Spectroscopic Investigation of the Interaction of FADH in DNA Photolyase with UV-Damaged DNA

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Spectroscopic Investigation of the Interaction of FADH$^*$ in DNA Photolyase with UV-Damaged DNA

By

Kyle Luke Williams

A Master’s Thesis Submitted to the Faculty of Montclair State University

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Skin cancer is the most prevalent malignancy found in humans, with the diagnosis rate continuing to steadily increase. The primary cause of this disease is overexposure to harmful ultraviolet (UV) radiation from sunlight, which can induce damage to the nitrogenous bases in DNA via dimerization. The most prevalent UV-induced photoproducts in DNA are cyclobutane pyrimidine dimers (CPDs), most commonly between adjacent thymidines.

Organisms have implemented methods by which to repair these CPDs, the primary of which are nucleotide excision repair (NER) and photoreactivation by photolyases. Photolyases are blue-light activated flavoproteins that are more efficient at recognizing and repairing CPDs than the NER enzymes relied on by humans. Thus, understanding the interactions between photolyase and DNA may lead to improved treatments for the harmful effects from UV radiation.

UV-irradiated oligothymidylates are the most commonly used models for studying DNA-bound photolyases, as it is possible to quickly form a high concentration of CPDs. However, these strands present a random distribution of CPDs and other photoproducts can be present within the strands. This research examines potential differences between single and double strand oligothymidine interactions with the neutral radical semiquinone flavin adenine dinucleotide (FADH') cofactor within the active site of \textit{E. coli} photolyase. This study also investigates whether photolyase-DNA interactions are satisfactorily modeled by using UV-irradiated oligothymidines.

An oligothymidine decamer, p(dT)$_{10}$, was irradiated with 254 nm UV light to form an average of \(~1.5\) CPDs per strand. An oligonucleotide dodecamer, 5'-CGGCATTACGGC-3', was irradiated with 302 nm UV light in the presence of acetophenone, a photosensitizer, and the CPD-containing strands were purified using reverse phase HPLC. Single strand UV-p(dT)$_{10}$, double strand UV-p(dT•dA)$_{10}$, and the CPD-oligonucleotide were complexed with \textit{E. coli} photolyase and analyzed using electronic absorption spectroscopy and resonance Raman spectroscopy, with excitation at 532 nm to enhance the vibrations of the FADH'.

All studied oligonucleotides induced very similar electrochromic shifts within the absorption spectrum of photolyase. The resonance Raman spectrum of photolyase exhibits similar changes in the presence of both the single and double strand UV-p(dT)$_{10}$, indicating comparable interactions within the active site. The Raman spectrum of the CPD-oligonucleotide-photolyase complex differs significantly from that of the UV-p(dT)$_{10}$ complex, indicating that oligothymidylates may not serve as good models in photolyase-DNA studies. The flavin cofactor exhibits a dramatically faster rate of reduction in the presence of the CPD-oligonucleotide than the UV-oligothymidylate. However, this may be a side effect of triethylamine from the HPLC.
SPECTROSCOPIC INVESTIGATION OF THE INTERACTION OF FADH' IN
DNA PHOTOLYASE WITH UV-DAMAGED DNA

A THESIS

Submitted in partial fulfillment of the requirements
For the degree of Master of Science

by

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1. INTRODUCTION

1.1 Skin Cancer from DNA Damage

The most widely occurring malignancy in humans is skin cancer, with over two million people diagnosed in the United States per year, and the rate of occurrence has more than doubled since 1994. While the majority of these cancers are curable nonmelanomas, there is a significant cost associated with treatment. It is thus increasingly important to gain a better fundamental understanding of the causes of skin cancer and to explore possible methods of prevention or treatment. The primary cause of skin cancer is overexposure to harmful ultraviolet (UV) radiation from sunlight. Ultraviolet light in the UVB (280–315 nm) range is sufficiently energetic to directly induce photoproducts in DNA strands, while the UVA (315–400 nm) range is capable of inducing photoproducts via photoelectron transfer or photosensitization. The most damaging UV-light, UVC (100–280 nm), does not reach the Earth's surface due to atmospheric absorption by ozone. Dimerization of adjacent pyrimidine bases (thymine or cytosine) is the most prevalent type of UV-induced photoproduct, primarily resulting in cis-syn cyclobutane pyrimidine dimers (CPDs) and 6,4 pyrimidine-pyrimidones, which can photoisomerize to Dewar valence isomers (Figure 1.1).

![Diagram of DNA damage](image)

**Figure 1.1** – Dimerization of adjacent thymines to a CPD, (6,4) pyrimidine-pyrimidone, and Dewar valence isomer, adapted from J.-S. Taylor.
1.2 Cyclobutane Pyrimidine Dimers

Of the primary UV-induced photoproducts, *cis-syn* CPDs are more likely to ultimately lead to genetic mutation.\(^2\) This is because simulated sunlight induces CPD, (6,4), and Dewar photoproducts at a relative rate of 1.00:0.18:0.06, respectively.\(^3\) Neighboring thymines are more photoreactive than neighboring cytosines, so that the most prevalent photoproduct is the *cis-syn* cyclobutane thymine dimer (T<>T).\(^4\) It has been shown that CPDs are removed nearly ten times slower than (6,4) photoproducts by nucleotide excision repair.\(^2\) As many as 76% of UV-induced CPDs can still remain in cells 24 hours after UV exposure, compared to only 10% of (6,4) photolesions still present just 6 hours after exposure (and virtually none present after 24 hours).\(^2,3\) CPD lesions are therefore many times more likely to be encountered by DNA polymerase during DNA replication.

The presence of photolesions within DNA strands have an impact on the overall structure of the DNA, introducing bending and partial unwinding in duplex DNA. How severely a CPD bends the DNA helix has been shown to vary based on the environment of the DNA. Gel mobility assays and solution-state NMR structures suggest a tilt of \(\sim 7-10^\circ\),\(^2,5\) theoretical calculations have predicted a range from virtually no bending to nearly \(27^\circ\),\(^6\) while circularization assays and x-ray crystallography show an angle of \(\sim 30^\circ\) (*Figure 1.2*).\(^6\) The unwinding of the strands has been determined to range from \(\sim 9-15^\circ\), based on crystallographic structure and mobility studies, respectively.\(^2,6\) These significant distortions within the structure of CPD-containing DNA are thought to potentially facilitate recognition of damage by DNA repair enzymes.\(^5\)

![Figure 1.2](image-url) - Illustration of the bending \((30^\circ)\) induced by a CPD, from the crystallographic data of Park et al.\(^6\) Views are of a CPD-containing strand overlaid on an undamaged strand, perpendicular to the helical axis (a) and along it (b).

