Chapter 3

Charge-Transfer Properties of Two Y82 Position Mutations of *Escherichia Coli* MutY

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Abstract

MutY from *Escherichia coli* is a [4Fe-4S] cluster containing DNA repair glycosylase that excises adenine mispaired with 8-oxo-7,8-dihydrodeoxyguanine. The human equivalent of MutY, MUTYH, has attracted the attention of cancer researchers because mutations in MUTYH predispose patients towards developing colorectal cancer. One mutation that occurs frequently in cancer patients is Y165C. Y165 in MUTYH aligns with Y82 in *E. coli* MutY, making Y82 position mutants intriguing candidates for experiments that examine the protein's ability to detect and repair DNA damage. Here, we characterized the electrochemical properties of MutY variants Y82C and Y82L. Y82C exhibits a weaker cyclic voltammetry signal on DNA-modified electrodes than wild-type MutY, and it is less able to regenerate a nitroxyl radical signal through DNA-mediated CT, as detected by EPR spectroscopy. By contrast, Y82L performed within error of WT MutY in both sets of experiments. These data demonstrate that Y82 may mediate protein-to-DNA CT *in vivo* in MutY and support the model for DNA-mediated signaling between DNA repair enzymes as a means of DNA damage detection.

Introduction

MutY is a [4Fe-4S] cluster-containing glycosylase that excises adenine mispaired with 8-oxo-7,8-dihydrodeoxyguanine [1, 2]. It is a member of the base excision repair (BER) family of DNA glycosylases [3] and shares structural homology to the helix-hairpin-helix family of DNA-binding proteins [4]. MutY

and related proteins have received much attention recently because mutations in the human version of MutY, MUTYH, are found in many colorectal cancer patients and have been associated with a predisposition towards developing this disease [5–10]. One of the mutations that occurs most frequently in cancer patients is Y165C [7, 8]. Y165 in human MUTYH aligns with Y82 in *E. coli* MutY, making Y82C a relevant mutant for research into how MUTYH Y165C leads to colorectal cancer progression. Y82L was chosen because *E. coli* EndoIII, which is also structurally similar to *E. coli* MutY, contains a leucine residue at the position equivalent to Y82 in MutY. Enzymatic assays of Y82C and Y82L have already been performed and indicate that Y82C exhibits a higher K_D and lower enzymatic turnover rate than WT MutY [9]. Y82L, by contrast, performed within error of WT MutY in both of these assays [9]. Yet, one feature of these mutants that has yet to be examined is their charge-transfer capability.

MutY contains a [4Fe-4S] cluster that is redox-active when bound to DNA [11]. The midpoint potential is within a physiologically relevant range, suggesting that the DNA-bound form of this enzyme could participate in electron-transfer reactions *in vivo* [11]. In our model for DNA damage detection, a bound enzyme could transmit an electron to another protein bound to a distal location on the DNA. Upon reduction, this "distal" protein would lose some of its DNA affinity, dissociate, and re-bind to a different region of the genome. However, if a lesion is present in the DNA sequence between the two bound proteins, then CT will not proceed efficiently, both proteins will remain oxidized and DNA-bound, and they

will localize to the site of damage between them. This model is supported by the fact that DNA-mediated charge-transfer (CT) can occur efficiently over long distances, but is attenuated by mismatches and damage sites in the DNA helix [12, 13]. We have also shown that the affinity of certain BER enzymes for DNA is stronger in their oxidized 3^+ form than in their reduced 2^+ form [14].

For this model of DNA damage detection using DNA-mediated CT to function, CT must proceed efficiently from the DNA helix to the [4Fe-4S] cluster of a bound enzyme. We wondered whether Y82 could mediate DNA-to-protein CT in MutY. In addition to its biomedical relevance, Y82 is a good candidate for CT studies because the equivalent residue in *Bacillus stearothermophilus* MutY has been shown to intercalate into the DNA [15] (Figure 3.1). If the same occurs in *E. coli*, this intercalation would allow residue Y82 to act as an important mediator for CT from the DNA into the MutY protein. Herein, two sets of experiments were performed to test the CT capabilities of MutY Y82C and Y82L.

