Supporting Information

UvrC Coordinates an O_2-Sensitive [4Fe4S] Cofactor

Rebekah M. B. Silva[1], Michael A. Grodick[1,2], Jacqueline K. Barton[1,*]

[1]*Division of Chemistry and Chemical Engineering*
California Institute of Technology, Pasadena, CA 91125, USA
[2]*Current address: Intel Corporation, Hillsboro, OR 97124, USA*

[*]E-mail: jkbarton@caltech.edu

Contents **Page**

Supporting Experimental Section

Primers and DNA Substrates. All oligomers were purchased from Integrated DNA Technologies (IDT) and all plasmids were purchased from Addgene. Dry oligomers were received on a 1 μmol scale and resuspended 600 μL in DNA Buffer (5 mM sodium phosphate, 50 mM NaCl, pH 7.0), and 300 μL of this stock was diluted in DNA buffer in a 1:1 ratio before purification.[1] ssDNA strands were purified on a reverse phase PLRP-S 300 Å column (Agilent) by HPLC using a linear gradient from 97% 50 mM ammonium acetate in ACN (pH 6.8) to 85% ammonium acetate over a period of 45 min at a flow rate of 2 mL/min. Peaks were collected, dried on a lyophilizer, ethanol precipitated, resuspended in DNA Buffer, quantified by UV-Vis, and stored at -20 °C until use unless otherwise indicated.[2] Sequences of DNA used here are given in **Table S1**. The fluorescein substrate was shipped dried and pre-purified, so this substrate was resuspended directly in DNA buffer to at a concentration of 300 μM. In DNA Buffer, duplexes were formed by mixing equimolar quantities of complementary strands at ≥ 30 μM, heating to 90°C for 10 minutes, and cooling to 20 °C over a linear gradient for 90 minutes.[2]

Thiol-modified oligonucleotide was resuspended in 10 mM Tris-HCl (pH 8.5, Qiagen) and reduced in 100 nmole batches with an excess of dithiothreitol (DTT, 50 to 100 mgs) to cleave the disulfide protecting group and reveal the thiol moity.[1,2] The deprotected oligonucleotide was purified on an Illustra NAP-5 column (GE Life Sciences) and then purified by HPLC as above. Deprotection of the electrochemistry substrate was confirmed on an Autoflex matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker). The thiol-modified strand was quantified by UV-Vis as above, sparged with Ar (1 s of sparging per μL of solution), and sealed with Teflon tape before storing. Duplexes including thiol-modified strands

were sparged and sealed with Teflon tape before annealing. Duplexes were also stored at -20 °C until use.

Liquid and Solid Growth Media.[1] Liquid media was prepared by adding 25 g of powered lysogeny broth (Miller, Mo BIO) in 1 L of MilliQ water and sterilizing the solution using the Amsco® Century™ SV-1262 Prevac Steam Sterilizer (Steris) on the Liquid 30 cycle.[1] Solid media was prepared by combining 12.5 g of powered lysogeny broth and 7.5 g of agar (Beckman Dickinson) in 0.5 L of MilliQ and sterilizing the solution by autoclaving. Once agar media had cooled to ~55 °C, ampicillin was added to a final concentration of 50 mg/L, and media was aliquoted into sterile, plastic petri dishes. Ampicillin was added to sterile, liquid media cultures immediately before inoculation.

Colony PCR.[1] To isolate the *uvrC* gene (1830 bases), a colony of the MG1665 *E. coli* strain grown on LB/agar plate was resuspended in 100 μL of ddH_2O, and 10 μL was mixed with Gibson Assembly (GA) primers (0.5 μM each), Expand High Fidelity dNTPs (200 μM; Roche), Expand High Fidelity 10x Buffer (1x, Roche), and Expand High Fidelity Polymerase in a total reaction volume of 50 μL.[1] Temperature cycling is a follows: 1. 5 minutes at 95 °C; 2. 34 cycles of 30 seconds at 95 °C, 1 minute at 55°C, and 4 minutes 72° C; 3. a final elongation for 10 mintues at 72 °C. Amplified products were purified using a PCR purification kit (Qiagen), mixed 1:1 with Ultraclean Gel Dye (MO Bio), resolved at 150 V for 1 hour on a 0.8% agarose gel (Invitrogen) containing 0.5 μg/mL EtBr, visualized with a transilluminator, purified using the gel extraction kit (Qiagen), purified again using the PCR purification kit (Qiagen), and quantified by UV-Vis.

