SUPPORTING INFORMATION

DNA Protection by the Bacterial Ferritin Dps via DNA Charge Transport

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Materials

All chemicals and starting materials were purchased from commercial sources and used as received. $[Ru(phen)(dppz)(bpy')]Cl_2$, where phen = 1,10-phenanthroline, dppz = dipyrido[3,2-a:2′,3′-c]phenazine and bpy′ = 4-butyric acid-4′-methyl-2,2′-bipyridine, was synthesized according to published methods (*1*), purified by reversed-phase chromatography, and characterized by NMR and ESI mass spectrometry (expected for the +2 ion: 409.62 m/z, observed: 410.2 m/z).

DNA synthesis and purification

Oligonucleotides were synthesized using standard phosphoramidite chemistry on an Applied Biosystems 3400 DNA synthesizer or purchased from Integrated DNA Technologies (Coralville, IA), purified using reversed-phase HPLC with a gradient of increasing acetonitrile, and characterized by MALDI-TOF mass spectrometry. The DNA sequences included a 70-mer: 5′-CTCTCGTCGTCAGTTGTCAAGTGTGCATGAAAGTAGAGTCTCGCTAAGCTCGGGA GAACAGAGAGATGCT-3′, and complementary 55-mer: 5′-TCCCGAGCTTAGCGAGACTCT ACTTTCATGCACACTTGACAACTGACGACGAGAG-3′, and 15-mer sequences: 5′-AGCAT CTCTCTGTTC-3′. In order to covalently couple racemic $[Ru(phen)(dppz)(bpy')]Cl_2$ to the 5′ end of the 15-mer, the DNA was modified (while on the solid support after DNA synthesis) with a 9-carbon saturated hydrocarbon tether terminating in an amine group to allow for amide coupling to the carboxylic acid moiety of the bpy′ ligand (*2*). Significant changes from published methodology include the following: after tether addition, the samples were lyophilized to ensure dryness; 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 11.6 μmol) was used in addition to previously described reagents in the ruthenium coupling step; metal coupling was allowed to proceed for 24 hours; and the final conjugates were

cleaved from the solid support by incubating in NH_4OH for 4 hours at room temperature, followed by 3 hours at 60°C. The Ru-DNA conjugates were then purified by reversed-phase HPLC, characterized by MALDI-TOF mass spectrometry (expected: 5479 g/mol, observed: 5487.59, 5487.47, 5488.39, and 5487.80, for the 4 eluting isomers, respectively), and quantified based on their absorbance at 440 nm ($\varepsilon_{440} = 1.9 \times 10^4\ M^{-1}cm^{-1}$).

Site-directed mutagenesis

The H51G/H63G *E. coli* Dps double mutant was made with a Quikchange II-E Site-Directed Mutagenesis Kit (Stratagene) using the pBAD18-dps plasmid (containing the WT *E. coli dps* gene and an ampicillin resistance cassette) donated by Dr. Roberto Kolter (*3*) as a template. The H51G mutation was made first, and this mutagenized plasmid was used as a template to make the H63G mutation. Primer sequences are shown in Table S1. Primers were purchased from Integrated DNA Technologies. All mutagenized plasmids were sequenced (Laragen) to confirm the desired sequences. After successfully creating the mutant pBAD18-dps plasmid, the *E. coli* cell line ZK2471 (dps::kan ΔrecA-(Cam) Δ*ara*714, leu::Tn10) donated by Dr. Roberto Kolter (*3*) was made electrocompetent and the plasmid was transformed via electroporation into these cells.

Dps overexpression and purification

WT and H51G/H63G *E. coli* Dps were purified according to published procedures with a number of modifications (*4*,*5*). A single colony of *E. coli* cell line ZK2473, which consists of ZK2471 (detailed above) with the pBAD18-dps plasmid, was used to inoculate 50 mL of LB media containing 100 μg/mL ampicillin and 50 μg/mL kanamycin which was grown overnight at 37°C with 200 rpm shaking. The subsequent day, 10 mL of the overnight culture was used to inoculate 1 L cultures of LB containing the same concentrations of antibiotics, which were