The first experiment was an EPR-based study that examined how effectively the protein mutants could regenerate a free-radical signal. DNA was prepared that contains a uridine base modified with a stable nitroxyl radical [16]. Following chemical oxidation of the nitroxyl radical to the corresponding N-oxo-ammonium ion using $IrCl_6^{2-}$, addition of MutY or UDG results in the regeneration of the nitroxyl radical, as followed by electron paramagnetic spectroscopy (EPR). This signal regeneration is consistent with electron trapping from the reduced (2+) to the oxidized (3+) form of the cluster. The second set of experiments was

electrochemically based and examined the mutants on a DNA-modified electrode. The signal strength of different mutants' cyclic voltammogram signals were quantified relative to WT protein. An attenuated electrochemical signal indicates a reduced ability to mediate CT. Through EPR and cyclic voltammetry, Y82 of *E. coli* MutY was examined as a facilitator of CT between the DNA and the [4Fe-4S] cluster of MutY.

Materials and Methods

Oligonucleotide synthesis. The sequences used for quantitative electrochemistry experiments were SH-(C_6H_{12})-(RR)- 5'-GA GAT ATA AAG CAC GCA-3' and complement, SH-(C_6H_{12})- 5'-AGT ACA GTC ATC GCG-3' and complement, and SH-(C_6H_{12})- 5'-AGT ACA GTC ATC GCG-3' paired with RR- 3'- TCA TGT CAG TAG CGC- 5'. "RR" stands for Redmond red, a redox active probe used to quantify the amount of DNA on the electrode surface [17]. Synthesis of the spin-labeled deoxyuridine and its incorporation into a 10-mer oligonucleotide (10-mer: 3' –GATG**U***CAGCA-5', where U* = spin-labeled uridine through acetylene linker) was previously reported [16]. Complementary DNA sequences include a 26-mer oligo 3'- CATAGCCGCAATCGCCGACTAGAGCC-5' annealed to a 36-mer oligo 5'- GTATCGGCGTTAGCGGCTGATCTCGGCTACAGTCGT-3'. All DNA sequences were prepared on an AB 3400 DNA Synthesizer from ABI using standard phosphoramidite chemistry. Phosphoramidites were purchased from Glen

Research. Oligonucleotides were purified by HPLC on a C18 reverse phase column and characterized by MALDI- TOF mass spectrometry.

DNA Annealing. For electrochemistry experiments, complementary strands were mixed in a 1:1 ratio in DNA Buffer (10 mM NaPi, 50 mM NaCl, pH = 7.5), heated to 90ºC for several minutes, and then allowed to cool slowly to room temperature over 1–4 hours. For the EPR experiments, DNA annealing was carried out initially by heating to 90ºC a solution containing the 26:36-mer strands in DNA Buffer (10 mM NaPi, 50 mM NaCl, pH = 7.5) at a 1:1 ratio, and then slowly cooling this solution to room temperature over at least 90 minutes. Next, the 10-mer spin labeled DNA (0.9:1 ratio to the 26:36-mer duplex) was added to the preassembled 26:36-mer duplex by heating the solution to 45ºC for two minutes and allowing it to cool to ambient temperature. Duplex formation with the 10-mer spin labeled DNA was verified by EPR spectroscopy.

Preparation of DNA-Modified Electrodes. After hybridization to their complement, oligonucleotides duplexes were deposited onto an Au surface in phosphate buffer (50 mM NaCl, 5 mM sodium phosphate, pH 7.0) and incubated for 24–36 hours. Au substrates were purchased from Molecular Imaging. The electrode surface was then washed, and further passivated by incubation with 100 mM mercaptohexanol in phosphate buffer for 30–45 minutes. The electrode surface was washed again in phosphate buffer, and in protein buffer (see below).

Cyclic Voltammetry. Preparation of the DNA-modified Au electrode and subsequent cyclic voltammetry experiments were performed as described

previously [11, 12, 18–21]. The DNA-modified Au electrode served as the working electrode, a Pt wire served as the auxiliary electrode, and the reference was either an Ag/AgCl electrode modified with an agarose tip or a 66-EE009 Ag/AgCl reference electrode (ESA Biosciences). Unless otherwise noted, all scans were taken at a rate of 50 mV/s on a CH Instruments 760 potentiostat. Each experiment used 50 μL of 50–200 μM of protein in MutY buffer (20 mM sodium phosphate pH=7.5, 100 mM NaCl, 5% glycerol, 1 mM EDTA). For MutY measurements, the protein signal intensity was normalized to DNA concentration using the intensity of the Redmond red signal. For each comparison, the protein samples were measured consecutively on the same electrode surface.

