consistent with trace amounts of non-covalently bound FAD. It was concluded that the BTI azoreductase cofactor is non-covalently bound FMN.

Cation sensitivity. Reduction of MeO was assessed in MES pH 6.0 at 0-500 mM KCl and NaCl and at 0-50 $MgCl_2$. The decolorization rate of MeO was moderately enhanced by Mg^{2+} up to 10 mM, possibly due to increased NADPH availability (24). KCl and NaCl 80 mM and above slightly decreased the reduction rate. Subsequently, ammonia was used to adjust the pH of all buffers (See supplemental methods).

Substrate solubility and NADPH sensitivity. The disappearance of absorbance at the dyes' λ_{max} was monitored at pH 5.5-8.3 over time in buffer alone, or in buffer with NADPH. Alizarin Yellow and *p*-Methyl Red (*p*-MeR) could not be assessed for enzymatic reduction due to insufficient solubility. Both Nitronaphthyl-DMA and Orange II were unaffected by NADPH, but their absorbance spectra changed within 30 min of dilution into aqueous buffers indicating aggregation, also noted by others (2). BTI10 did not reduce Nitronaphthyl-DMA, and Orange II only slowly, possibly due to such aggregation. Basic Blue 41 and Orange I remained soluble, but were unstable to NADPH; absorption at their maxima faded within 30 minutes.

SUPPLEMENTAL REFERENCES

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