grown at 37ºC with 150 rpm shaking until OD_{600} = 1. Overexpression of Dps was then induced with 0.3% L-arabinose. After 4 hours of incubation at 37 ºC with continued shaking, the cells were harvested by centrifugation for 10 minutes at 7,000 *g*. Cells were resuspended in Buffer A (50 mM Tris, pH 8, 500 mM NaCl, 0.1 mM EDTA) containing a protease inhibitor cocktail (Roche) and lysed via microfluidization. The cell lysate was clarified by centrifugation at 15,000 *g* for 15 minutes, followed by a second round of centrifugation of the supernatant at 27,000 *g* for 20 minutes. The resulting supernatant was fractionated by ammonium sulfate (AS) precipitation by slow addition of solid AS up to 60% saturated AS, centrifugation for 15 minutes at 10,000 *g*, and collection of the supernatant. Next, more solid AS was added to the supernatant up to 90% saturated AS in order to precipitate Dps. The protein was recovered by centrifugation for 20 minutes at 27,000 *g*, and the pellet resuspended in approximately 5 mL of Buffer A. The partially-purified Dps was then dialyzed against a low ionic strength buffer (20 mM Tris, pH 8), where it precipitated while colored contaminants remained in solution. Precipitated protein was isolated by centrifugation at 26,000 *g* for 10 minutes, and resuspended in Buffer B (50 mM Tris, pH 8, 2 M NaCl, 0.1 mM EDTA). Dps was separated from DNA and other contaminants by loading onto a HiLoad 16/600 Superdex 200 pg size exclusion column (GE Healthcare), and running with Buffer B. Fractions were pooled that had an A_{260}:A_{280} ratio of 0.57, indicating no nucleic acid contamination. Finally, excess salt was removed by exchanging Dps into Buffer C (50 mM Tris, pH 7, 150 mM NaCl, 20% glycerol) using a HiPrep 26/10 desalting column (GE Healthcare). From 4 L of cell culture, the yield of purified Dps is approximately 130 mg. Protein purity was confirmed by SDS-PAGE (data not shown).

Iron loading and oxidation of Dps

Buffer (50 mM Tris, pH 7.00, 150 mM NaCl) was placed in a Schlenk flask and deoxygenated with 4 cycles of freeze-pump-thaw. Tris buffer was utilized because MOPS buffer was found to disrupt flash-quench chemistry, resulting in lower overall yields of oxidative DNA damage. Dps solutions were placed in a 5 mL Schlenk tube and deoxygenated by 4 sets of 10 cycles each of rapid dynamic vacuum followed by argon filling while constantly stirring, letting the protein stir under argon for 15 minutes between sets. Solid ferrous iron (as iron sulfate heptahydrate) was placed in a separate Schlenk tube and put under vacuum for at least 2 hours. Solid sodium dithionite was dispensed under an anaerobic atmosphere into a sealed vial. All deoxygenated components were then brought into an anaerobic chamber (Coy); subsequent steps were accomplished within the anaerobic chamber. Dithionite was used to scavenge residual dioxygen.

First, buffer was prepared with sufficient dithionite (800 μM) such that when the dithionite-containing iron solution is added to the Dps solutions, the final dithionite concentration was approximately in 5-fold excess over the protein concentration. This dithionite-containing buffer was added to the solid ferrous iron to make a solution of approximately 6 mM. To load the ferroxidase sites of Dps with ferrous iron, Dps solutions (20 μM) were incubated with 2-fold excess ferrous iron (48 Fe^{2+}/Dps) for 2 hours in the presence of 100 μM dithionite. Excess iron and dithionite were then removed with small size exclusion columns (Micro Bio-Spin 6, Biorad) that had been exchanged into deoxygenated buffer. While as-purified Dps was considered to be Apo-Dps (with reliably ≤ 1 Fe/Dps), in order to control for the possibility of residual dithionite in the ferrous iron-loaded protein, Apo-Dps was treated with dithionite in the same manner as ferrous iron-loaded Dps. Specifically, Apo-Dps was incubated

with buffer containing 100 μM dithionite for 2 hours and subsequently passed through size exclusion columns in the same manner as above. The UV-Visible absorbance spectrum of the ferrous iron-loaded protein was measured (Figure S4); no change is expected for ferrous iron binding.

A sample of iron-loaded Dps was removed from the anaerobic chamber in order to determine the Fe/Dps dodecamer. Protein concentrations were measured using the Bradford assay (Sigma). The iron concentration was determined based on the absorbance of $[Fe(bpy)_3]^{2+}$ (*6*). Protein (50 μL) was first denatured by addition of 2 M HCl (125 μL), and then precipitated with addition of 20% w/v trichloroacetic acid (125 μL). Precipitated protein was removed by centrifugation at 16,000 *g* for 20 minutes, and saturated sodium acetate (250 μL) was added to buffer the resulting supernatant. Iron was reduced with thioglycolic acid (6 μL) and chelated with 2,2′-bipyridine (50 μL of 0.4% w/v solution in 5% acetic acid). The absorbance of the resulting $[Fe(bpy)_3]^{2+}$ solution was measured at 522 nm and concentrations calculated using a molar absorptivity value of 8,789 $M^{-1}cm^{-1}$ derived from a standard curve of ferrous sulfate heptahydrate solutions (*7*).