Plasmid Construction.[1] The Gibson assembly method was used for plasmid construction. All plasmid samples were submitted to Laragen (Culver City, CA) for sequencing

to confirm integration of the desired gene into the plasmid using the procedures described.[1] In a total volume of 50 μL, 2.5 μg of pBAD plasmid (Addgene 37503), 50 units each of SspI and BamHI, and 10x Cutsmart Buffer (New England Biolabs) were restricted at 37 °C for 16 hours, heat inactivated at 65 °C for 20 min, and held at 4 °C. The restricted plasmid was purified as described. In a total volume of 42.6 μL, 100 ng of restricted plasmid was combined in a 1:1 ratio with the PCR-amplified gene and 2x GA Master Mix (New England Biolabs). The Gibson assembly was completed at 50 °C for 1 hour, diluted three-fold, and 2 μL of diluted samples were transformed into a vial of One Shot® TOP10 Electrocomp™ *E. coli* cells (Invitrogen) using a MicroPulser Electroporation Unit (Bio-Rad) with a 1.8 kV pulse. Cells were recovered by growing in 1 mL of SOC Media (Invitrogen) for 1 hour at 240 rpm at 37 °C. To an LB/Amp plate (50 mg/LB), 250 μL transformed cells were plated and grown at 37 °C overnight. Individual colonies were picked and grown in liquid cultures overnight as above, miniprepped using a Qiagen kit, and sequenced by Laragen. Samples containing the desired plasmids were transformed into TOP10 cells, grown, cell stocks were made with 25% glycerol, and cells were flash frozen in liquid N_2 before storing -80 °C.

Cysteine mutants were generated using site-directed mutagenesis. Primers (**Table S1**) were designed according to the guidelines included in QuikChange Kits (Agilent), and IDT's OligoAnalyzer was used to design primers with minimal hairpins. The QuikChange II-E Site-Directed Mutagenesis Kit was used to generate the Cys154Ala mutant. 50 ng of WT pBAD-UvrC plasmid and 125 ng of each primer was used. Temperature cycling was as follows: 1. 30 seconds at 95 °C; 2. 16 cycles of 30 seconds at 95 °C, 1 minute at 60 °C, and 10 minutes 68 °C; 3. a final extension according the manufacturer's instructions. Once the amplification was

complete, 30 U of Dpn I was added to the reaction mixture and then incubated for 3 hours at 37°C. Subsequent steps were completed as instructed by Qiagen.

The QuikChange II XL Site-Directed Mutagenesis Kit was used to generate the Cys166Ala, Cys174Ala, and Cys178Ala mutants. For the Cys166Ala reaction 50 ng of WT pBAD-UvrC plasmid was used; for the Cys174Ala and Cys178Ala reactions, 10 ng of plasmid was used. 125 ng of each primer was used for all reactions. Temperature cycling is a follows: 1. 30 seconds at 95 °C; 2. 16 cycles of 30 seconds at 95 °C, 1 minute at 60 °C, and 10 minutes 68 °C; 3. a final extension according the manufacturer's instructions. Once the amplification was complete, 10 U of Dpn I was added to the reaction mixture then incubated for 2 hours at 37°C. Subsequent steps were completed as instructed by Qiagen.

Induction Trials.[1] Overnight starter cultures of pBad plasmid containing UvrC or Cys→Ala mutants from a single colony were grown in Lb/Amp as above.[1] A large 1 L culture was inoculated as above, grown to an OD_{600} of ~0.6, and protein expression was induced by arabinose (10 mg/L). Time points were taken between 0 and 16 hours (0.5 mL), centrifuged at 9000 rpm for 2 min, and stored at -80 °C after discarding the supernatant. For SDS-PAGE gel analysis, these whole cell samples were resuspended in 0.5 mL of Blue Loading Buffer (New England Biolabs), vortexed, heated at 95 °C for 5 minutes, vortexed again, and heated at 95 °C for 5 minutes. Samples were developed at 200 V for 35 min on 7.5% Precast Protein Gels (Bio-Rad) in SDS-PAGE buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) with High Molecular Weight Dual Color Protein Standards (Bio-Rad). Gels were imaged on the ChemiDoc™ Imaging System (Bio-Rad).

Molecular Weight Determination.[3–5] Stock solutions of proteins from the Gel Filtration HMW Calibration Kit (GE Healthcare) were made anaerobically from lyophilized powder in

UvrC buffer according the manufacturer's instructions. Standards were flash frozen and stored at -80 ºC until use. Once standards were thawed on ice in a glove bag, all subsequent steps were completed at room temperature. Samples were diluted to recommended concentrations, passed through a 0.22 μM syringe filter, loaded into a Hamilton syringe, and injected into a 250 μL superloop. All chromatography was done anaerobically at a flow rate of 0.35 mL/min on a Bio-Rad NGC using a Superdex 100/300L analytical size exclusion column (GE Healthcare) in UvrC buffer. All standards were completed in triplicate. UvrC was thawed on ice in an anaerobic chamber, buffer-exchanged into UvrC buffer, quantified by UV-Vis (by cluster concentration), and 250 μL of UvrC (between concentrations of 5 and 10 μM by cluster for each replicate) was assessed by analytical size exclusion chromatography.