Repair of DNA photoproducts is critical, as UV-induced CPD formation has several negative biological consequences, ranging in severity from transient to chronic. Extended UVB
exposure from sunlight leads to erythema (sunburn), which has been directly linked to the formation of excessive cyclobutane dimers within the skin cells. The inflammation fades over time as the CPDs in the DNA within the cells are repaired. CPDs, along with other UV-photoproducts, have also been linked to the transient immunosuppression experienced by mammals exposed to UV light. The most hazardous consequence of DNA damage is the potential for formation, and then propagation, of genetic mutations, as CPDs can lead to misreading and replication errors by DNA polymerase. For example, cis-syn cyclobutane dimerization of adjacent cytosines, while less likely than T<>T formation, leads to a remarkable increase in the estimated rate of deamination, ~12 hours compared to ~30,000 years for monomeric cytosine. This deamination results in uracil dimers, which ultimately leads to replication of thymine-adenine pairs in duplex DNA. These mutations can have grave consequences, such as skin cancer. It is therefore critical that UV-exposed organisms incorporate mechanisms by which to repair UV–damage in DNA.

1.3 Photolyase: DNA Repair Enzyme

There are two primary methods by which living organisms repair DNA damage, nucleotide excision repair (NER) and photoreactivation. NER is a general process by which organisms remove damaged nucleotides from DNA. It involves excision of a short damage-containing section of the strand followed by replication of the excised portion. The other method by which UV-damaged DNA can be repaired is via the reversal of photolesions by blue light-activated enzymes called photolyases, in a process known as photoreactivation. Photolyases have a high degree of substrate specificity, such that there are CPD photolyases to repair solely CPDs while (6,4) photolyases are specific to the repair of (6,4) photoproducts. CPD photolyases are much more prevalent than (6,4) photolyases and, having been discovered first, are traditionally referred to just as photolyase, a nomenclature which will be followed throughout the remainder of this text. Photolyase shows equal affinity for both single and double strand DNA, whereas NER requires a duplex so as to have a template to replicate the excised nucleotide sequence.

Photolyases vary in sequence across species, consisting of a monomeric protein chain between 450–550 amino acids in length and two different cofactors. All photolyases contain a flavin adenine dinucleotide (FAD) within the active site, which is required for the photolyase to function, and either 5,10-methenyltetrahydrofolate polyglutamate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deazariboflavin (8–HDF), which do not play a significant role in enzymatic activity.
The flavin within the active site can adopt three different oxidation states via electron transfer: fully oxidized quinone form (FAD\textsubscript{ox}), one-electron reduced neutral radical semiquinone form (FADH\textsuperscript{*}), and the two-electron reduced anionic hydroquinone form (FADH\textsuperscript{-}) (Figure 1.4). Both the quinone and semiquinone oxidization states of the protein are inactive, so that only the fully reduced hydroquinone is catalytically active. The oxidation state of the flavin cofactor has no significant impact on the binding affinity of photolyase, such that (when oxidized) the protein will bind to the substrate but will not catalyze repair.

![Figure 1.3](image-url)  

**Figure 1.3** – Crystal structure of *Eschericia coli* photolyase with cofactors labeled, from A. Sancar.

![Figure 1.4](image-url)  

**Figure 1.4** – The three redox states of the flavin cofactor within the active site of photolyase, with regions of interest circled in red.

Most photolyases can only be purified in either the FAD\textsubscript{ox} or FADH\textsuperscript{-} form, as FADH\textsuperscript{*} generally undergoes rapid oxidation upon cell lysis. The protein can be reduced from FADH\textsuperscript{*} to FADH\textsuperscript{-} by electron transfer from a nearby tryptophan (\textsuperscript{382}Trp). In *E. coli* photolyase this occurs
via the tryptophan triad pathway of 306-Trp, 359-Trp, and 382-Trp (Figure 1.5), which is conserved throughout all known photolyases.\textsuperscript{12} This is initiated by a photoinduced proton-coupled electron transfer from the 306-Trp, likely in a concerted fashion at biological pH.\textsuperscript{12}

$$FADH^* + 306\text{Trp}H \leftrightarrow FADH^- + 306\text{Trp}^* + H^+$$

**Figure 1.5** – Tryptophan photoreduction pathway in *E. coli* photolyase, from Schelvis et al.\textsuperscript{12}

In its active hydroquinone form, photolyase repairs cyclobutane dimers via a photoinduced electron transfer mechanism upon 350–450 nm blue light exposure.\textsuperscript{8,13} The pertinent steps in the repair pathway are summarized in the following reactions,

$$FADH^- \rightarrow FADH^{**}$$

$$FADH^{**} + Pyr \leftrightarrow Pyr \rightarrow FADH^* + Pyr \leftrightarrow Pyr^{**}$$

$$Pyr \leftrightarrow Pyr^{**} \rightarrow Pyr - Pyr^{**}$$

$$FADH^* + Pyr - Pyr^{**} \rightarrow FADH^- + Pyr - Pyr$$

with the overall mechanism illustrated in Figure 1.6. An incoming photon excites the reduced flavin cofactor, which proceeds to transfer an electron to the pyrimidine dimer on the picosecond timescale.\textsuperscript{13} The CPD then undergoes [2+2] cycloreversion of the 5–5 and 6–6 bonds,\textsuperscript{8} likely in a sequential fashion,\textsuperscript{13} to yield an anionic radical pyrimidine. An electron is transferred back to the flavin cofactor to fully reduce it back to the hydroquinone state, at which point the substrate can ultimately be released from the enzyme.\textsuperscript{8,13}

**Figure 1.6** – Mechanism of cyclobutane thymine dimer photoreactivation by photolyase, adapted from A. Sancar.\textsuperscript{8}
1.4 Photolyase–DNA Interactions

The active site of photolyase is tailored to specifically fit a CPD lesion, while the rest of the strand binds in a structure specific manner (rather than sequence specific), such that the phosphate backbone of the DNA lies along the exterior of the protein. Elucidation of the crystal structure of *Anacystis nidulans* photolyase bound to a CPD analog on an oligonucleotide by Mees et al. corroborates the induced fit of a lone CPD within the active site (Figure 1.7). While the CPD-like lesion was repaired during the measurement, the crystal structure shows the repaired dinucleotide within the active site, sitting just 3.1 Å from the FADH cofactor and thus within hydrogen bonding distance of the adenine base on the cofactor.