Ferrous-iron loaded Dps was chemically oxidized within the anaerobic chamber. Solid sodium ferricyanide was brought into the anaerobic chamber and deoxygenated buffer was added to make a 10 mM solution. 4-fold excess ferricyanide (based on 12 Fe^{2+}/Dps) was added to the ferrous-iron loaded Dps solutions and incubated for approximately 10 minutes (Figure S4). Ferricyanide was then removed by spin columns as described above. The UV-Visible spectrum of the iron-loaded protein was measured to confirm iron oxidation, characterized by increased absorbance at 310 nm (*5*).

Anaerobic Fe(II) fluorescence titration of Dps

Dps solutions, solid iron sulfate heptahydrate, and buffer solutions were deoxygenated as described above. MOPS buffer was used to allow comparison with iron loading results in Tris buffer. Samples were prepared in an anaerobic chamber (Coy), and measured in sealed cuvettes. Each sample contained 1 μM Apo-Dps with either buffer alone (50 mM MOPS, pH 7.00, 150 mM NaCl) or additionally 6, 12, 18, 24, or 36 Fe^{2+}/Dps. Dps solutions were incubated with ferrous iron for 10 minutes before spectra were taken. As a control, the spectra of 36 Fe^{2+}/Dps was retaken after an additional 2 hours of incubation, and was found to be almost identical (data not shown). Using an ISS-K2 spectrofluorometer, the solutions were excited at 280 nm, and emission measured from 300 to 400 nm (Figure S1). A spectrum of buffer alone has been subtracted from all traces.

Circular dichroism

Protein concentrations (5 μM) were determined using a calculated molar absorptivity value for the *E. coli* Dps dodecamer of ε_{280} = 185,640 $M^{-1}cm^{-1}$ (calculated using ExPASy ProtPram tool, http://web.expasy.org/protparam/). Spectra were recorded at 25°C on a 62A DS circular dichroism spectrometer (AVIV) in a buffer consisting of 50 mM Tris, pH 7.00, 150 mM NaCl (Figure S2). The spectra shown are the average of three individual scans, with a buffer alone spectrum subtracted.

Guanine oxidation experiment

69-mer single-stranded DNA, with the same sequence as the 70-mer listed above but lacking the 3′-thymine, was radiolabeled with α-^{32}P-dTTP (Perkin Elmer) using standard protocols, treated with 10% piperidine for 30 minutes at 90°C to cleave any abasic sites, and purified by 20% denaturing PAGE as previously described (*8*). The radiolabeled 70-mer strand

was then annealed with unlabeled 70-mer and complementary 55-mer DNA by heating at 90°C for 5 minutes, followed by slow cooling to room temperature, to result in an approximately 10% statistical radiolabel with roughly 1,000,000 cpm in each sample. After this first annealing step, the Ru-15-mer conjugate was added to the solution, heated to 65°C for 5 minutes, and again slowly cooled to room temperature. The annealed Ru-DNA samples were dried, brought into the anaerobic chamber, and resuspended with deoxygenated buffer (50 mM Tris, pH 7.00, 150 mM NaCl). Solid $[Co(NH_3)_5Cl]^{2+}$ was brought into the anaerobic chamber and also resuspended in deoxygenated buffer. Buffer and Dps solutions were deoxygenated and prepared as described above in "Iron loading and oxidation of Dps." Samples were prepared within the anaerobic chamber and transferred to septa-sealed NMR tubes. Protein-containing samples were incubated with DNA for 30 minutes prior to irradiation to allow time for DNA binding. In order to induce DNA damage via the flash-quench technique, the anaerobic samples were irradiated at 442 nm using a Hg/Xe arc lamp (Oriel) equipped with a monochromator and 320 nm long-pass filter, with total incident powers of approximately 3700 mJ/sample (2.4 – 3.2 mW, 20 – 25 minutes irradiation). Within each experiment, all samples were irradiated with identical lamp power. Samples containing both Dps and cobalt quencher precipitated upon irradiation; such precipitation was independent of Dps iron-loading. Following irradiation, samples were aerobically treated with 10% piperidine for 30 minutes at 90°C to cleave the DNA at sites of oxidative guanine damage and subsequently dried on a centrifugal evaporator. Samples were resuspended with denaturing loading dye (80% formamide, 10 mM NaOH, 0.025% xylene cyanol, 0.025% bromophenol blue in 90 mM Tris-borate). After heating at 90°C for 2 minutes, identical scintillation counts were loaded for each sample onto a 20% denaturing polyacrylamide gel, and electrophoresed at 90 W for 2 hours. DNA damage was quantified by phosphorimagery

(ImageQuant TL). Specifically, oxidative DNA damage was quantified for each lane by the ratio of guanine triplet damage to the undamaged parent band. Each protein set (i.e. Apo-Dps 0, 2, 4, and 6 μM) was then normalized to the lane with no added protein (normalized lane profiles are shown in Figure 2). The fold damage attenuation was calculated by dividing the normalized level of DNA damage in the no protein lane by that in the lane with maximal protein concentration (i.e. "0 μM Apo-Dps" / "6 μM Apo-Dps"). Values given in the text are the average of three separate experiments; errors are given as standard deviation.