Radiolabeling of DNA Substrates.[6–9] For 5' end labeling phosphorylation, 14 μL water, 2 μL T4 PNK buffer (New England Biolabs), 1 μL ssDNA 100 μM stock solution, 2 μl T4 polynucleotide kinase solution and 1 μL of [γ-^{32}P] ATP (NEG035C005MC, 6000 Ci/mmol, 150 mCi/mL, PerkinElmer) were combined in a 1.7 mL microcentrifuge tube, the tube was clipped, and incubated at 37 °C for 30 min followed by heat inactivation at 5 °C for 20 min. Solutions were cooled, centrifuged, and the volume of the solution was brought up to > 25 μL (or two labeling reactions were combined). Micro Bio-Spin 6 column (Bio-Rad) were used to purify radiolabeled ssDNA followed by a Monarch PCR & DNA Cleanup Kit (New England Biolabs) with a modified protocol for short oligomers. The filtrate from the Micro Bio-Spin 6 column was combined with 100 μL Monarch DNA Cleanup Binding Buffer and 300 μL ethanol. The resulting solution was loaded into a Monarch DNA Cleanup Column (New England Biolabs) and spun at 16,000 g for 1 min. After discarding the flow-through, 500 μL of a 1:4 solution of Monarch DNA Cleanup Binding Buffer and ethanol were added to the column and spun at

16,000 g for 1 min. This washing procedure was repeated once more and flow through was discarded each time. The column was spun at 16,000 g for 1 min to remove residual ethanol. Radiolabeled ssDNA stocks were stored at -20 °C.

Annealing Titrations.[8,9] A 100% yield was assumed following radiolabeling. A solution of or 95:5 cold:radiolabeled between 30 to 40 μM dsDNA was generated by heating complementary strands to 95 °C for 10 min and cooling to room temperature over a linear gradient. Titrations from 75%-125% were prepared by varying the complement concentration DNA buffer in order to verify the duplex character of the substrate (and to detect any pipetting errors). Radioactivity was measured using the LS 6000SC Scintillation Counter (Beckman). DNA was electrophoresed at 50 V for 90-105 minutes at RT using Bio-Rad Mini-PROTEAN 4-20% TGX Precast Gels (Native PAGE) in Tris-Glycine buffer.[6] Phosphorimaging screens (GE Healthcare or Molecular Diagnostics) were exposed according to the guideline that samples with 300,000 counts require 1 hour of exposure. The exposed screens were scanned using the Typhoon FLA 9000 (GE Healthcare). Images were analyzed using Image Lab™ software (Bio-Rad).

Figure S1. The UvrC sequence contains additional sequence motifs suggestive of a [4Fe4S] center. Two putative LYR tripeptide motifs (shaded) are located N-terminal (VYR, IFK, or LFK) and between (VYR or VFK) the conserved cysteine residues (Cys154 and Cys166). A highly-conserved aromatic residue (Tyr169) and two conserved proline residues (Pro165 and Pro177) are also located in the vicinity of the conserved cysteines. A partial sequence is shown.