This model of CPD binding has been termed the dinucleotide flipping model, as the pyrimidine dimer must flip from a base-stacked conformation within the duplex to a solvent-exposed conformation on the exterior of the strand (Figure 1.8), in order to access the active site. Although the mechanism by which photolyase recognizes CPD lesions is still poorly understood, there are two possible methods that the binding can occur. Either the photolyase recognizes the damage, binds to the site, and the CPD is then flipped into the active site for repair, or the CPD lesion flips out of the strand and is then recognized and bound by the
Photolyase. Recent thermodynamic evidence by Gindt et al. strongly indicates that the CPD lesion flips out of the duplex prior to complexation with photolyase. This suggests that the extrahelical CPD plays a role in recognition of the DNA damage by photolyase.

Figure 1.8 – Computational model displaying the flipped-out extrahelical CPD (left) versus base-stacked intrahelical CPD (right), from O’Neil et al.

1.5 Potential Applications of Photolyase

The rapidly increasing occurrence of skin cancer poses a significant hazard, and is therefore of tremendous concern. Humans rely on nucleotide excision repair to restore DNA damage due to sun exposure and therefore prevent genetic mutation. However, NER does not appear to recognize cyclobutane pyrimidine dimers well, and correspondingly CPDs are repaired much slower than other UV-induced photoproducts. Photolyase is not found in placental mammals, but recognizes and repairs CPDs much more efficiently than NER, and consequently dramatically reduces the likelihood of CPD-induced mutagenesis. As a result, photolyase may hold the key to reducing the harmful effects from ultraviolet radiation. Investigation of how photolyase recognizes and interacts with UV-damaged DNA can potentially lead to the development of better methods for the treatment and prevention of UV-induced CPDs in DNA.

Photolyase has been shown to more effectively remove CPD photolesions from the skin cells of humans in vivo than T4 endonuclease V, an enzyme that performs NER of CPDs. Photolyase-containing liposomes are even effective when applied topically, potentially making them good candidates for use in skin care products. In fact, a combination of sunscreen with a low concentration of liposomal photolyase has been shown to reduce the formation of CPDs by 93% when applied to human skin, compared to a 62% reduction by sunscreen alone.
Photolyase-containing sunscreen also reduces cell apoptosis from repetitive UV-exposure by 82%, compared to a 40% reduction by standard sunscreen.\(^{18}\)

1.6 Research Interests and Goals

Short chain oligonucleotides can be used to model DNA for photolyase studies, as oligonucleotides of \( n \geq 9 \) exhibit a stability similar to DNA when bound to photolyase in the enzyme-substrate complex (ES).\(^{19}\) The majority of photolyase-DNA studies are performed using oligothymidylates, \( p(dT)_n \), as UV-irradiation of polythymidine strands can quickly yield an average of greater than one \( cis\text{-}syn \) CPD per strand.\(^{20}\) This degree of CPD production, because of the high substrate selectivity of photolyases,\(^{21}\) circumvents any need for purification of the irradiated polythymidines. The damaged polythymidines, however, have a random distribution of CPDs along the chains and some strands will contain more than a single CPD. Moreover, other photoproducts will be produced as well, some within CPD-containing oligothymidylates.

This research investigates the suitability of these oligothymidines for photolyase-DNA studies by examining the interactions with the FADH\(^*\) cofactor near the active site of \( E. coli \) photolyase. In order to do this, the flavin interaction with a UV-damaged oligothymididine decamer, UV-\( p(dT)_{10} \), were compared to the interaction with a CPD-containing oligonucleotide dodecamer of sequence 5'-CGGCAT<>TACGGC-3', where (<> indicates a CPD linkage. DNA sequence does not have a significant impact on binding or repair,\(^{22}\) but nevertheless may have an effect on the interactions within the active site environment. All of these oligonucleotide strands also contain a single CPD of known location, with no other photoproducts along the strand lying along the strand.

The UV-\( p(dT)_{10} \) was prepared via ultraviolet irradiation at 254 nm. The corresponding duplex was prepared by annealing a complementary \( p(dA)_{10} \) to the UV-\( p(dT)_{10} \). The dodecamer was irradiated in the presence of an acetophenone photosensitizer at 302 nm to obtain a higher yield of CPD production.\(^{23}\) The CPD-containing dodecamer strands were then purified using reverse-phase high performance liquid chromatography (RP-HPLC). The oligonucleotides were complexed with \( E. coli \) photolyase to characterize the changes induced around the flavin cofactor. The FADH in the photolyase was present in its inactive semiquinone oxidation state to prevent repair of the cyclobutane dimers during investigation.
1.7 Analytical Methods

Investigation of the FADH\(^+\) cofactor and the surrounding protein environment was accomplished using resonance Raman spectroscopy. A Raman spectrum can provide useful information about the vibrational modes of a molecule in a biological relevant environment.\(^2\) Resonance Raman can enhance signal intensities by a factor of \(10^2\) to \(10^6\) and also allows for vibrational selectivity,\(^4\) making it an ideal method for investigation of the flavin cofactor within the photolyase.\(^5\) The photolyase-substrate complexes were therefore analyzed using this powerful technique with an excitation wavelength of 532 nm, so as to gain resonance enhancement of the vibrations of the FADH\(^+\).\(^5\) This allowed for probing of the protein environment around the flavin, near the CPD bound within the active site. Possible electrochromic shifts induced by the oligonucleotides were examined using electronic absorption spectroscopy, in order to investigate the impact on the Raman excitation profile and thus resonance enhancement.