Gel-shift assays

In an anaerobic chamber (Coy), 10 nM of 100% 5′-^{32}P-radiolabeled 70-mer DNA with the complementary 70-mer strand was incubated with 0 – 600 nM Apo-Dps, ferrous iron-loaded Dps or ferric iron-loaded Dps for 25 minutes in 50 mM Tris, pH 7.00, 150 mM NaCl, 20% glycerol. Deoxygenation procedures for all components were the same as described above in "Iron loading and oxidation of Dps." Within the anaerobic chamber, samples were then loaded onto 10% TBE gels and electrophoresed for 1 hour at 50 V at room-temperature in 0.5 X TBE. The DNA embedded in the gel matrix was aerobically transferred to an Amersham Hybond-N membrane (GE Healthcare) via electroblotting and quantified by phosphorimagery (ImageQuant TL). Dps-DNA complexes did not enter the gel matrix; thus, gels were quantified by the disappearance of the free DNA band with increasing titration of Dps. The fraction of free DNA, relative to the amount of DNA with no added protein, was plotted versus protein concentration for each iron loading condition of Dps (data not shown). These data were fit sigmoidally, and the fit used to solve for the protein concentration where the fraction of free DNA was 0.5. The K_d values extracted in this manner are 260 nM for Apo-Dps, 230 nM for ferrous iron-loaded Dps, and 310 nM for ferric iron-loaded Dps.

Ruthenium luminescence

Samples were prepared in an anaerobic chamber (Coy), and measured in sealed cuvettes. Each sample contained 8 μM duplexed Ru-DNA (70-mer DNA with complementary Ru-15-mer and 55-mer strands) with either buffer alone (50 mM Tris, pH 7.00, 150 mM NaCl) or additionally 8 μM Dps solutions with varying iron loading (Apo-Dps and Dps loaded with either ferrous (11.8 ± 0.2 Fe/Dps dodecamer) or ferric iron). Using an ISS-K2 spectrofluorometer, the solutions were excited at 440 nm, and emission measured from 500 to 800 nm. A spectrum of buffer alone has been subtracted from all traces. Slight precipitation occurred for the sample containing Ru-DNA and ferric iron-loaded Dps, resulting in a raised baseline.

Table S1. Primers used to make H51G/H63G *E. coli* Dps double-mutant via site-directed mutagenesis, with mutagenized codons underlined and bolded.

Mutation	Primer sequence
H51G-forward	5′-GAT CTT TCT TTG ATT ACC AAA CAA GCG **GGC** TGG AAC ATG C-3′
H51G-reverse	5′-GCA TGT TCC A**GC** **C**CG CTT GTT TGG TAA TCA AAG AAA GAT C-3′
H63G-forward	5′-GCT AAC TTC ATT GCC GTA **GGT** GAA ATG CTG GAT GGC TTC-3′
H63G-reverse	5′-GAA GCC ATC CAG CAT TTC **ACC** TAC GGC AAT GAA GTT AGC-3′

