SUPPORTING INFORMATION

Analysis of the Structure and Function of YfcG from *Escherichia coli* Reveal an Efficient and Unique Disulfide Bond Reductase

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A. Materials

E. coli K-12 genomic DNA was from ATCC (Manassas, VA)[1]. Primers were custom ordered from Invitrogen (Carlsbad, CA). Restriction enzymes were from New England Biolabs (Ipswich, MA). The pET20b(+) vector and monobromobimame was from EMB Chemicals, Inc (Gibbstown, NJ). BL21-Gold (DE3) cells were from Stratagene (La Jolla, CA). Luria broth, ampicillin, IPTG[2], HEPES, DTT, streptomycin sulfate, sodium chloride, MES, glycerol, ammonium sulfate, and GSH were all from Research Products International (Mt. Prospect, IL). EDTA, ammonium acetate, DTNB, glutathione disulfide, CDNB, ethacrynic acid, trans-4-phenyl-3-butene-2-one, 1,2-epoxy-3-(4-nitrophenoxy) propane, 1,4-dichloro-2-nitrobenzene, sodium borohydride, dichloromethane, diamide, *tert*-Butyl hydroperoxide, cumene hydroperoxide, benzoyl peroxide, lauroyl peroxide and 2-hydroxyethyl disulfide were ordered from Sigma Aldrich (St. Louis, MO). DEAE-Sepharose and SP-Sepharose resins were obtained from GE Healthcare (Piscataway, NJ). The hydroxyapatite resin and 15% SDS-PAGE gels were obtained from Biorad (Hercules, CA). Potassium phosphate (monobasic), magnesium chloride, hydrogen peroxide and acteonitrile were all from Fisher Scientific (Pittsburgh, PA). The

spermidine and *S. cerevisiae* glutathione reductase were obtained from VWR International (West Chester, PA).

B. Cloning and Expression *yfcG* and Purification YfcG

The *yfcG* gene was amplified from *Escherichia coli* K-12 genomic DNA by PCR using a forward primer AAAAAAACATATGATCGATCTCTATTTCGCCCCGAC containing the restriction site *Nde1* and a reverse primer TTTTAAAGCTTAACTATCCGAACGCTCATCACCGAG containing the restriction site *HindIII*. The amplified and digested *yfcG* gene was subcloned into the pET20b(+) expression vector. The pET20b(+) plasmid containing the *yfcG* gene was transformed into BL21(DE3) cells and grown overnight at 37° C on LB agar containing ampicillin. A single colony was transferred to liquid LB media supplemented with 0.1 mg/mL ampicillin and grown overnight at 37° C with shaking at 225 rpm. Cells were diluted 100 times in fresh LB media containing 0.1 mg/mL ampicillin and grown under the same conditions until an OD_{600} of 0.8 was reached. The cultures were induced with 0.5 mM IPTG and the temperature was reduced to 18° C and grown an additional 18 hours. Cells were harvested by centrifugation at 4,000 x g for 10 minutes at 4° C. The liquid media was removed and the pellets frozen at -20° C. The frozen cell pellet was suspended in 50 mM HEPES buffer containing 1 mM EDTA and 1 mM DTT at pH 7.0 and lysed by sonication (4 cycles at 70% output for 3 minutes with 7 minutes of gentle stirring between each cycle). The cellular debris was removed by centrifugation at 31,000 x g for 30 minutes at 4° C. The supernatant was removed and treated with 10 mg/mL streptomycin sulfate, then centrifuged again at 31,000 x g for 30 minutes at 4° C. The supernatant was dialyzed overnight in 20 mM HEPES buffer (pH 7.0) containing 1 mM EDTA and 1mM DTT (pH 7.0). The protein

solution was loaded on a DEAE-Sepharose column equilibrated in the dialysis buffer and eluted from the column with a linear gradient of 0 to 0.4 M NaCl in the same buffer. Protein-containing fractions, determined using A_{280} and SDS-Page, were combined and dialyzed overnight against 20 mM MES buffer (pH 6.0) containing 1 mM EDTA and 1 mM DTT. The protein was loaded on a SP-Sepharose column equilibrated in the dialysis buffer and eluted from the column with a linear gradient of 0 to 0.4 M NaCl in the same buffer. Protein containing fractions were determined by the same procedure and combined and dialyzed overnight against 20 mM KH_2PO_4 buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT. For final purification the protein was loaded onto a HA column preequilibrated in the dialysis buffer and fractions were collected immediately. The YfcG protein eluted in the flow through and those fractions containing protein were pooled, and dialyzed in 20 mM KH_2PO_4 buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT. Protein used in the HED assay was dialyzed against buffer in the absence of DTT. To prevent protein precipitation in the absence of DTT, the final dialysis buffer contained 300 mM NaCl and 10% glycerol in addition to KH_2PO_4 and EDTA. The purified protein (estimated to be ≥99% pure by SDS-PAGE) was concentrated to ~ 5 mL (70 mg/mL) under nitrogen using an Amicon stirred cell fitted with a YM10 membrane.