1.8 Experimental Findings

The effects of the UV-p(dT)\(_{10}\) on the vibrational spectrum were consistent with previous findings.\(^5\) The duplex UV-p(dT\(\bullet dA\))\(_{10}\) exhibited changes to the Raman spectrum of photolyase similar to those of the single strand, with only a few minor differences. This indicates that the duplex interacts with photolyase in a manner similar to the single strand, with some minor differences possibly due to its increased rigidity. The intensity changes associated with the enzyme-substrate primarily correspond to altered resonance enhancement, due to a modified Raman excitation profile induced by a blue electrochromic shift of the two flavin electronic transitions at 496 and 584 nm.\(^5\)

Collection of the Raman spectrum of the CPD-oligonucleotide-photolyase complex proved significantly more challenging than the oligothymidinolate spectrum. A contamination within the DNA, which was not removed during the HPLC purification, introduced a significant fluorescent background, leading to a dramatically reduced signal-to-noise ratio. A significant increase in the rate of reduction of the photolyase was also observed, leading to a rapid loss of resonance enhancement. This curious reduction behavior is of great interest and merits further investigation. The collected Raman spectrum differs significantly from that of the UV-oligothymidylate-photolyase complex, seemingly indicating that oligothymidines may not serve
as appropriate models for DNA-photolyase studies. Further investigation is necessary to obtain a clearer Raman spectrum in order to verify these findings.
2. MATERIALS AND METHODS

2.1 Materials

Chemicals were purchased from either Sigma-Aldrich or Acros Organics. All oligonucleotides were purchased from TriLink BioTechnologies, Inc as desalted, lyophilized solids. The inactive radical semiquinone *Escherichia coli* DNA Photolyase was kindly provided at a concentration of 456 μM by Dr. Yvonne Gindt in 20 mM potassium phosphate buffer pH 7.0 and 0.40 M potassium sulfate, and stored at -80 °C.

2.2 Preparation of Oligonucleotide Substrate

The oligothymidine decamer [p(dT)10] substrate was prepared by dissolving solid p(dT)10 in ultrapure water to a concentration of 200 μM, verified with a Perkin-Elmer Lambda 40 UV/VIS spectrometer using the p(dT)10 extinction coefficient of 81,600 M⁻¹cm⁻¹ at 260 nm. The solution was purged with nitrogen gas for 15 minutes and irradiated with 254 nm light from a 6W Spectroline ENF-260C dual wavelength lamp in a 7Q quartz cuvette, over ice, for approximately 4 hours. Formation of 1.5 CPDs per strand was estimated by measuring the absorbance of the thymidylate decamer solution at 265 nm and the absorbance of the oligothymidine (6-4)-photoproduct at 325 nm and obtaining a ratio of $\frac{A_{265}}{A_{325}}$ between 20 and 25. Photolyase exhibits a high affinity for *cis-syn* CPD-containing substrates, so the UV-irradiated p(dT)10 solution was not purified further. The UV-p(dT)10 solution was stored in refrigeration between 0–5 °C. Double stranded damaged p(dT•dA)10 was prepared by combining equimolar UV-damaged p(dT)10 and p(dA)10 solutions, heating the mixture to 50 °C, slowly cooling to room temperature, and then storing in refrigeration between 0–5 °C.

The oligonucleotide dodecamer (5’-CGGCATTACGGC-3’) substrate was prepared by dissolving the dodecamer in ultrapure water containing 20 mM acetoephone, acting as a photosensitizer to increase the yield of *cis-syn* modifications, to a concentration of 200 μM. The solution was purged with nitrogen gas for 15 minutes and the oligonucleotide dodecamer solution was then irradiated with 302 nm light from an 8W UVP UVM-28 twin lamp in an 11SOG special optical glass cuvette, over ice, for 2.5 hours.
2.3 Purification of Oligonucleotide Substrate

The 302 nm-irradiated dodecamer solution was concentrated approximately five-fold at 50 °C in a Labconco Refrigerated CentriVap Concentrator with CentriVap Cold Trap. The CPD-containing oligonucleotide strands (5’-CGGCAT<>TACGGC-3’) were then purified from the irradiated dodecamer solution by high performance liquid chromatography (HPLC) with a triethylammonium acetate/acetonitrile (TEAA/ACN) gradient using a system consisting of two Jasco PU-987 intelligent preparation pumps, a Jasco HG-980-31 solvent mixing module, a Rheodyne 7725i analytical scale sample injector with a 500 µL injection loop, a Varian Microsorb-MV 100-5 C18 250 x 4.6 mm column or Phenomenex Prodigy 5 C8 250 x 4.60 mm column, a Jasco MD-910 multiwavelength detector, and a Pharmacia Biotech Rec 101 chart recorder or Vernier LabPro with Logger Pro software. The purified CPD-oligonucleotide solution was concentrated to near-dryness and again purified by HPLC using an ultrapure water/acetonitrile gradient. The elution was once again concentrated in the CentriVap to a millimolar-scale concentration of CPD-oligonucleotide. The concentration was determined by absorption spectroscopy with the CPD-oligonucleotide extinction coefficient of 96,000 M⁻¹cm⁻¹. The 5’-CGGCAT<>TACGGC-3’ solution was stored in refrigeration between 0-5 °C.

The identity of the CPD-containing oligonucleotide was verified by performing a repair assay with photolyase. A solution was prepared containing the suspected CPD-oligonucleotide, ~6 mM dithiothreitol (DTT) as a reducing agent, and 39.4 µM *E. coli* photolyase. This solution was irradiated in a 7Q quartz cuvette with 365 nm light from the 6W Spectroline ENF-260C dual wavelength lamp in 5 minute intervals, with 50 µL aliquots extracted at each interval. The aliquots were immediately heated in a hot water bath at ~85 °C for approximately 10 minutes. The samples were then centrifuged in an Eppendorf AG miniSpin 5452 at 13,400 revolutions per minute for 10 minutes, in order to spin down the denatured protein. Each supernatant was then run through the HPLC to verify repair of the CPD lesion (data not shown).