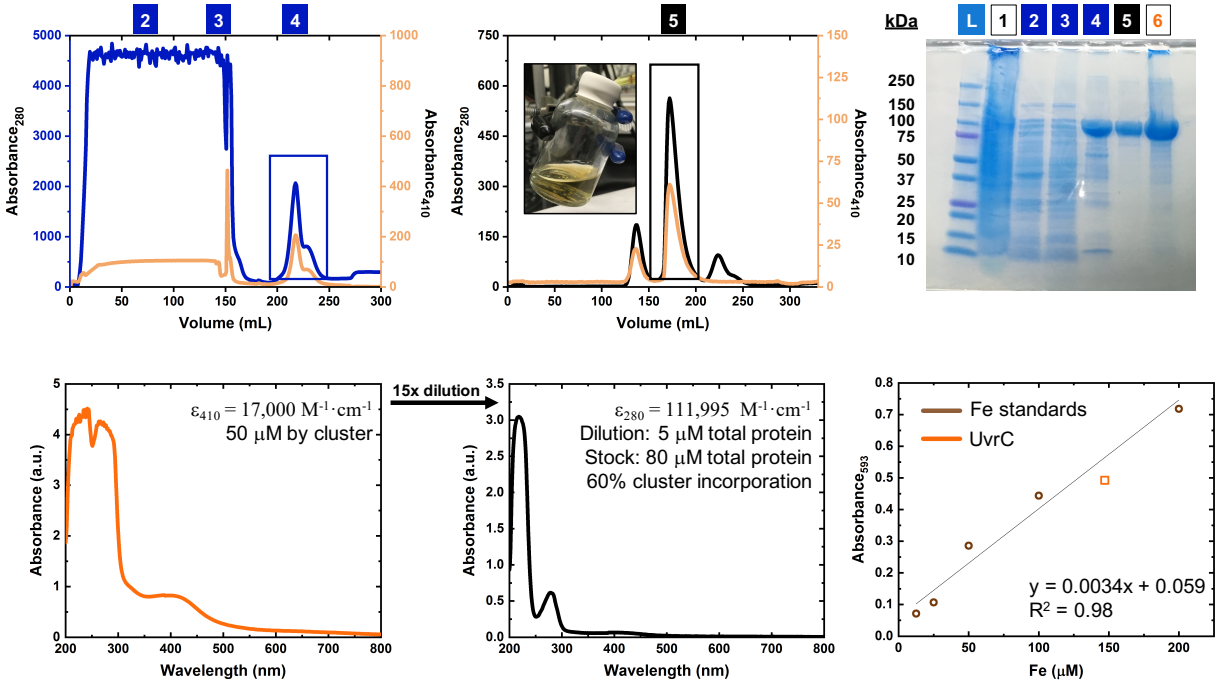


Figure S2. UvrC is purified using affinity and gel filtration chromatography. Shown are representative chromatograms. (*Top Left*) Cell lysate is first loaded onto a 10 mL Histrap column, washed with 10% elution buffer to remove non-specifically bound debris, and then eluted with a linear gradient of elution buffer (Materials and Methods). The collected peak (boxed, dark blue) is then concentrated and further purified on a Superdex 200 column. (*Top Middle*) The yellow and clear peak (boxed and pictured) was concentrated, stored in 250 μL aliquots at concentrations between 20 and 30 μM. All steps were completed in a glove bag or using standard Schlenk line technique under a positive pressure of N_2 or Ar gas. (*Top Right*) After concentration, the protein was analyzed by SDS-PAGE to assess purity. Labels with a blue background correspond to fractions taken from the Histrap column. The label with a black background corresponds to the fraction taken from the Superdex. L: Ladder; 1: Insoluble pellet (black box and text); 2: Histrap flow through; 3: Histrap 10% wash; 4: Collected Histrap fraction; 5: Collected Superdex fraction: 6: Concentrated UvrC (black box, orange text). (*Bottom Left and Middle*) Given is an example of how percent incorporation is calculated. (*Bottom Right*) A representative standard curve and data point for UvrC from an independent trial of the ferene assay is shown. All buffers are at pH 7.5. Lysis Buffer: 25 mM Tris-HCl, 0.5 M KCl, 10% v/v glycerol supplemented with 6-8 tablets of crushed cOmplete™ protease inhibitor cocktail tablets (Roche), DNAse (15 kU, Sigma), and DTT (1 mM); Nickel Elution Buffer: 25 mM Tris-HCl, 0.5 M KCl, 20% v/v glycerol, 0.5 M Imidazole, 1 mM DTT; Size Exclusion Buffer: 25 mM Tris-HCl, 0.5 M KCl, 20% v/v glycerol, 1 mM DTT.

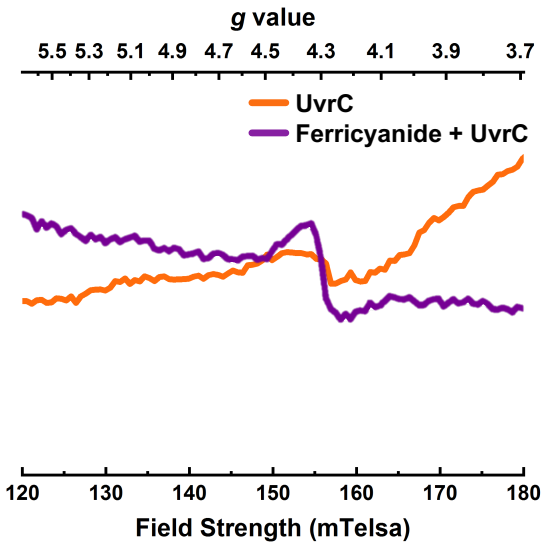


Figure S3. Oxidation of UvrC results in release of ferric iron. Oxidation of UvrC with ferricyanide results in release of a ferric iron species seen at a *g* value of 4.3 (purple), which is not observed above the background signal for UvrC in the native state (orange). EPR conditions are as in Figure 2.