C. Synthesis of Glutathionylspermidine and its Disulfide.

Preparation of the C59A mutant of glutathionlyspermidine synthetase/amidase. Glutathionylspermidine (GspSH) was synthesized and purified using a procedure similar to that described by Bollinger et al. (*3*). The *gss* gene was amplified from genomic *E. coli* DNA and subcloned into the pET20b(+) expression vector as described for *yfcG*. The following primers and restriction sites were used: forward primer,

AAAAAAGAATTCATGAGCAAAGGAACGACCAGC (*EcoRI*) and reverse primer, TTTAAGCTTACTTTTTCACCACAATTAACGGTT (*HindIII*). To prevent hydrolysis of the conjugate by the enzyme, cysteine 59 was mutated to alanine with the QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) according to the manufactures protocol. The following primers were used, GGC CAC AAG TGG CAA **TCC** GTT GAA TTT GCT CGC CG (forward) and CGG CGA GCA AAT TCA AC**G GA**T TGC CAC TTG TGG CC (reverse). The pET20b(+) plasmid containing the *gss* gene with the C59A mutation was transformed in BL21(DE3) cells and the protein expressed per the procedure described earlier. After harvesting, the cells were sonicated and a streptomycin sulfate precipitation was performed. An ammonium sulfate precipitation at 65% saturation was also performed. The pellet containing the precipitated proteins was collected by centrifugation at 22,100 x g for 40 minutes at 4° C. The pellet was dissolved in 20 mM HEPES buffer (pH7.0) containing 1 mM EDTA and 1mM DTT and dialyzed against the same buffer overnight. The protein was partially purified by DEAE-Sepharose column equilibrated in the dialysis buffer. The GSS (C59A) protein was eluted from the column with a linear gradient of 0 to 0.4 M NaCl in the same buffer. Fractions containing the partially purified protein were combined and concentrated.

Enzymatic Synthesis of Glutathionylspermidine. A 30 mL reaction containing 5 mM GSH, 10 mM spermidine, 15 mM $MgCl_2$ and 30 μM GSS (C59A) in 100 mM KH_2PO_4 (pH 6.5) buffer was initiated by the addition of 15 mM ATP. The reaction was allowed to proceed at room temperature overnight and was terminated by heating the mixture to 95° C for 3 min. The precipitate was removed by centrifugation and the supernatant was loaded onto a SP-Sepharose column equilibrated with 50 mM KH_2PO_4 (pH 7.0). The column was washed with the equilibration buffer and the protein was eluted by a linear gradient (0-300 mM) of ammonium

acetate, pH 5.2. A small volume of each fraction was removed and mixed with 5 mM DTNB to check for the presence of a thiol (as indicated by a yellow color change). A 5 μL aliquot of the fractions containing thiols was reacted with monobromobimane and the fluorescent product analyzed by reverse phase HPLC with on a Beckman Ultrasphere C18 column (4.6 mm x 25 cm). The mobile phase was an CH_3CN and 140 mM ammonium acetate (pH 5.0) gradient (15-25% over 20 min). Peaks were identified by the retention times of GspSH and GSH standards at 3.9 min and 14.5 min, respectively. Fractions containing only GspSH were pooled, lyophilized and stored at -20° C.

Preparation of GspSH-disulfide. A solution of 20 μmol glutathionylspermidine [8.7 mg in 10 mL of water (pH 7.0)] was mixed with 20 μmol of diamide (3.4 mg in 10 mL of water) in the manner described by Kosower and Kosower (*4*). The reaction was allowed to proceed for ~3 min at room temperature. The reagent was removed by several extractions with dichloromethane (1 volume of CH_2Cl_2/3 volume of reaction solution). After reaction with diamide no remaining GspSH could be detected upon reaction with DTNB. The complete conversion of GspSH to GspSH-disulfide was confirmed by reduction of the GspSH-disulfide back to GspSH in the presence of sodium borohydride. A small volume of GspSH-disulfide was incubated with 1.5 molar excess sodium borohydride at pH 9.0. The reaction was allowed to proceed for ~3 min before the pH was adjusted to 6.0, to decompose the excess $NaBH_4$. The concentration of GspSH in the final stock solution was measured by titration with DTNB. GspSH concentration for the reduced GspSH-disulfide agreed with the concentration of GspSH used in the initial diamide oxidation reaction within 5%.