2.4 Electronic Absorption Spectroscopy

The absorption spectra of *E. coli* DNA Photolyase were obtained using a Perkin-Elmer Lambda 40 UV/VIS spectrometer with a Thermo Electron Corp. Neslab RTE-10 liquid cooler. The sample holder was cooled to 5 °C and the chamber was purged with dry air for 15 minutes before each measurement to eliminate condensation. The instrument was set to scan from 800.0 to
200.0 nm in 1.0 nm intervals at a rate of 480 nm/min with a slit width of 2.0 nm and was zeroed with 20 mM K$_2$HPO$_3$ pH 7.0 and 0.40 M K$_2$SO$_4$ in a 7Q quartz cuvette. A 40.0 μM semiquinone photolyase solution was prepared and the absorption spectrum from 800.0 to 200.0 nm was collected. The enzyme-substrate complex was prepared by pipetting 10.00 μL of highly-concentrated damaged p(dT)$_{10}$ into the photolyase solution to yield a three-fold excess (120 μM) of oligothymidylate, compared to the minimally diluted concentration of photolyase (39.6 μM). The absorption spectrum was then measured. A similar method was used to collect the absorption spectra of the photolyase-UV-p(dT*dA) and photolyase-CPD-oligonucleotide complexes.

2.5 Resonance Raman Spectroscopy

Resonance Raman spectra were collected using a system consisting of a HORIBA Jobin Yvon TRIAX 550 spectrograph, a Spectrum One N$_2$(l)-cooled CCD detector with a UV-enhanced 2048 x 512 English Electric Valve microchip, a CCD-3000 controller, and SpectraMax for Windows version 3.1. A 300 mW 532 nm Lambda Pro diode-pumped solid-state laser with an LPS-300 power supply was used for excitation. The intensity of the laser was attenuated using an ND filter with an optical density of 1.0, and was measured to be approximately 23 mW using a Newport 1815-C power meter. The laser was focused onto the sample using a 50. mm focal length lens. All samples were analyzed in a spinning quartz cell that required a minimum volume of 70 μL of solution and was sealed with a rubber septum. The scattered light from the sample was collected through a Nikon Nikkor 50 mm f/1.2 camera lens at an angle of 90° from the excitation, and then passed through a Kaiser Optical Systems, Inc. 532.0 nm holographic notch filter to remove Rayleigh scattering and unwanted laser light. The Raman scattered light was then focused with a 300. mm lens through a wedge depolarizer onto the slit of the spectrograph. The spinning cell was enclosed in a plastic chamber that was cooled by passing dry air through a dry ice/denatured ethanol bath and into the chamber, so that the sample remained between 5–10 °C.

2.6 Data Analysis

All data analysis and plotting was performed using Origin® 7 by OriginLab Corp. All Raman spectra were calibrated to the standard Raman spectrum of toluene via 2nd order polynomial fit. All Raman spectra were corrected via subtraction of 4th to 6th order polynomial baselines.
3. RESULTS

3.1 Purification of CPD-Oligonucleotide

The RP-HPLC purification parameters were adapted from Mu et al.,\textsuperscript{27} in order to obtain baseline separation between the CPD-oligonucleotide and the undamaged oligonucleotide. A 100 mM pH 7 TEAA buffer was used as the primary component of the mobile phase, with a gradient of increasing ACN during the purification. The HPLC detector was to 260 nm, corresponding to the maximum absorbance oligonucleotides. The following gradient was used for the initial trial: Initial – 7% ACN, 5 min. – 7% ACN, 35 min. – 10% ACN, 45 min. – 12% ACN, 50 min. – 70% ACN. The gradient was adjusted iteratively to achieve a shorter runtime with good resolution, for a final gradient of: Initial – 7% ACN, 5 min. – 7% ACN, 15 min. – 8% ACN, 20 min. – 10% ACN, 25 min. – 75%. This program was adjusted depending on the stationary phase in use. The gradient provided a significant separation between the elution times of the \textit{cis-syn} CPD-containing oligonucleotides and the undamaged oligonucleotides. The CPD-oligonucleotide was eluted from ~9-10 minutes, while the undamaged oligonucleotide was eluted from ~16.5-18 minutes (\textit{Figure 3.1}).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{chromatogram.png}
\caption{HPLC Chromatogram of Irradiated Oligonucleotide Solution.}
\end{figure}
The retention order was verified using UV/visible absorption spectroscopy and corresponded with the work of Mu et al.\textsuperscript{27} Both the 9-10 minute elution and the 16.5-17 minute elution showed the presence of oligonucleotide with strong absorption bands at ~260 nm. The 9 to 10 minute elution was determined to be the CPD-oligonucleotide via a photolyase repair assay (data not shown). The 16.5 to 17 minutes elution was determined to be the original undamaged oligonucleotide dodecamer. The strongly absorbing elution from 28.5-29.5 minutes was determined to be the acetophenone. After collection and concentration, the CPD-oligonucleotide solution was again purified by HPLC using an ultrapure water/ACN gradient to separate the strands from the TEAA buffer of the initial purification. The program was modified to achieve a retention time similar to that of the TEAA/ACN purification.

3.2 Electronic Absorption Spectroscopy

The electronic absorption spectrum of semiquinone \textit{E. coli} photolyase from 300 to 700 nm (Figure 3.2) shows four distinct electronic transitions, a band at 383 nm primarily from the MTHF cofactor, with a broad underlying contribution from the FADH\textsuperscript{+}, and bands at 496, 584, and 623 nm from the FADH\textsuperscript{+} cofactor.\textsuperscript{25} These electronic transitions allow for selective resonance Raman enhancement of the flavin vibrations, depending on the wavelength of excitation. The primary transitions enhanced by the excitation wavelength of 532 nm used in this study are the 496 and 584 nm bands from the FADH\textsuperscript{+}.

Upon binding to photolyase, the interaction of the single strand (ss) UV-p(dT)\textsubscript{10} with the FADH\textsuperscript{+} cofactor induces a blue electrochromic shift in the absorption bands at 496 and 584 nm. The baselines were adjusted to zero, but no normalization was conducted as the measurements were conducted at nearly identical concentrations. The extinction coefficient of the 496 nm transition also decreases slightly, while the extinction coefficient of the 584 nm transition increases significantly in the ES complex. Binding of the double strand (ds) UV-p(dT$^*$.dA)\textsubscript{10} to the photolyase induces changes within the absorption spectrum of the photolyase similar to those of the ssUV-p(dT)\textsubscript{10}. The CPD-oligonucleotide-photolyase complex exhibits similar changes within the absorption spectrum as those witnessed with both the ss and ds UV-p(dT)\textsubscript{10}-photolyase complexes.