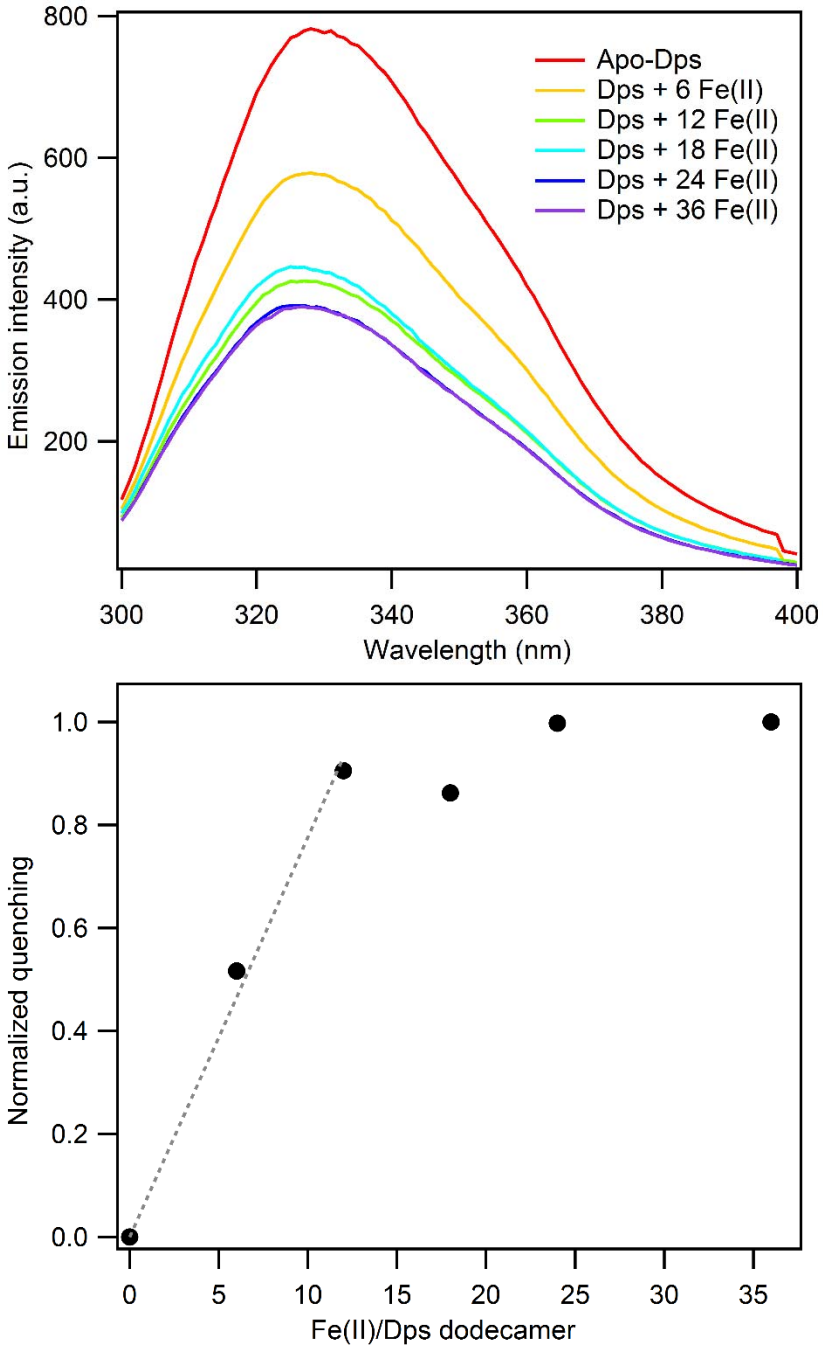

Apo-Dps
Dps + 6 Fe(II)
Dps + 12 Fe(II)
Dps + 18 Fe(II)
Dps + 24 Fe(II)
Dps + 36 Fe(II)
Emission intensity (a.u.)
800
600
400
200
300
320
340
360
380
400
Wavelength (nm)
Normalized quenching
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
Fe(II)/Dps dodecamer

Figure S1. Anaerobic titration of *E. coli* Dps with ferrous iron, which quenches the intrinsic protein fluorescence. *Upper*: Emission spectra of Apo-Dps compared to Dps that has been incubated with 6, 12, 18, 24 or 36 Fe(II)/Dps dodecamer (excitation wavelength = 280 nm). Protein concentration was 1 μM in a buffer of 50 mM MOPS, pH 7.00, 150 mM NaCl. *Lower*: Plot of fluorescence quenching, normalized to quenching observed with 36 Fe(II)/Dps, versus the number of Fe(II)/Dps. A linear fit to the 0 to 12 Fe(II)/Dps data yields a line with the equation $y = 0.0776x$, with an R^2 value of 0.99.

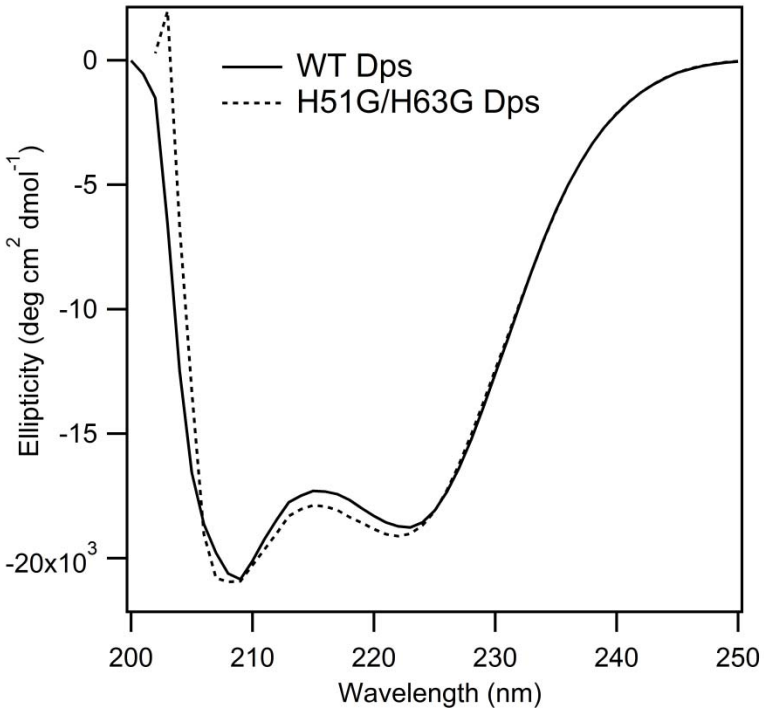


Figure S2. Circular dichroism spectra of wild-type (WT) (solid line) and H51G/H63G *E. coli* Dps (dashed line). Protein concentration was 5 μM in a buffer of 50 mM Tris, pH 7.00, 150 mM NaCl.