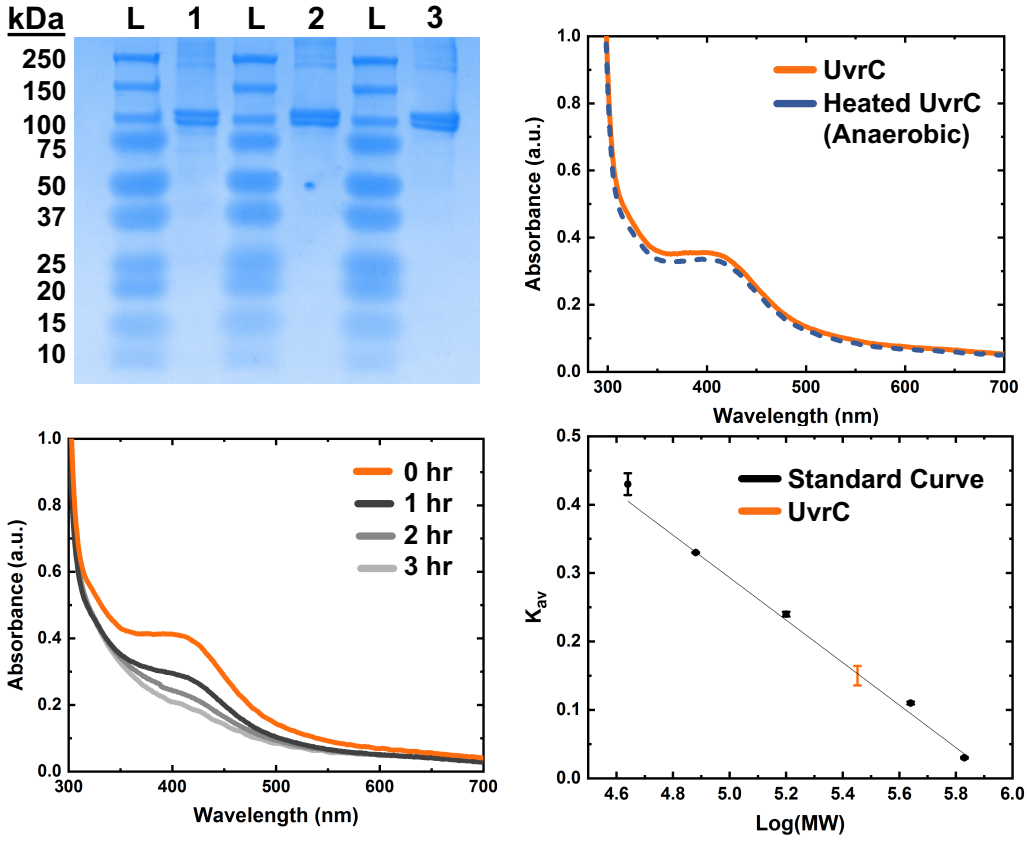


Figure S4. Aerobic incubation of UvrC at 37 °C results in degradation that is centered at the [4Fe4S] cluster. (*Top Left*) Aerobic incubation does not result in degradation of peptide backbone of UvrC.[10] L: Ladder; Lane 1: Purified UvrC; Lane 2: Aerobically-degraded UvrC; Lane 3: Aerobically-degraded UvrC incubated with the WM 30mer. (*Top Right*) The [4Fe4S] cluster of UvrC is stable during incubation at 37°C in the absence of O_2 over the course of 4 hours (blue dashed line). (*Bottom Left*) Binding to the duplexed WM substrate does not slow the aerobic degradation of the [4Fe4S] cluster. (*Bottom Right*) Relative to a standard curve of elution volumes for globular proteins, UvrC elutes at a volume consistent with a protein that is a dimer. Elution volumes were measured in triplicate for each protein. Average elution volumes are shown, and the error bars represent the standard deviation. Buffer and chromatography conditions are as in Figure 3.