The difference between the substrate-bound photolyase spectra and the unbound photolyase spectrum (Figure 3.2 inset) highlight the changes induced by interaction with the
oligonucleotide substrates. The net change in absorption at the 532 nm excitation wavelength is negligible upon binding of the ssUV-p(dT)$_{10}$, dsUV-p(dT)$_{10}$, and CPD-oligonucleotide.

![Figure 3.2](image_url)

**Figure 3.2** – Electronic absorption spectra of photolyase (black), single strand UV-p(dT)$_{10}$-bound photolyase (red), duplex UV-p(dT•dA)-bound photolyase (blue), and single strand CPD-oligonucleotide-bound photolyase (green) without normalization. Inset: Difference of the photolyase-substrate spectrum minus photolyase spectrum for each substrate, with the 532 nm Raman excitation wavelength highlighted.

### 3.3 Resonance Raman Spectroscopy

The resonance Raman spectra of photolyase and UV-p(dT)$_{10}$-bound photolyase (*Figure 3.3*) are consistent with previous results. The spectra were normalized using the prominent peak from the FADH' at 1607 cm$^{-1}$. The presence of the UV-p(dT)$_{10}$ substrate induces significant intensity changes in many of the vibrational bands, most notably at the 1223, 1261, 1305, 1334, 1351, 1380, and 1394 cm$^{-1}$ modes. The most dramatic change in intensity is seen at the shoulder around 1351 cm$^{-1}$, as it appears to completely disappear. There are also minor frequency shifts produced upon interaction with the single strand substrate, the most prominent of which is a shift of +3.2 cm$^{-1}$ in the 1236 cm$^{-1}$ band. The resonance Raman spectrum of the photolyase with
double strand UV-p(dT•dA)$_{10}$ also experiences significant intensity changes and small frequency shifts similar to those induced by those of ssUV-p(dT)$_{10}$.

![Resonance Raman spectra](image)

**Figure 3.3** – Resonance Raman spectra of photolyase (black), single strand UV-p(dT)$_{10}$-bound photolyase (red), and duplex UV-p(dT•dA)-bound photolyase (blue) with 532 nm excitation.

A comparison of single and double strand UV-p(dT)$_{10}$ (**Figure 3.4**) shows that there are a few slight differences within the Raman spectra. The most prominent differences in intensity can be seen at the 1236 and 1380 cm$^{-1}$ bands, although they are relatively minor. There are also a few minor frequency differences between the single and double strand oligonucleotides, notably at the 1223, 1236, 1261, 1305, and 1334 cm$^{-1}$ bands. The majority of these shifts are very minor (within ~1 cm$^{-1}$), with a notable change at the 1236 cm$^{-1}$ vibration. Interaction with the dsUV-p(dT)$_{10}$ appears to induce a smaller shift at 1236 cm$^{-1}$ than the 3.2 cm$^{-1}$ caused by the ssUV-p(dT)$_{10}$. The difference spectra compare the differing intensity and wavenumber changes induced by the presence of single strand and double strand oligonucleotides (**Figure 3.4 inset**).
The resonance Raman spectrum of photolyase with the CPD-oligonucleotide dodecamer also shows significant changes from that of the unadulterated photolyase (Figure 3.5). There are notable intensity changes in the bands at 1223, 1261, 1305, 1334, and 1350 cm⁻¹. There are also small frequency shifts observed at the 1223, 1261, 1305, 1334, and 1380 cm⁻¹ vibrations. The most prominent frequency shift occurs at the 1223 cm⁻¹ vibration, with a change of -3.4 cm⁻¹.

Measurement of the resonance Raman spectrum of photolyase with CPD-oligonucleotide proved to be more difficult than the oligothymidine complex. A fluorescent contaminant within the oligonucleotide sample presented high background in the Raman spectrum, leading to poor signal-to-noise. Also contributing to the poor quality was a dramatically increased reduction rate of the FADH⁺ in the CPD-oligonucleotide-photolyase complex, leading to a loss of resonance enhancement. HPLC analysis showed that some repair of the CPD-oligonucleotide occurred during collection of the Raman spectrum, indicating reduction to FADH⁻. The photolyase alone shows a signal loss of ~33% after 30 minutes of 532 nm laser exposure, photolyase with UV-p(dT)₁₀ shows a loss of ~75% after 30 minutes, while photolyase with CPD-dodecamer shows a loss of ~80% after just 14 minutes (Figure 3.6).
Figure 3.5 – Resonance Raman spectra of photolyase (black) and CPD-oligonucleotide-bound photolyase (green).

Figure 3.6 – Raman spectra of photolyase (top left), photolyase with UV-p(dT)₁₀ (top right), and photolyase with CPD-oligonucleotide (bottom). Initial spectrum is shown in red with subsequent spectrum after indicated time shown in blue.
The fluorescence seen within the oligonucleotide sample decreased significantly with prolonged exposure to the 532 nm laser. In an attempt to reduce the fluorescent background, CPD-containing oligonucleotide was exposed to the laser beam for approximately 3 hours before combining it with photolyase. The resonance Raman spectrum was then collected for the photolyase with irradiated-CPD-oligonucleotide. The resulting spectrum (Figure 3.7) did not show an appreciable improvement in signal-to-noise, and more importantly appears to exhibit additional, unexpected vibrational bands.

A comparison of the Raman spectrum of the photolyase containing CPD-oligonucleotide and the spectrum of the photolyase with ssUV-p(dT)$_{10}$ (Figure 3.8) shows that there are significant differences between them. There are notable dissimilarities between the frequency shifts observed within the UV-p(dT)$_{10}$ complex and those observed within the CPD-dodecamer complex, highlighted within the difference spectra (Figure 3.8 bottom).
Figure 3.8 – Top: Resonance Raman spectra of photolyase (black), single strand UV-p(dT)₁₀-bound photolyase (red), duplex UV-p(dT•dA)-bound photolyase (blue), and single strand CPD-oligonucleotide-bound photolyase (green). Bottom: Difference of the photolyase-substrate spectrum minus photolyase spectrum for each substrate.
4. DISCUSSION

4.1 Impact of Oligonucleotide Interaction on Electronic Absorption

The electrochromic shift of the 496 and 584 nm electronic transitions in the semiquinone photolyase upon interaction with UV-p(dT)$_{10}$ and CPD-oligonucleotide leads to a different Raman excitation profile. While the overall impact on absorption at 532 nm due to substrate interaction is negligible, the percent contribution from each of the primary transitions changes significantly. At 532 nm there is less contribution from the 496 nm transition and more contribution from the 584 nm transition. This leads to a decreased enhancement of the vibrations associated with the 496 nm electronic excitation and an increased enhancement of the vibrations associated with the 584 nm electronic excitation within the Raman spectra.