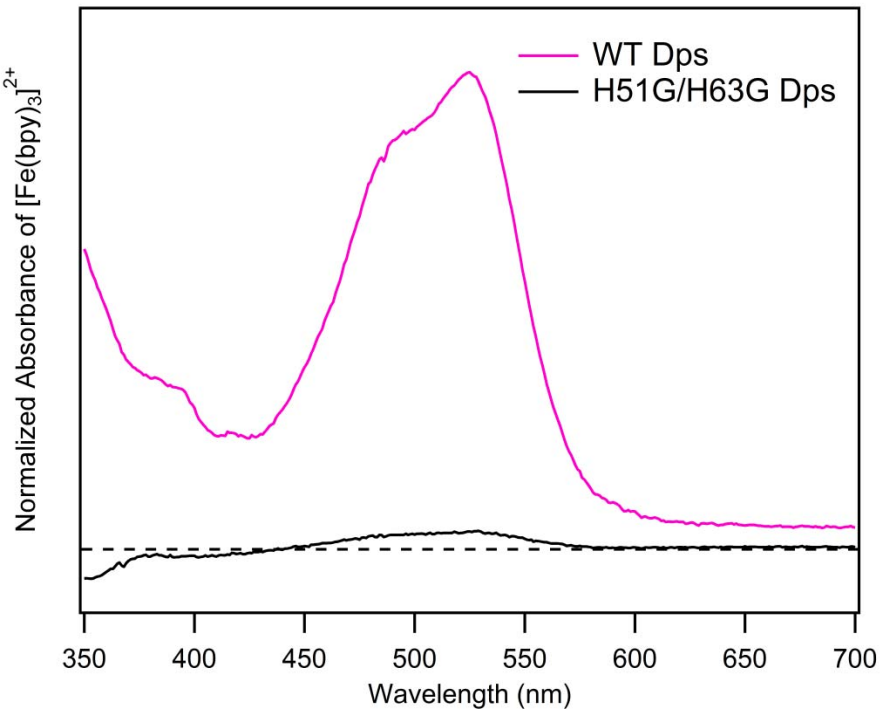
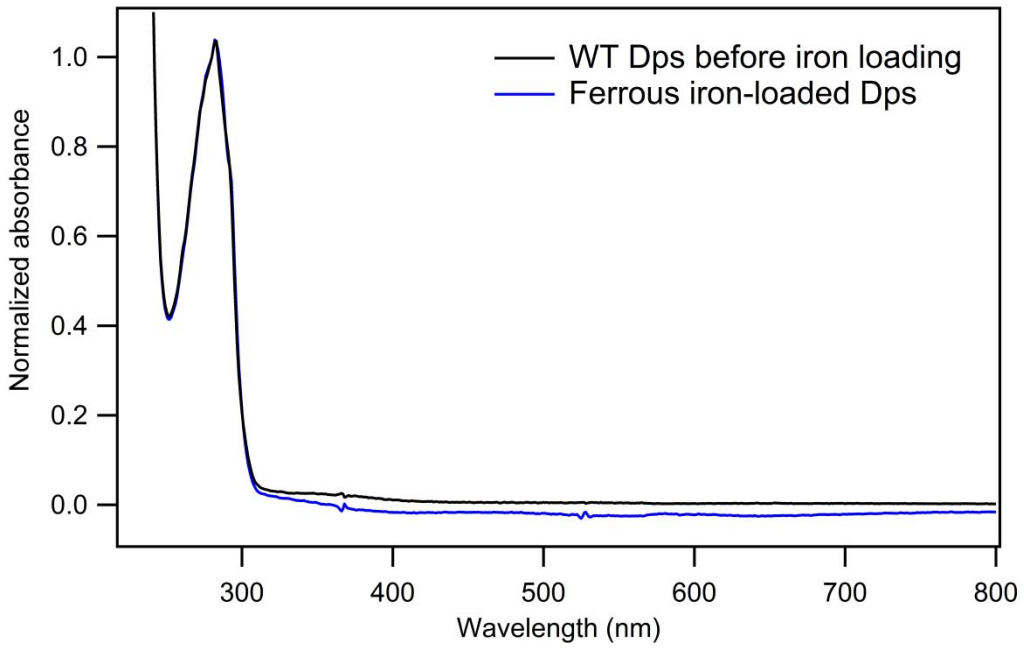