Protein Purification. MutY was prepared as documented previously [22]. The concentration was quantified using ε_{410}= 17,000 $M^{-1}cm^{-1}$.

EPR Spectroscopy. X-band EPR spectra were obtained on an EMX spectrometer (Bruker, Billerica, MA) equipped with a rectangular cavity working in the TE_{102} mode. A quartz flat cell (100 μL) was used in all ambient–temperature experiments. A frequency counter built into the microwave bridge provided accurate frequency values. DNA samples consisted of 26–36 preassembled duplexes (8 μM) and 10-mer spin-labeled DNA (7.2 μM) in 75 μL of buffer (10 mM NaPi, 29 mM NaCl, 2.6 mM $MgCl_2$, pH = 6.2). Chemical oxidation of the spin-labeled probe was then accomplished by addition of 2 μL of $K_2[IrCl_6]$ (2.5 mM in 10 mM NaPi, 50 mM NaCl, pH = 7.5), as determined by a significant attenuation in the EPR signal. After this oxidation, protein was added to the

sample, and the spectrum was immediately recorded (typically after two minutes). EPR parameters were as follows: microwave power = 20 mW, receiver gain = $1x10^4$, and modulation amplitude = 4 G.

EcoRI Control Experiment. A stock solution of EcoRI (40 units/ 1 μL) was dialyzed in protein buffer (100 mM NaCl, 20 mM NaPi, pH=7.5, 1 mM EDTA, 5% glycerol) for four hours at 4°C. Protein activity post-dialysis was tested by incubating dialyzed EcoRI with genomic DNA (extracted from *HeLa* cells) at 37°C for 30 minutes, and then visualizing the digest on an agarose gel relative to cell extracts treated with un-dialyzed EcoRI. Both samples showed similar levels of activity as indicated by comparable DNA smears (data not shown). EcoRI concentration was then determined using a fluorescence-based detection kit (Nano-Orange/ Molecular Probes). An estimated 200 μM stock solution was prepared for the EPR assay.

Results

EPR-based detection of electrons generated from DNA-bound [4Fe-4S] cluster enzymes. The spectra were acquired at room temperature and are shown before and after the addition of the chemical oxidant, $IrCl_6^{2-,}$ and after addition of protein. Upon addition of Ir(IV), the EPR signal decreases substantially, indicating that the spin label was efficiently oxidized from the nitroxyl radical to the EPR silent *N*-oxo-ammonium ion.

Two MutY mutants, Y82C and Y82L, were also examined. Y82C initially

exhibits low signal regeneration relative to WT MutY (Figures 3.2a and 3.2b), although the EPR signal gradually increases to WT level over the course of 28 minutes (Figure 3.3). Y82L, however, regenerated the EPR signal to the same level as WT MutY (Figure 3.4). As a control, these experiments were also performed with EcoRI to verify that the observed EPR signal regeneration originated from the [4Fe-4S] cluster. No apparent signal regeneration was observed after EcoRI was added to the oxidized spin-labeled sample (Figure 3.5).

Quantitative electrochemistry of BER enzymes on DNA-modified electrodes. Quantitative electrochemistry experiments were used to examine the MutY variants Y82C and Y82L. Consistent with EPR results, the cyclic voltammetry signal produced by Y82C was much weaker than that of WT MutY (Figure 3.6a), whereas the signal produced by Y82L was within error of that produced by wild-type MutY (Figure 3.6b). Both mutants produce midpoint potentials within error of WT MutY (Table 3.2).