D. Crystallization and Determination of the Structure of YfcG

Crystallization of YfcG. Crystals were grown by the hanging-drop vapor diffusion method at room temperature (approximately 20 °C). The crystallization buffer in the well contained 20% w/v PEG 3000, 100 mM acetate at pH 4.5. The 10 μL drop contained a 50:50 mixture of the crystallization buffer and a solution of 33 mg/mL YfcG and 10 mM GSH (the final drop concentration of YfcG was 16.5 mg/mL and 5 mM of GSH). Crystals grew in about 2 weeks.

X-Ray Data Collection and Processing. Diffraction data were collected at the Vanderbilt University Center for Structural Biology Biomolecular Crystallography Facility. Crystals were screened initially with an Xcalibur PX2 Ultra sealed tube X-ray generator with an ONYX CCD area detector (Oxford Diffraction) and Oxford Cryojet (Oxford Instruments) cryostat to maintain crystals at 100K. Complete data sets were acquired using a Bruker Microstar rotating-anode X-ray generator and a Bruker Proteum PT135 CCD area detector. Crystals were maintained at 100K using a Bruker Kryo-Flex cryostat. Data collection sweeps were optimized using Cosmo (Bruker AXS, 2008) software and data integrated and scaled using SADABS (Bruker AXS, 2008) and XPREP (Bruker AXS, 2008) in the PROTEUM2 (Bruker AXS, 2008) package. The cryoprotectant used was the crystallization buffer supplemented with 30% glycerol. The data collection statistics are given in Table S-1.

Structure Determination. The structure was determined using the molecular replacement method. The Fold and Function Assignment System web server (*5*) was used to search for known structures to use as the molecular replacement model. The structure (PDBid 1PN9) gave the best score and was chosen as the model for molecular replacement. The Matthews coefficient for one polypeptide chain in the asymmetric unit is 2.6 $Å^3$/Da. The program PHASER (*6*) was used to place the model in the asymmetric unit and calculate the initial phases. Using the iterative script

Table S-1. Data Collection and Refinement Statistics for the Structure Determination of YghU. Values for the outer shell are given in parentheses.

Data	
PDB file name	3GX0
space group	$P4_12_12$
cell parameters (a,b,c) (Å)	68.49, 68.49, 111.99
wavelength of data collection (Å)	1.54
no. of unique reflections	12,571 (1,462)
resolution of data (Å)	18.1-2.3
highest resolution shell (Å)	2.4-2.3
R_{sym}	0.130 (0.551)
completeness (%)	99.8 (98.5)
redundancy	10.9 (6.8)
Refinement	
resolution limits (Å)	18.1-2.3 (2.4-2.3)
number of reflections used	11,342 (1486)
number of reflections for R_{free}	584 (93)
R-factor	0.190 (0.251)
R_{free}	0.248 (0.353)
rms deviation bond length (Å)	0.017
rms deviation angle (°)	1.663
average B: main-chain/side-chain/water/GSSG ($Å^2$)	27.3/28.7/26.9/30.7

(www.solve.lanl.gov) which includes cycles of density modification and automated model building with RESOLVE (*7,8*) and molecular refinement with REFMAC5 (*9*), 160 of the 215 residues were built and 119 side chains were placed. The model was completed by cycles of examining and building in the graphics display program COOT (*10*) and refining in REFMAC5. The final model included 205 residues, one GSSG ligand, and 93 water molecules. Ten residues at the C-terminal are not seen in the electron density map. The model was checked with the validation tools inside COOT and with Molprobity (*11*). The Ramachandran plot highlighted only one outlier, E71. The final refinement statistics are shown in Table S-1.

E. Steady State Fluorescence Titration

Equilibrium dissociation constants were determined for the complex of YfcG with GSH, GSO_3^-, GspSH, GspSH-disulfide and GSSG by titration of the intrinsic protein fluorescence. Measurements were made on a SPEX Fluorolog-3 spectrofluorimeter in constant wavelength mode. The samples were excited at 295 nm and the emission was collected at 340 nm and integrated over a period of 30 seconds. The change in fluorescence intensity of 1 μM YfcG in 100 mM KH_2PO_4 buffer at pH 7.0, was measured after 5 min preincubation with each addition of ligand. Fluorescence data for each concentration of thiol was collected three times and averaged. Three independent trials were completed for the GSH, GspSH and GSSG substrates. One trial was completed for the GSO_3^- and GspSH-disulfide ligands. All data were corrected for inner filter effects using the equation: $F_c = F^*\text{antilog}[(A_{295} + A_{340})/2]$, and for dilution. The data was fit to a one-site binding model according to the equation: $Y = Y_{max}/[1 + (K_D/X)]$, where Y = change in fluorescence and X = concentration of the ligand.