Figure S3. Ferrous iron loading of Dps ferroxidase mutant H51G/H63G compared to WT *E. coli* protein. Normalized UV-Visible spectra of $[Fe(bpy)_3]^{2+}$ produced from either WT Dps (pink) or H51G/H63G Dps (black). For normalization, the absorbance values were divided by protein concentration. The calculated number of iron atoms per Dps dodecamer is 11.8 ± 0.2 for WT Dps and 1.0 ± 0.3 for H51G/H63G Dps. Protein concentrations were determined via the Bradford assay; the concentration of $[Fe(bpy)_3]^{2+}$ was determined by A_{522} using a derived extinction coefficient of 8,789 $M^{-1}cm^{-1}$.


WT Dps before iron loading
Ferrous iron-loaded Dps
Normalized absorbance
1.0
0.8
0.6
0.4
0.2
0.0
300
400
500
600
700
800
Wavelength (nm)

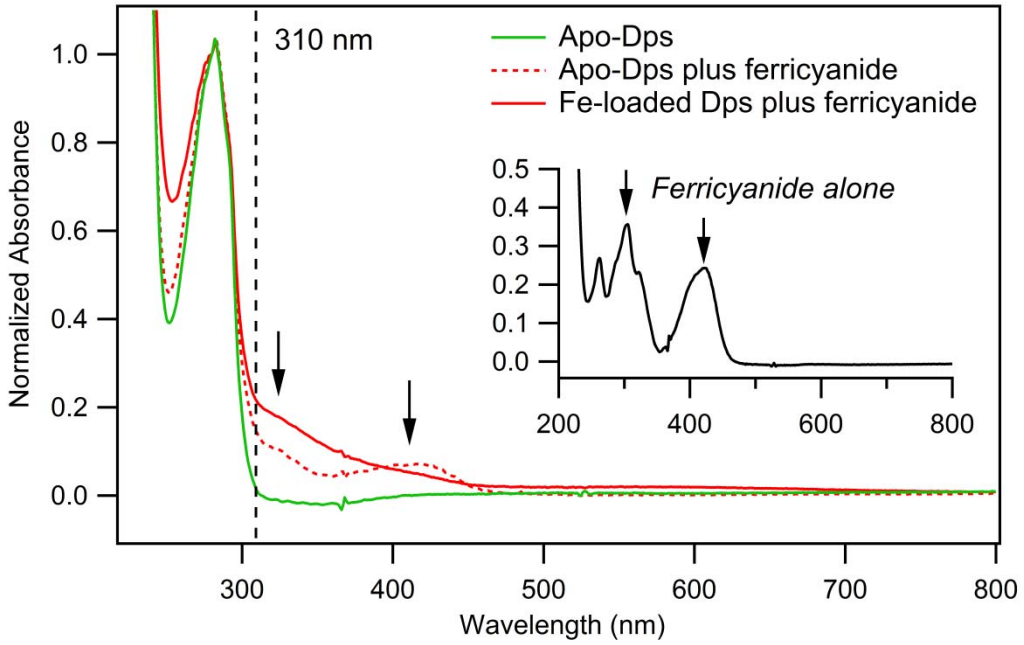

310 nm
Apo-Dps
Apo-Dps plus ferricyanide
Fe-loaded Dps plus ferricyanide
Ferricyanide alone
Normalized Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0.5
0.4
0.3
0.2
0.1
0.0
200
400
600
800
300
400
500
600
700
800
Wavelength (nm)

Figure S4. Ferrous iron loading of *E. coli* Dps and subsequent oxidation by ferricyanide. All protein spectra were normalized to A_{280}. *Upper*: Comparison of UV-Visible spectra of Dps before (black) and after (blue) anaerobically loading with ferrous iron; no change in the UV-Visible spectra of Dps is expected upon binding of ferrous iron. *Lower*: Dps oxidation with stoichiometric potassium ferricyanide. Apo-Dps (4 μM), shown in green, has minimal spectral features other than the characteristic protein absorbance at 280 nm. Apo-Dps with ferricyanide (4 μM each), shown as dashed red line, additionally shows characteristic spectral features of ferricyanide (inset, arrows). Ferrous-iron loaded Dps (4 μM), shown as solid red line, that has been incubated with stoichiometric ferricyanide displays a distinct increase in absorbance at 310 nm (dashed black vertical line), as expected for iron oxidation. Note that ferrocyanide is spectroscopically silent. A similar A_{310} increase was observed for oxidation by dioxygen (data not shown).