Previous work by Schelvis et al. has shown that the vibrational intensity differences within the resonance Raman spectrum of UV-p(dT)$_{10}$-bound photolyase can be primarily explained by the changes within the Raman excitation profile near 532 nm. There are two bands with significant intensity differences between the single and double strand substrate complexes, at 1236 and 1380 cm$^{-1}$. These intensity differences are likely induced by the altered Raman excitation profiles upon binding, as the 1380 cm$^{-1}$ band has been linked to the electronic transition at 496 nm, which exhibits a slightly larger extinction coefficient in the double strand complex.

CPD-oligonucleotide-bound photolyase exhibits changes within its absorption spectrum similar to those of the UV-p(dT)$_{10}$-bound photolyase, so it also should exhibit significant intensity changes within its resonance Raman spectrum due to the altered excitation profile. In the CPD-oligonucleotide-bound photolyase there are differences in intensity in many of the same bands as the UV-p(dT)$_{10}$-bound photolyase, although the changes differ significantly. For instance, the 1261 and 1334 cm$^{-1}$ bands are reduced in the presence of UV-p(dT)$_{10}$, while they exhibit an enhancement in the presence of CPD-oligonucleotide (Figure 3.8). The absorption band at 496 nm is likely responsible for the resonance enhancement of these vibrational modes, and this band does appear to differ slightly from the UV-p(dT)$_{10}$ complex (Figure 3.2). There appear to be other minor differences in the Raman intensities of the UV-p(dT)$_{10}$ and CPD-oligonucleotide complexes, but the poor signal-to-noise ratio makes definitive comparison difficult.
4.2 Comparison of Single and Double Strand UV-Polythymidine

The frequency shifts in the resonance Raman spectrum of *E. coli* photolyase induced by the presence of the single strand UV-p(dT)$_{10}$ are fairly consistent with previous work by Schelvis et al.$^{25}$ Most of the affected vibrational bands have been shown to be sensitive to hydrogen-deuterium exchange, and are therefore related to changes in the protein environment, such as hydrogen bonding, around the flavin cofactor within the active site.$^{25}$ Changes within the remaining vibrational modes are suspected to be induced by the dipole moment of the substrate (vibrational Stark effect).$^{25}$ There are only very minor differences observed within the resonance Raman spectra of the ssUV-p(dT)$_{10}$-bound photolyase and the dsUV-p(dT•dA)$_{10}$-bound photolyase (*Figure 3.4*). The prominent disappearance of the shoulder around 1350 cm$^{-1}$ in both spectra is a good indicator that the photolyase is bound to the substrate, signifying interaction with the double strand oligothymidylate.

The small frequency differences observed in the UV-p(dT•dA)$_{10}$-bound photolyase compared to those of the UV-p(dT)$_{10}$-bound photolyase indicate that there are some minor disparities in the protein environment within the active site, as all of the bands that exhibit frequency disparities between the two enzyme-substrate complexes are deuterium-sensitive. As the difference between the substrates is the presence of the hydrogen-bonded p(dA)$_{10}$ complementary strand, the disparities within the active site environment are likely due to the much greater persistence length of duplex DNA,$^{29}$ compared to that of single strand DNA. Overall, the single strand and duplex UV-p(dT)$_{10}$ induce very similar changes in the interactions between the protein and the FADH$^+$ the active site of photolyase.

4.3 Comparison of UV-Polythymidine and CPD-Oligonucleotide

The presence of CPD-containing oligonucleotides induces a number of small frequency shifts in the resonance Raman spectrum of photolyase (*Figure 3.5*). The CPD-oligonucleotide-bound photolyase shows some significantly different shifts in frequency than those induced by the UV-p(dT)$_{10}$ (*Figure 3.8*). These bands that exhibit differences undergo frequency shifts due to changes in the interactions between the protein and FADH$^+$ in the active site of photolyase.$^{25}$

The disparities between the resonance Raman spectra of the UV-oligothymidylate and CPD-oligonucleotide complexes appear to indicate that the two strands interact with the active site differently. It is therefore plausible that oligonucleotide sequence, CPD location, and/or
auxiliary intrastrand photoproducts play a role in the induced fit of the CPD within the active site of *E. coli* DNA photolyase. This also suggests that UV-irradiated oligothymidylates may not actually serve as good models for UV-damaged DNA in photolyase studies. While these results were reproducible using the same batch of purified CPD-oligonucleotide, the poor quality of the resonance Raman spectrum of the CPD-oligonucleotide-bound photolyase prevents a definitive conclusion. In order to verify these findings, better signal-to-noise needs to be obtained within the Raman spectrum.

4.4 Investigation of Fluorescent Contamination

Attempts to improve the signal-to-noise ratio of the Raman spectrum for the CPD-oligonucleotide-bound photolyase, by prolonged irradiation of the CPD-oligonucleotide with the 532 nm laser, did not yield the desired result. Not only was a higher quality spectrum not obtained, but this procedure also presented an entirely new concern with the addition of unexpected bands in the resonance Raman spectrum (*Figure 3.7*). A vibrational band appears at 1550 cm⁻¹ in the Raman spectrum of the irradiated-CPD-oligonucleotide-bound photolyase, which does not appear to have a corresponding peak in either the photolyase or UV-p(dT)₁₀-bound photolyase spectra. There are two vibrational modes at 1361 and 1370 cm⁻¹ in the irradiated-CPD-oligonucleotide-bound photolyase spectrum that do not appear in either the photolyase or UV-p(dT)₁₀-bound photolyase spectra. A shoulder also appears next to the 1334 cm⁻¹ band at about 1322 cm⁻¹, which is not present in the other spectra.