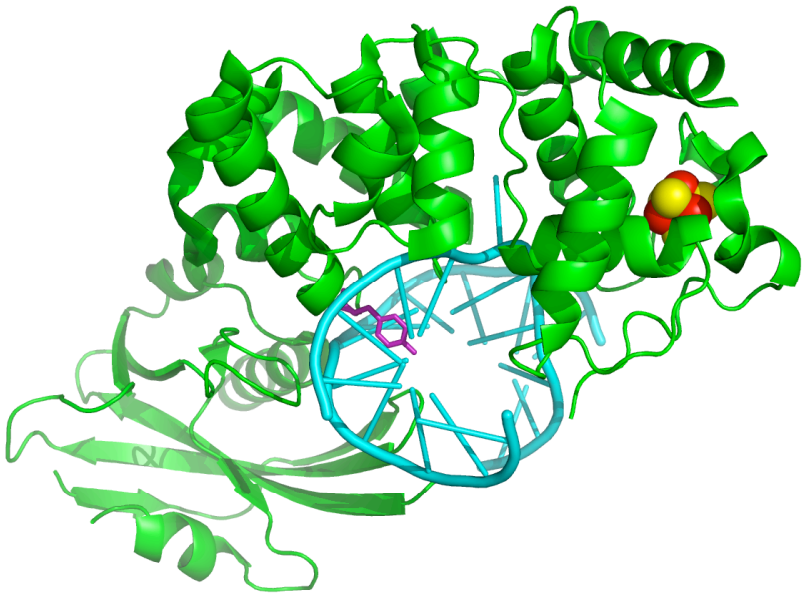
Discussion

The MutY mutants examined were both chosen based on relevance to cancer research, sequence conservation, and the ability to intercalate into the DNA π-stack. Y82C displayed both an attenuated electrochemical signal and a reduced ability to regenerate a nitroxyl radical EPR signal. This result is consistent with the hypothesis that MUTYH Y165C mutants are less able to electrochemically detect and repair DNA damage, thus causing patients with this mutation to develop

colorectal cancer. Y82L produced an electrochemical signal and EPR signal strength within error of that of WT MutY, indicating that the leucine substitution does not dramatically impede DNA-to-protein CT in MutY.

These data on Y82 position mutants establish Y82 as a CT-relevant residue in MutY. They also indicate that bulky residues that can intercalate into the DNA helix, such as tyrosine and leucine, are best able to facilitate CT in this enzyme, whereas shorter side chains, such as the thiol of cysteine, are less capable of mediating CT. It should be noted that although Y82C exhibits a higher K_D than WT MutY, we expect that it bound the DNA just as well as WT during these experiments because the amount of protein used far exceeded the amount of DNA. Future work on this system could include research on other site-directed mutants to detect other segments of the CT pathway between DNA and the iron-sulfur cluster of MutY. If such studies are undertaken, special attention could be given to residues with long side chains that could intercalate into the DNA. Aromatic amino acids are also regarded as good mediators of protein CT [23–26]. The discovery of additional pathway residues would increase support for the hypothesis that MutY detects DNA damage through long-range DNA mediated signaling.

Figure 3.1: Crystal structure of MutY from *Geobacillus stearothermophilus* bound to DNA. Tyr88 (equivalent to Tyr82 in *E. coli* MutY) is highlighted in purple. Mutations at this position in the human equivalent of MutY are associated with a predisposition to colorectal cancer. The sulfur and iron atoms of the [4Fe-4S] cluster are annotated in yellow and red, respectively. Adapted from [15] and formatted in PyMOL (PBD: 1RRQ).



Figures 3.2a and 3.2b: EPR spectra before and after addition of WT-MutY (left, 5.8 μM) **and MutY-Y82C** (right, 5.8 μM). The DNA duplex (6.7 μM) was initially treated with $IrCl_6$ 2 μL (60 μM). Spectra shown are before oxidation (black), after oxidation before protein addition (red), and then four minutes after protein addition (green).