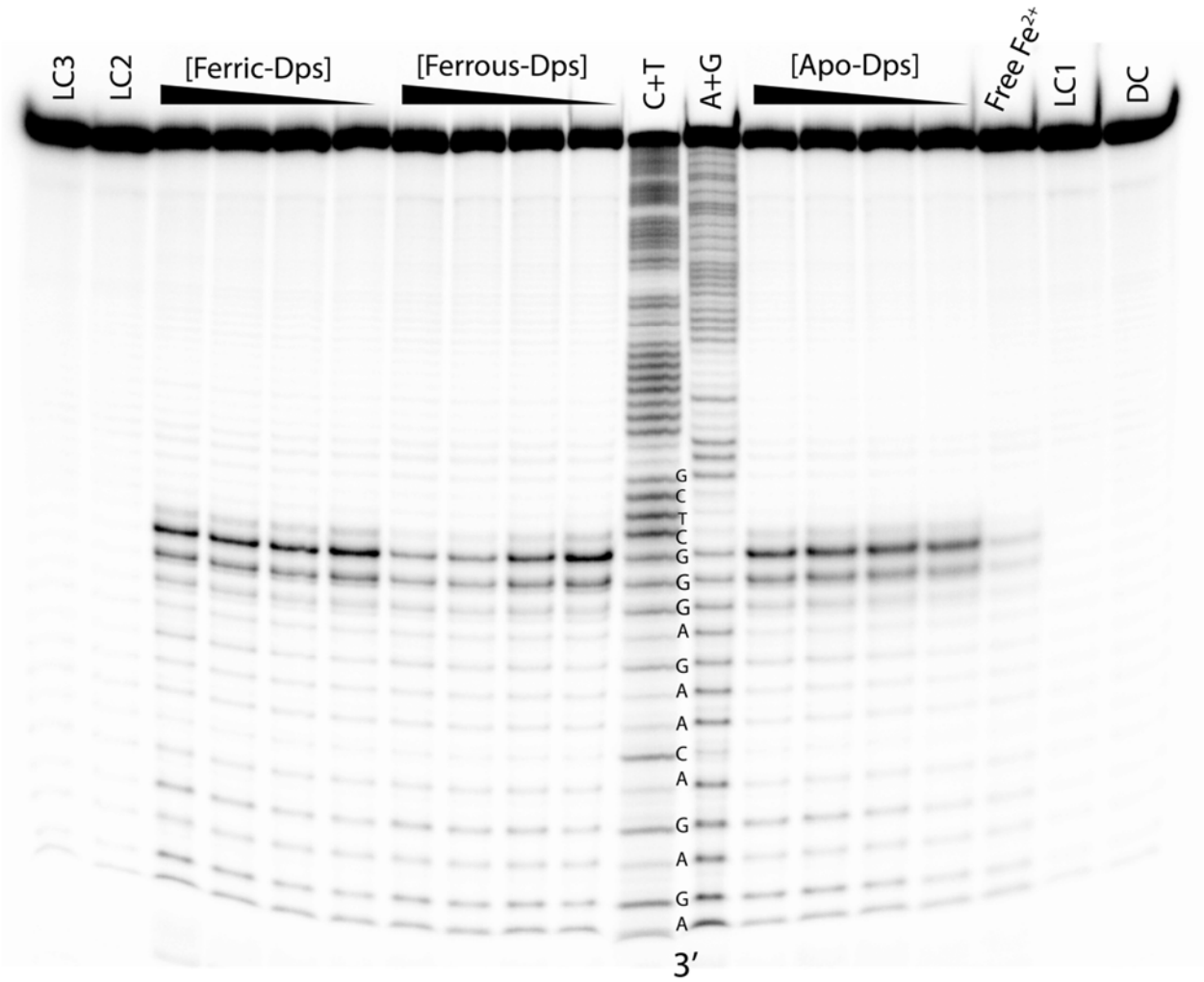


Figure S5. Autoradiogram of gel showing effect of increasing *E. coli* Dps with varying iron loading. A+G and C+T are Maxam-Gilbert sequencing lanes. Controls: Dark control (DC) contains all components (DNA, photooxidant, and quencher) but is not irradiated; Light control 1 (LC1) is irradiated but lacks quencher; LC2 is irradiated, lacking quencher, but contains 6 μM Dps loaded with ferric iron; LC3 is irradiated, lacking quencher, but contains 6 μM Dps loaded with ferrous iron; "Free Fe^{2+}" is irradiated, containing all components, but additionally free ferrous iron at a concentration equivalent to that in ferrous iron-loaded Dps. The number of Fe/Dps in ferrous iron-loaded Dps was 13.6 ± 0.6.

Supporting references

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7. This derived value is comparable to the reported molar absorptivity for $[Fe(bpy)_3]^{2+}$ of 8,650 $M^{-1}cm^{-1}$ (Ford-Smith, M.H.; Sutin, N. *J. Am. Chem. Soc*. **1961**, *83*, 1830-1834).

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