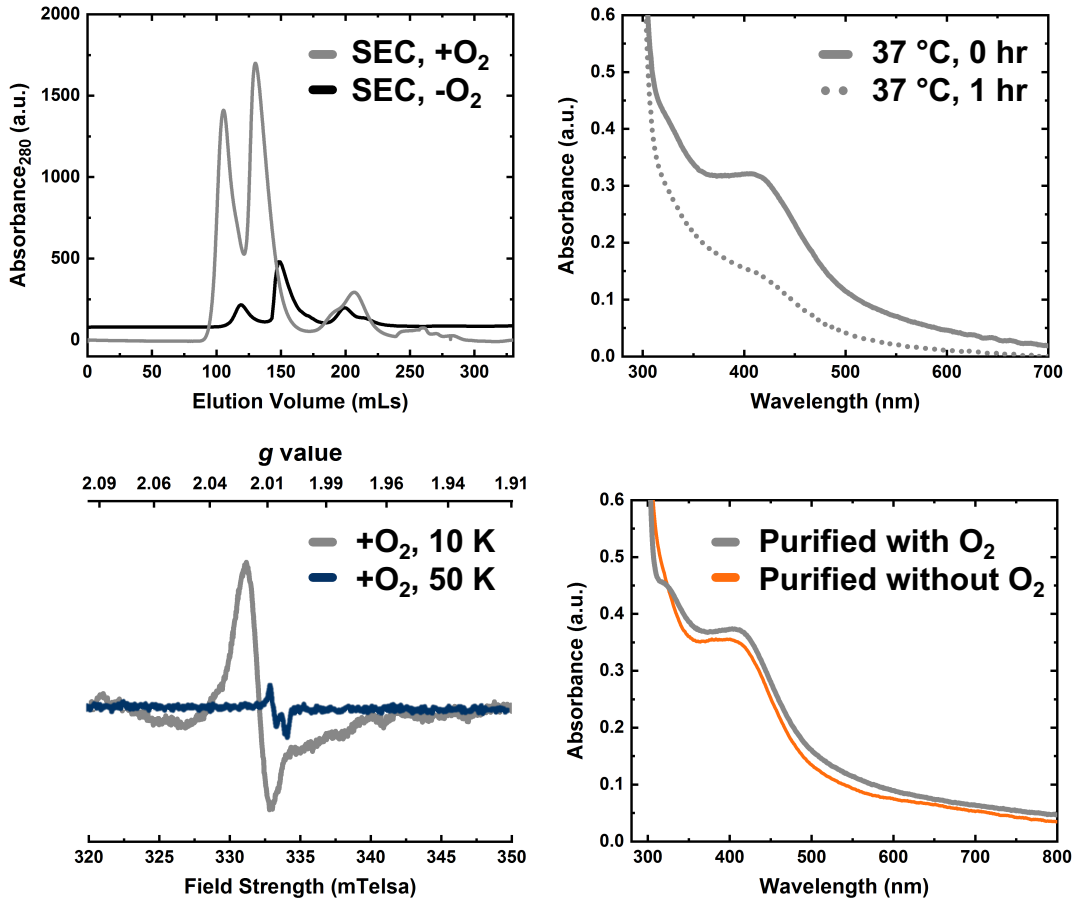


Figure S5. UvrC isolated aerobically exhibits distinct properties from anaerobically-isolated UvrC. (*Top Left*) During an aerobic purification at 4 °C (from ~30 g wet pellet, gray), UvrC elutes at an earlier volume from a prep grade size exclusion column than UvrC isolated during anaerobic purification (~10 gram wet pellet, black). The ratio of aggregated:solube protein is also much improved for the anaerobic trace. Flow rates and buffer components for the aerobically-purified samples are the same as those described in Figure S2, except size exclusion buffer does not contain DTT. (*Top Right, Bottom Left*) UvrC that had been aerobically-purified was yellow in color with absorption band at 410 nm and exhibited a temperature-dependent EPR signal at 10 K (gray) and 50 K (dark blue). (*Top Right*) Incubation of aerobically-purified UvrC at 37 °C in aerobic UvrC buffer results in near complete degradation of the cluster in 1 hour (dotted line). Degradation of the [4Fe4S] cluster results in elution at the void volume of a size exclusion column (not shown). (*Bottom Right*) When comparing of the UV-Vis spectra of aerobically and anaerobically-isolated UvrC (both in UvrC buffer), an extra shoulder centered near 325 nm (black) can be seen for UvrC purified in atmosphere.

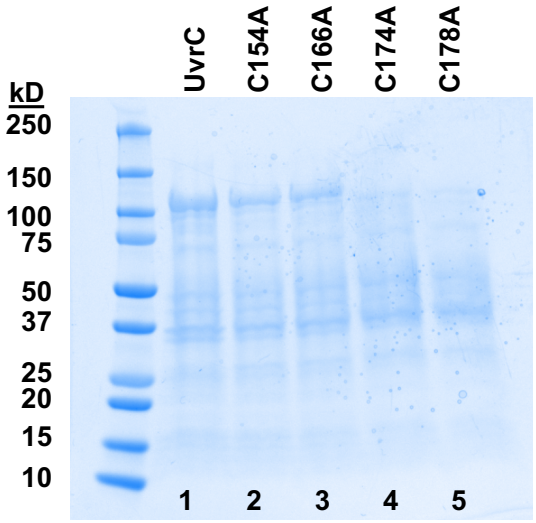


Figure S6. Mutation of coordinating cysteines affects overexpression of UvrC. Whole cell lysate was analyzed by SDS-PAGE following protein overexpression. MBP-UvrC (top band) expression can be seen in WT, Cys154Ala, and Cys166Ala lanes. Cys154Ala and Cys166Ala oligomerized and precipitated (not shown), and overexpression could not be detected for Cys174Ala or Cys178Ala.