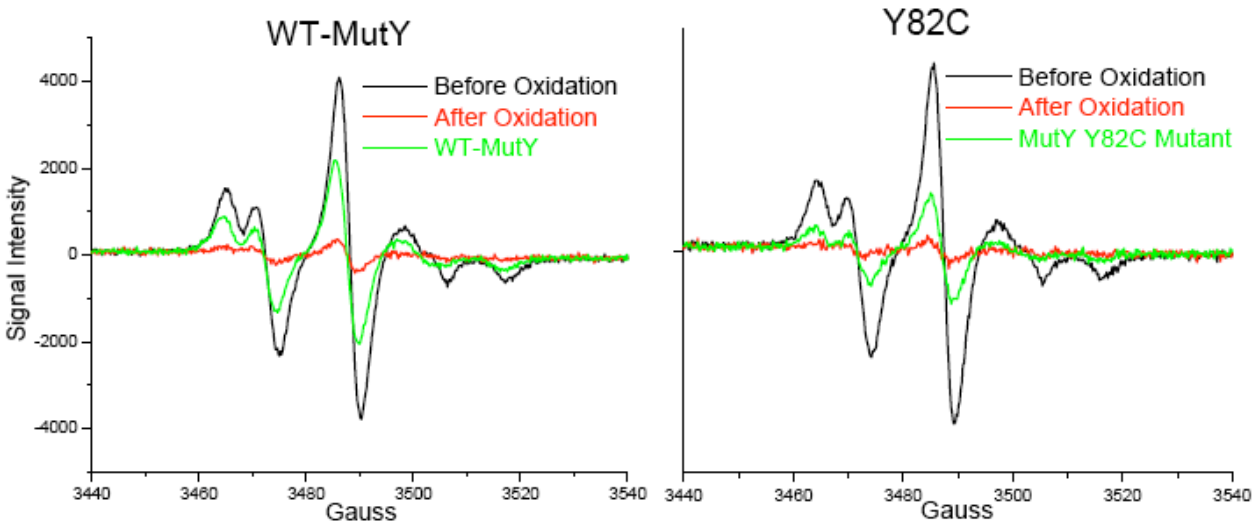


Figure 3.3: EPR spectra before and after addition of MutY-Y82C (5.7 μM) .The DNA duplex (6.7 μM) was initially treated with $IrCl_6$ 2 μL (60 μM). Spectra shown are before oxidation (black), after oxidation before protein addition (red), and then throughout a time course of 28 minutes after protein addition (green to blue).

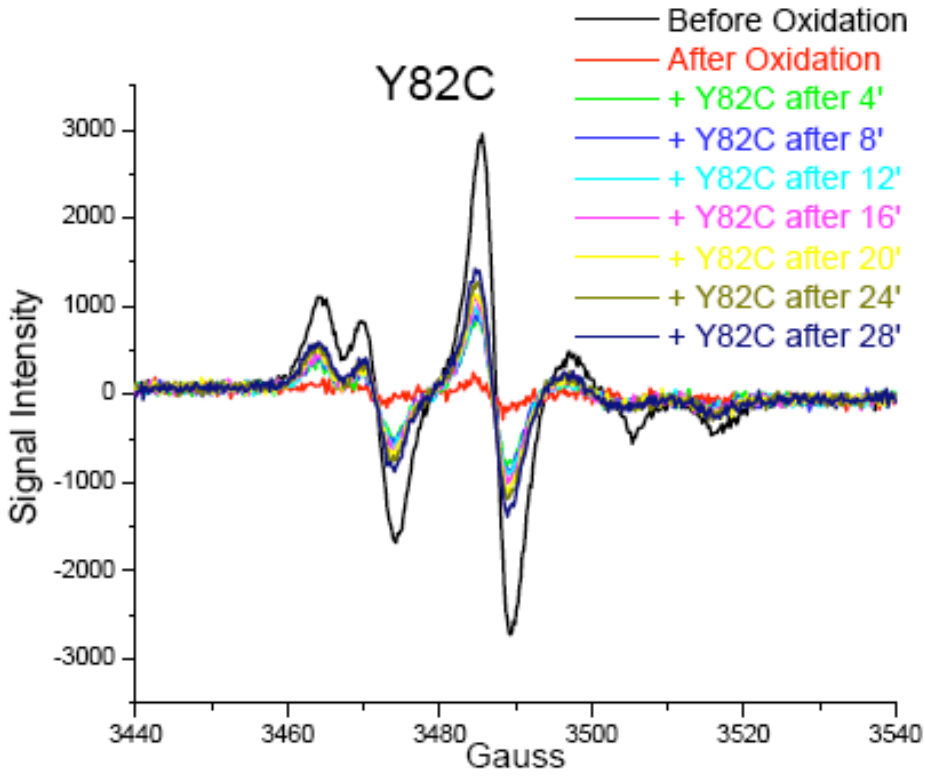


Figure 3.4: EPR spectra before and after addition of MutY-Y82L (5.7 μM). The DNA duplex (6.7 μM) was initially treated with $IrCl_6$ 2 μL (60 μM). Spectra shown are before oxidation (black), after oxidation before protein addition (red), and then after protein addition (green).