F. Steady State Enzyme Kinetics

Glutathione Transferase Activity Assays. The GSH transferase activity of YfcG was measured with a variety of electrophilic substrates including CDNB, ethacrynic acid, trans-4-phenyl-3-butene-2-one, 1,2-epoxy-3-(4-nitrophenoxy) propane and 1,4-dichloro-2-nitrobenzene. Measurable activity was only detected for the CDNB substrate. Reactions were followed at 340 nm using a Perkin-Elmer Lambda 45 Spectrophotometer. In each reaction 1 μM YfcG was pre-incubated with varying concentrations of GSH (0.5-25 mM) for 5 minutes in 100 mM KH_2PO_4 buffer, pH 6.5. The reactions were started by addition of CDNB in acetonitrile (1 mM final concentration). All measurements were made at 25°C. Initial rate measurements for the production of the GSDNB conjugate were calculated using the slope of the increase in absorbance at 340 nm ($\Delta\varepsilon = 9{,}600\ M^{-1}cm^{-1}$). The data was corrected for the spontaneous reaction between GSH and CDNB. All measurements were made in triplicate and averaged. The data was fit to the Michaelis-Menten equation using GraphPad Prism (La Jolla, CA) and values of V_{max} and K_m were determined.

Glutathione-dependent Peroxidase Activity Assay. The GSH-dependent peroxidase activity of YfcG was measured using a coupled assay with GSH reductase similar to the method described previously (*12*). A variety of peroxides including hydrogen peroxide, benzoyl peroxide, lauroyl peroxide, cumene hydroperoxide and *tert*-Butyl hydroperoxide were tested. Activity was only detected with the cumene hydroperoxide substrate, however, the background reaction was significant (73-90% of enzyme catalyzed reaction). Reactions were followed at 340 nm using a Perkin-Elmer Lambda 45 Spectrophotometer. Each reaction contained 1 μM YfcG, varying concentrations of GSH (0.1 - 3.0 mM), 1 mM EDTA, 0.15 mM NADPH, 1 unit (6 μg/mL) glutathione reductase in 100 mM KH_2PO_4 buffer (pH 7.0). The reactions were started

by addition of 1.2 mM cumene hydroperoxide. All measurements were made at 37 °C. The data was corrected for the spontaneous reduction of cumene hydroperoxide by GSH. The decrease in absorbance at 340 nm was monitored over 3 minutes ($\Delta\varepsilon_{NADPH}$ = 6,220 $M^{-1}cm^{-1}$) and the slope of this line was used to calculate the initial rate. The data was fit to the Michaelis-Menten equation using GraphPad Prism and values of V_{max} and K_m were determined.

Disulfide Bond Reductase Assay. The disulfide bond reductase activity of YfcG was measured by a coupled assay with glutathione reductase similar to the method described previously (*13*). Briefly, reactions containing 2 mM EDTA, 0.2 mM NADPH, 6 μg/mL (1 unit) glutathione reductase, 0.1 μM YfcG and varying concentrations of GSH (0.1 to 4 mM) were prepared in 100 mM Tris-Cl (pH 8.0). Reactions were started by addition of 1 mM HED. All measurements were made at 25 °C. The decrease in absorbance at 340 nm was monitored over 4 minutes ($\Delta\varepsilon_{NADPH}$ = -6,220 $M^{-1}cm^{-1}$) and the slope of this line was used to calculate the initial rate. All measurements were done in duplicate and averaged. The data was fit to the Michaelis-Menten equation using GraphPad Prism and values of V_{max} and K_m were determined.

REFERENCES AND NOTES

1. Certain commercial materials, instruments, and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments, or equipment identified is necessarily the best available for the purpose.
2. Abbreviations used: IPTG, isopropyl-D-thiogalactopyranoside; PCR, polymerase chain reaction; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA,

ethylenediaminetetraacetic acid: GSH, glutathione; GspSH, glutathionylspermidine; GspSH-disulfide, glutathionylspermidine disulfide; GSO_3^-, glutathione sulfonate; HEPES, N-2-Hydroxyehtyl piperizine-N'-ethanesulfonic acid; LB, Luria Broth; PEG, polyethyleneglycol; HA, hydroxyapatite; SP, sulphopropyl; ATP, adenosine-5'-triphosphate; DTNB, 5, 5'-dithiobis-(2-nitrobenzoic acid); PEG, polyethyleneglycol; CDNB, 1-chloro-2,4-dinitrobenzene; HED, 2-hydroxyethyldisulfide; NADPH, nicotinamide adenine dinucleotide phosphate

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