These contaminating vibrational bands show up only after irradiation of the CPD-oligonucleotide solution with the 532 nm laser. As the fluorescence decreases with irradiation, it seems that there may be photodegradation of the fluorescent compound induced by the 532 nm light. The possible formation of photoproducts presents a reasonable explanation for the appearance of the additional vibrational modes within the resonance Raman spectrum. Because of these contaminating bands, laser irradiation is not a practical means of reducing the fluorescence witnessed from the oligonucleotide dodecamer.

Further work must be performed in order to obtain a dodecamer sample that is free of the fluorescent contaminant. Purification by RP-HPLC does not separate the contaminating compound from the oligonucleotide, so it is possible that the contaminant is actually coupled to the strand in some manner. One possible solution to the problem may be to employ other purification methods that may separate the fluorescent compound from the CPD-oligonucleotide.
Alternatively, a sample of the dodecameric oligonucleotide can be purchased from other suppliers for comparison, as the stock lyophilized oligonucleotide from TriLink Biotechnologies exhibits this fluorescence. Elimination of the interfering fluorescence will allow a longer exposure time during collection of the Raman spectrum, which will provide a better signal-to-noise ratio.

4.5 Rate of FADH* Reduction

Excitation of photolyase with the 532 nm laser slowly reduces the FADH* by photoinduced electron transfer from $306^\text{TrpH}$ until it reaches equilibrium. This accounts for much of the signal loss over time in the resonance Raman spectrum of photolyase (Figure 3.6), as reduction to FADH* leads to a loss in resonance enhancement. The photolyase-UV-p(dT)$_{10}$ complex exhibits a faster rate of reduction than the photolyase by itself, nearly 3 times faster. This indicates that perhaps the fully reduced hydroquinone flavin cofactor is more stable in the enzyme-substrate complex than the enzyme alone. Interestingly, the photolyase-CPD-oligonucleotide complex exhibits an even more dramatic increase in the rate of reduction of the flavin cofactor, more than twice as fast as the UV-p(dT)$_{10}$-bound photolyase.

A possible explanation for this behavior may be linked to the TEAA buffer from the HPLC purification. If all of the triethylamine (TEA) was not removed from the purified CPD-oligonucleotide solution, it could interfere with the photoreduction process between flavin and the tryptophan triad. The reduction potential of triethylamine is -0.96 V, while the reduction potential of the tryptophan residue at pH 7.0 is 0.895 V. Therefore the triethylamine would act as a reducing agent in the following reaction.

$$\text{TEA} + 306^\text{Trp}^* + H^+ \rightarrow \text{TEA}^+ + 306^\text{TrpH} \quad E = 1.86 \text{ V}$$

If the oxidized tryptophan radical is reduced by TEA, then the fully reduced FADH* can no longer be oxidized back to the semiquinone FADH* by the tryptophan triad pathway and is essentially trapped within the reduced state.

The dramatic increase in the rate of reduction of photolyase with CPD-oligonucleotide could also possibly be induced by a change in the reduction potential of the FADH* cofactor upon interaction with the substrate. Further investigation is required to determine whether the mobile phase of the HPLC purification process is responsible for the increased rate of reduction witnessed in the presence of CPD-oligonucleotide. As the UV-oligothymidylicate also exhibits an increase in reduction of the photolyase, the behavior may not be due solely to the presence of TEA, as the UV-oligothymidine does not undergo purification.
5. CONCLUSIONS

The single strand and double strand UV-oligothymidylates induce virtually identical electrochromic shifts in the electronic absorption spectrum of photolyase, indicating no significant difference in the Raman excitation profiles. There are a few minor intensity changes and frequency shifts, but both the single and duplex strand UV-oligothymidine-photolyase Raman spectra are largely the same overall. As the bands that do exhibit differences are related to the protein environment around the flavin cofactor, the single and double strand UV-oligothymidines appear to have slightly different impacts on the conformation of the active site. Because the CPD must flip into the catalytic pocket of the protein, regardless of whether the strand is duplexed or not, the differences are likely induced by the much greater rigidity of the duplex strand. However, the observed differences were minor, such that both single and double strand DNA appear to interact very similarly with photolyase near the flavin cofactor.

The single strand CPD-oligonucleotide induces electrochromic shifts similar to those of the UV-oligothymidines within the electronic absorption spectrum of photolyase, albeit possibly with slightly different extinction coefficients. A slightly different Raman excitation profile might account for some of the intensity differences in the resonance Raman spectrum compared to that of the single strand UV-p(dT)10-bound photolyase. However, there are also significantly different frequency shifts in many of the vibrational bands of the CPD-oligonucleotide-photolyase complex. This indicates that the CPD-oligonucleotide produces significantly different changes than the UV-p(dT)10 in the protein environment around the flavin cofactor. Because of the relatively poor signal-to-noise ratio of the resonance Raman spectrum of the CPD-oligonucleotide-bound photolyase, further investigation is necessary for verification of these findings.

Unfortunately, a high fluorescent background hindered the quality of the Raman spectrum for the CPD-oligonucleotide-photolyase complex, likely caused by a fluorescent contamination within the stock oligonucleotide dodecamer. Irradiation with the 532 nm laser significantly reduced the fluorescence, however new vibrational bands were detected in the resonance Raman spectrum after this laser irradiation. Implementation of additional purification techniques may be able to remove the fluorescent contaminant.

Interestingly, the CPD-oligonucleotide increases the rate of reduction of the flavin cofactor much more dramatically than the UV-p(dT)10 strand. This may potentially be due to contamination by triethylamine (TEA) from the mobile phase used in the HPLC purification. If
not all of the TEA was removed via HPLC purification using water or during the drying process in the centrivap, the TEA can act as a reducing agent to reduce the oxidized tryptophan within the reduced photolyase. This would essentially trap the flavin in the fully reduced, active hydroquinone state. However, the presence of UV-oligothymidine strands also increases the rate of reduction of the flavin, though less dramatically. Interaction with substrate may therefore potentially have an impact on the reduction potential of the flavin. Further study is necessary to investigate the impact of CPD-oligonucleotide on the rate of reduction of the flavin cofactor, in order to determine whether TEA is present at a concentration sufficient to rapidly reduce the photolyase.
6. REFERENCES