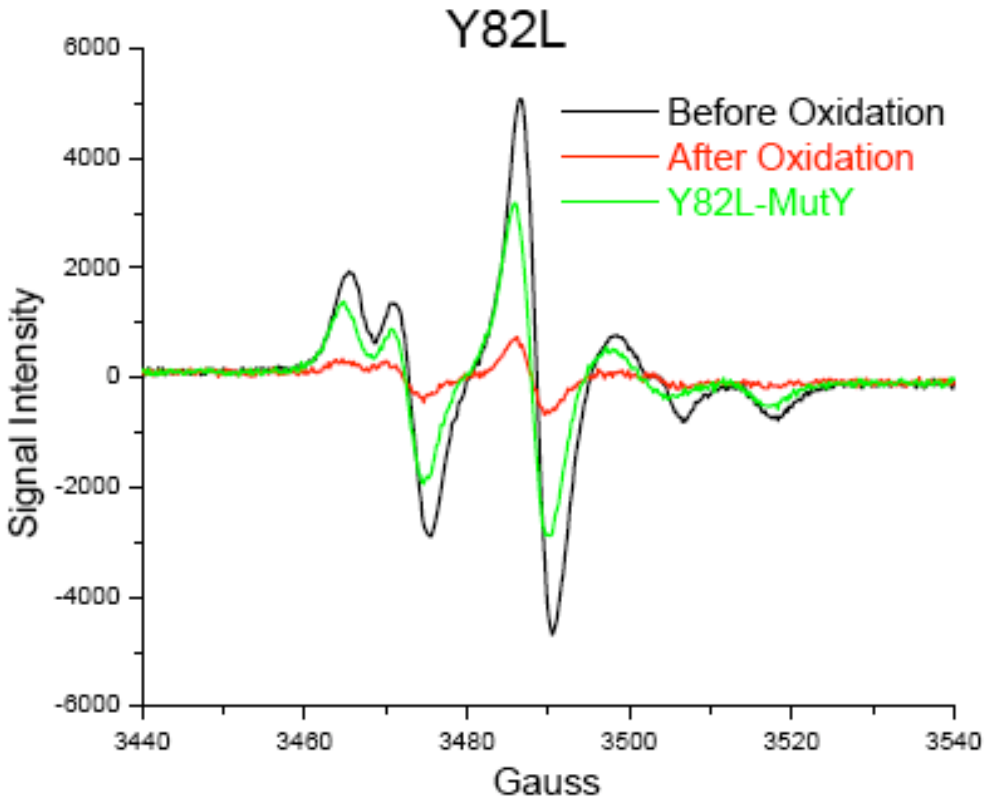


Figure 3.5: EPR spectra before and after addition of EcoRI (13.8 μM). The DNA duplex (6.7 μM) was initially treated with $IrCl_6$ 2 μL (60 μM). Spectra shown are before oxidation (black), after oxidation before protein addition (red), and then after protein addition (green).