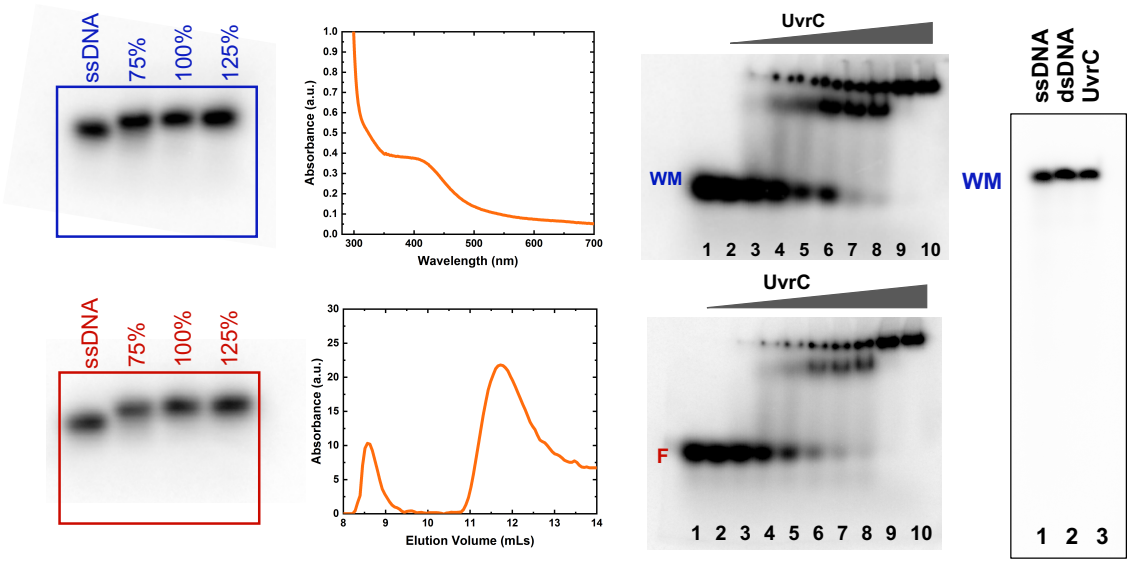


Figure S7. UvrC retains stability and function in low-salt buffer. (*Left*) Radiolabeled DNA (95:5 cold:labeled) was heated at 90 °C for 10 min in the presence of limiting, 1:1, or excess complement in a final concentration of 30 μM (by duplex) in DNA buffer (see Supplementary Materials and Methods) and assessed by native PAGE. Annealing titrations verify duplex character of annealed WM (blue) and F (red) substrates (top bands). (*Middle Left*) Prior to an activity assay, UvrC was buffer-exchanged into low-salt activity buffer (see Figure 5). The UV-Vis spectrum (top) and the size exclusion chromatogram (bottom) indicate that the overall integrity of the [4Fe4S] cluster and the protein were not compromised by buffer exchanging UvrC into a low-salt environment. (*Middle Right*) The binding pattern of UvrC to duplexed WM and F DNA substrates in low-salt activity buffer (with 0.1 M KCl) is comparable to the binding pattern of UvrC in high-salt UvrC buffer (with 0.5 M KCl). In the low-salt activity buffer, the apparent dissociation constants over three independent trials (reported with the standard error) are 100 ± 20 and 200 ± 20 nM for WM and F substrates, respectively. Hill coefficients were also found to be >1. (*Right*) UvrC does not exhibit enzymatic activity on WM duplexes. Lane 1: ssDNA, Lane 2: dsDNA, Lane 3: 1 μM UvrC (by cluster).

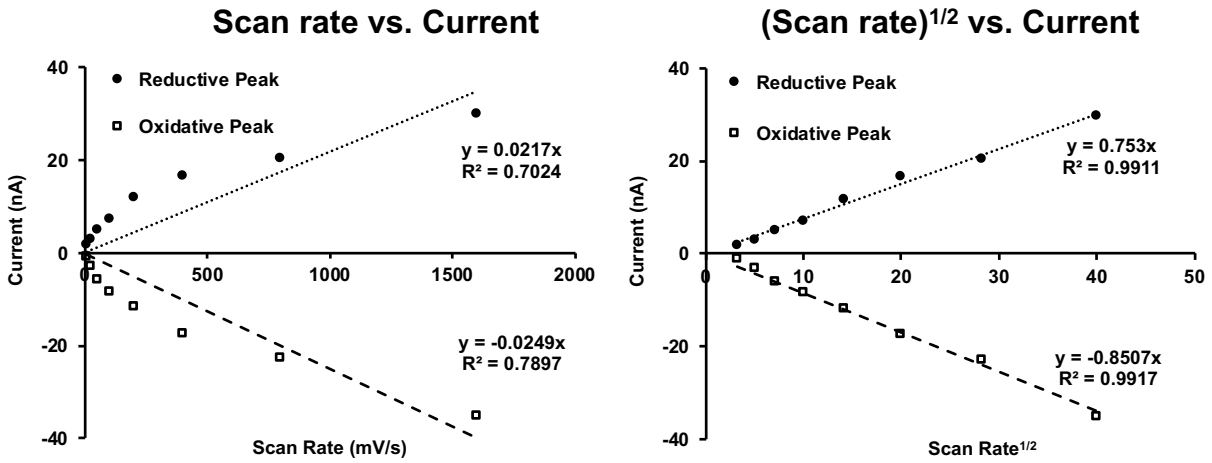
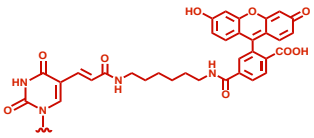
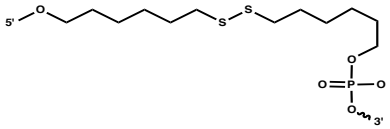


Figure S8. UvrC is diffusive on DNA monolayers. Scan rate was varied from 10 mV/s to 1600 mV/s, and the current at the anodic and cathodic peaks were quantified using software from CHI Instruments. A weak linear correlation between peak current and can rate can be seen on the left, while a strong linear relationship between peak current and the square root of scan rate can be seen on the right, indicating UvrC is diffusive on DNA monolayers. Experimental conditions are as in Figure 6.