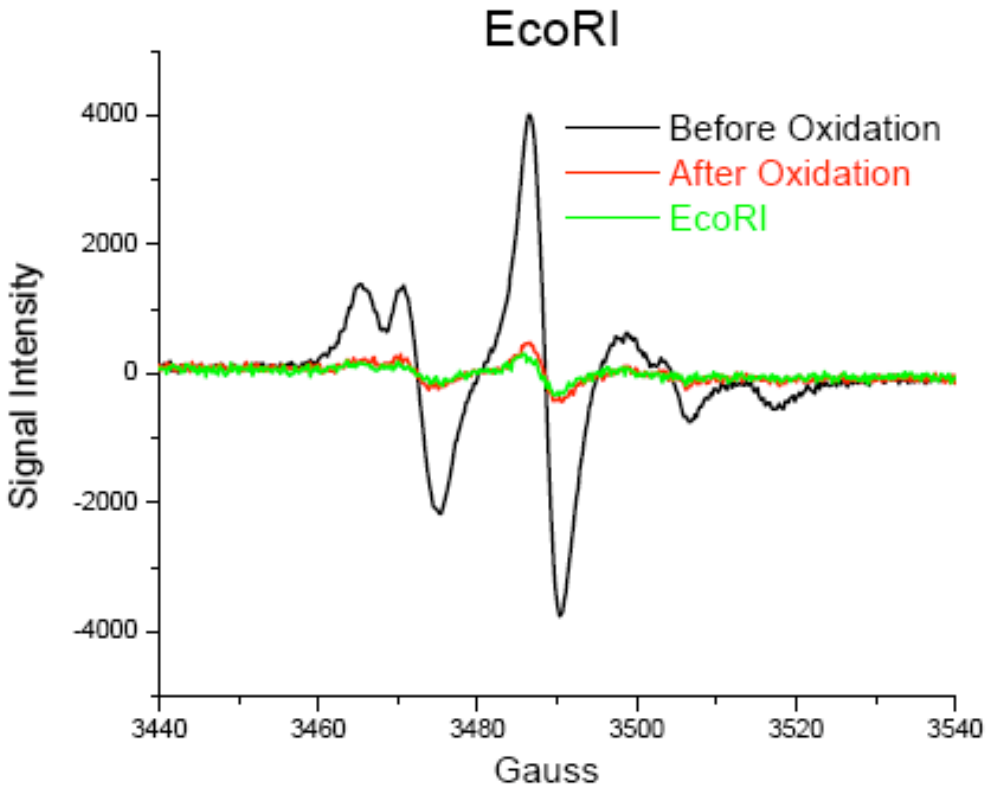


Table 3.1: Summary of the MutY data in terms of signal re-generation. The values are corrected for the non-zero value obtained after treating DNA samples with Ir(IV).

SAMPLE #	1	2	3	AVERAGE
WT-MutY	45[a]	45	47	46±1
Y82C (4')	27	27	23	26±2
Y82C (30')	39	42	42	41±2
Y82L	44	69	48	54±11

[a] Signal regeneration is based on the ratio of main EPR signal at g = 2.0 ($\Delta_{\text{peak to peak}}$) between the EPR signal observed after protein addition to that of untreated DNA.

Figure 3.6: Electrochemistry of MutY variants. The cyclic voltammograms of the MutY variants Y82C (pale blue) and Y82L (dark green) are shown relative to those of WT MutY (burgundy). The scan in buffer is also pictured (gray). Y82C is charge-transfer deficient relative to WT MutY and Y82L produces a signal within error of that of WT.

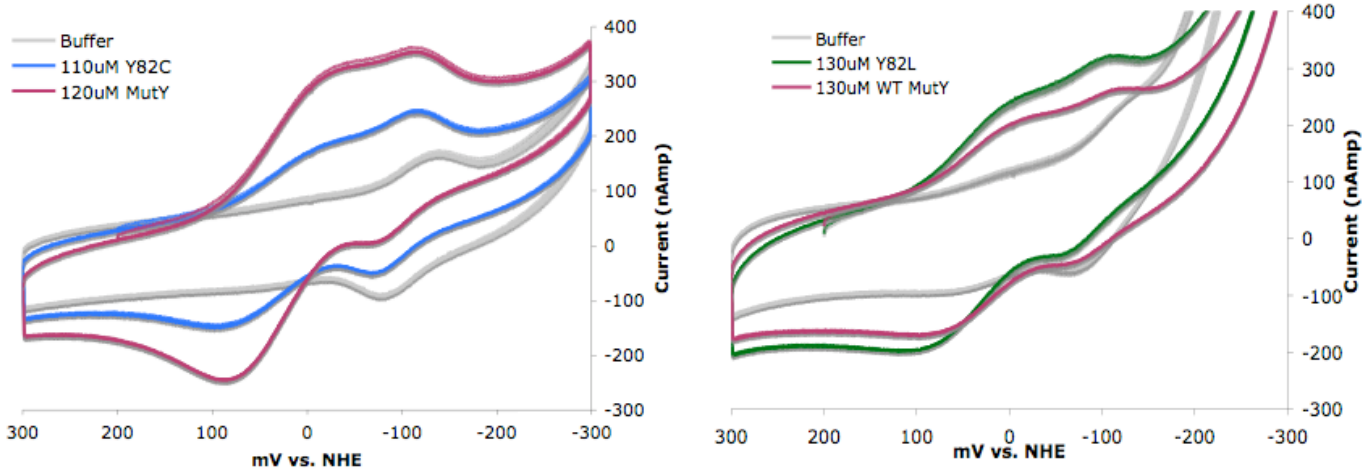


Table 3.2: Summary of quantitative electrochemical measurements of MutY variants

Variant	Midpoint Potential (mV vs. NHE)	Signal strength relative to WT (RR corrected)
Y82C	88 ± 8	0.25 ± 0.20
Y82L	88 ± 15	0.94 ± 0.17

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