Table S1. DNA Primers and Substrates

Name	Sequence (5′→3′)
Colony PCR Primer F	TCGGGATCGAGGAAAACCTGTACTTCCAATCCAATATTATGAGTGATCAGTTTGACGC
Colony PCR Primer R	TGAAAATCTTCTCTCATCCGCCAAAACAGCCAAGGGATCCTCAATGTTTCAACGACCAGA
C154A F	CTACTGCAAAAGATTTTCCCCATTCGCCAGGCCGAAAATA
C154A R	ACGCGAGCGATTGCGATAAACACTATTTTCGGCCTGGCG
C166A F	GAAAATAGTGTTTATCGCAATCGCTCGCGTCCGGCCCTGCAA
C166A R	GCCCTATCTGGTATTGCAGCGGCGGACGCGAGC
C176A F	CTGCAATACCAGATAGGGCGCGCGCTGGGACCG
C176A R	TCACTCACCAGTCCTTCAACGCACGGTCCCAGCGCGCGCCCT
C178A F	GATAGGGCGCTGTCTGGGACCGGCAGTTGAAGG
C178A R	GTATTCTTCTTCACTCACCAGTCCTTCAACTGCCGGTCCC
Well-matched substrate	CCGACTGAACTCTGTACCTGACACGACAGG
Complement	CCTGTCGTGCCATGGACAGAGTTCAGTCGG
Fluorescein-modified substrate	CCGACTGAACTCTGT  ACCTGACACGACAGG
Electrochemistry Stubstrate (Protected)	~S-S-$(CH_2)_6$-CCTGTCGTGCCATGGACAGAGTTCAGTCGG 

References

(1) Grodick, M. A.; Segal, H. M.; Zwang, T. J.; Barton, J. K. DNA-Mediated Signaling by Proteins with 4Fe-4S Clusters Is Necessary for Genomic Integrity. *J. Am. Chem. Soc.* **2014**, *136* (17), 6470–6478. https://doi.org/10.1021/ja501973c.

(2) Pheeney, C. G.; Arnold, A. R.; Grodick, M. A.; Barton, J. K. Multiplexed Electrochemistry of DNA-Bound Metalloproteins. *J. Am. Chem. Soc.* **2013**, *135* (32), 11869–11878. https://doi.org/10.1021/ja4041779.

(3) Orren, D. K.; Sancar, A. The (A)BC Excinuclease of Escherichia Coli Has Only the UvrB and UvrC Subunits in the Incision Complex. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86* (14), 5237–5241. https://doi.org/10.1073/pnas.86.14.5237.

(4) Tang, M. S.; Nazimiec, M.; Ye, X.; Iyer, G. H.; Eveleigh, J.; Zheng, Y.; Zhou, W.; Tang, Y. Y. Two Forms of UvrC Protein with Different Double-Stranded DNA Binding Affinities. *J. Biol. Chem.* **2001**, *276* (6), 3904–3910. https://doi.org/10.1074/jbc.M008538200.

(5) Singh, S.; Folkers, G. E.; Bonvin, A. M. J. J.; Boelens, R.; Wechselberger, R.; Niztayev, A.; Kaptein, R. Solution Structure and DNA-Binding Properties of the C-Terminal Domain of UvrC from E. Coli. *EMBO J.* **2002**, *21* (22), 6257–6266. https://doi.org/10.1093/emboj/cdf627.

(6) Ekanger, L. A.; Oyala, P. H.; Moradian, A.; Sweredoski, M. J.; Barton, J. K. Nitric Oxide Modulates Endonuclease III Redox Activity by a 800 MV Negative Shift upon [Fe4S4] Cluster Nitrosylation. *J. Am. Chem. Soc.* **2018**, *140* (37), 11800–11810. https://doi.org/10.1021/jacs.8b07362.

(7) Tse, E. C. M.; Zwang, T. J.; Barton, J. K. The Oxidation State of [4Fe4S] Clusters Modulates the DNA-Binding Affinity of DNA Repair Proteins. *J. Am. Chem. Soc.* **2017**, *139* (36), 12784–12792. https://doi.org/10.1021/jacs.7b07230.

(8) Schaefer, K. N.; Barton, J. K. DNA-Mediated Oxidation of P53. *Biochemistry* **2014**, *53* (21), 3467–3475. https://doi.org/10.1021/bi5003184.

(9) Schaefer, K. N.; Geil, W. M.; Sweredoski, M. J.; Moradian, A.; Hess, S.; Barton, J. K. Oxidation of P53 through DNA Charge Transport Involves a Network of Disulfides within the DNA-Binding Domain. *Biochemistry* **2015**, *54* (3), 932–941. https://doi.org/10.1021/bi501424v.

(10) Sancar, A.; Rupp, W. D. A Novel Repair Enzyme: UVRABC Excision Nuclease of Escherichia Coli Cuts a DNA Strand on Both Sides of the Damaged Region. *Cell* **1983**, *33* (1), 249–260. https://doi.org/10.1016/0092-8674(83)90354-9.