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DNA Photolyase: An Electron Paramagnetic Resonance Spectroscopy Study

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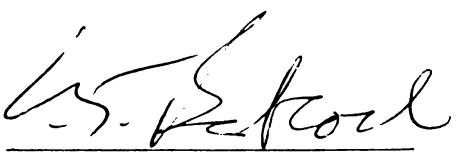

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DNA PHOTOLYASE: AN ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY STUDY

By

Kristi Lee Westphal

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ABSTRACT

DNA PHOTOLYASE: AN ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY STUDY

By

Kristi Lee Westphal

DNA photolyase is an enzyme that repairs thymine dimers formed by exposure of the DNA strand to far UV light. This enzyme is activated when it is in the presence of visible light. The basic mechanism of photolyase is as follows: (1) photolyase binds to the dimer in a light independent step (2) upon absorption of a photon the dimer is split (3) photolyase dissociates from the DNA. Photolyase has been successfully overproduced in *E. coli* cells and can now be isolated in a stable, nearly pure form.

Electron Paramagnetic Resonance (EPR) experiments have been conducted on photolyase in order to better understand the repair mechanism. A spin polarized EPR signal has been detected using time resolved spectroscopy. Labeling techniques have been used in order to identify the active species involved in the spin polarization process. Experiments which have been performed and future work in this area will be discussed.

ACKNOWLEDGMENTS

I would like to thank my colleagues for all of their support, technical and personal. I want to give a huge thank you to Yvonne Gindt who is a great teacher and has taught me all that I know on this research. I would also like to thank my advisor, Jerry Babcock, for his guidance and for teaching me the difference between 'which' and 'that'! A special thanks goes out to my mother whose words of encouragement always keeps me going when the only thing I want to do is give up. Thanks to all of you!

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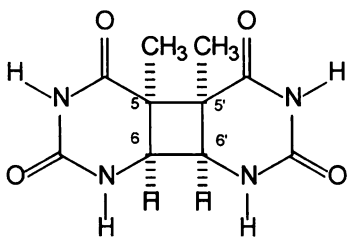
Abbreviations: pyr<>pyr, pyrimidine dimer; FADH, flavin adenine dinucleotide; MTHF, methenyltetrahydrofolate; E-FAD, enzyme apoenzyme bound to flavin; T<>T, thymine dimer; pyr, pyrimidine molecule; $pyr^{\cdot}$, pyrmidine charged radical; *tac*, promoter for transcription; *phr*, gene; NpT<>TpNpNp, DNA strand containing three of any bases and two thymine bases; T<c,s>T, *cis,syn* cyclobutane dimer; trp, tryptophan molecule; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital

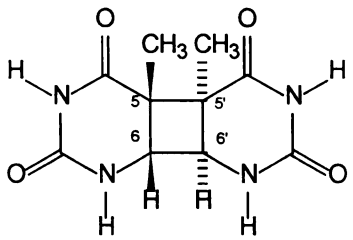
Chapter 1: Introduction

DNA photolyase is an enzyme that repairs cyclopyrimidine dimers found in DNA. These dimers are formed by adjacent bases on the same strand when the DNA has been exposed to far UV light (200-300nm). The two main bases that are affected are purine and pyrimidine that absorb at λ_{max} = 250-270nm, with pyrimidine (pyr, see page 25 for abbreviations) photoproducts predominating.[1] Four isomers of pyrimidine dimers are shown in Figure 1.1, with the *cis, syn* isomer being the natural substrate of photolyase. The pyr<>pyr kills cells by blocking replication and transcription and by causing a mutation at the site of the lesion. Cells protect themselves from these effects by eliminating photodimers from their genome either by excision repair or by photoreactivation. The latter process is carried out by DNA photolyase, which prevents the harmful effects (cancer, mutations, death) of far UV light by concurrent or subsequent exposure to near UV light (350-500nm).[2] The DNA photolyases are a group of enzymes that catalyze this photoreactivation of damaged cells by breaking the cyclobutane bonds of the pyrimidine dimer, thereby restoring the pyrimidine monomers. These light-activated repair enzymes are widespread in nature and have been found in many bacteria, blue green algae, fungi, higher plants, and all major groups of vertebrates with the possible exception of placental mammals. Its presence in humans has been a controversial issue with the most recent conclusion being that photolyase is found in humans.[3]

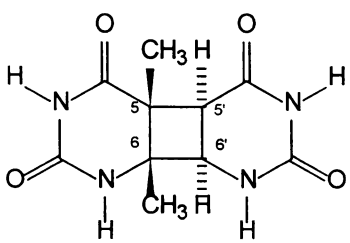
Figure 1.1. The four isomers of pyrimidine dimers.



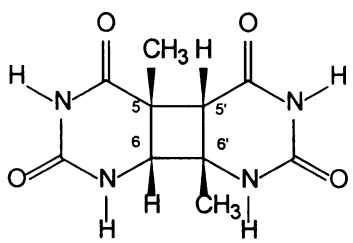
cis-syn



trans-syn (racemic)



trans-anti (racemic)



cis-anti

Heelis, P.F., Hartman, R.F., and Rose, S. *Chemical Society Reviews* **1995**, *24*, 290.

Figure 1.1

Photoreactivation was discovered in the late 1940's by Albert Kelner when he showed that after UV inactivation *Streptomyces Griseus* cells could be reactivated by subsequent irradiation with visible light.[4] In 1962, Rupert, et *al.* showed that photoreactivation restores pyrimidine dimers to the original pyrimidines in DNA.[5] By 1960 the basic mechanism was thought to occur in three steps: (1) Photolyase binds to pyrimidine dimers in DNA in a light independent step (2) upon absorption of a photon the cyclobutane dimers are split to yield two pyrimidines (3) photolyase then dissociates from the DNA. Figure 1.2 shows a diagram of this basic mechanism.

The photolyase gene was cloned by Sancar and Rupert in 1978.[6] In 1983 Sancar, et *al.* described the construction of a *tac-phr* plasmid that greatly over produces photolyase in *Escherichia coli.*[7] These achievements in the cloning and overproducing of photolyase made it possible to study this enzyme in considerable detail. Now with a more efficient method for purification (described in chapter 2) substantial quantities of the enzyme can be produced and isolated in a relatively short period of time.

DNA photolyases are monomeric proteins of molecular weight 55-65 kDa. Their action is described by classic Michaelis-Menten enzyme kinetics with the exception that the catalysis is light initiated. All contain stoichiometric amounts of two light absorbing cofactors, one of which is 1,5-dihydroflavin adenine dinucleotide (FADH) and the other either methenyltetrahydrofolate or 8-hydroxy-5-deazariboflavin.[8] The structures of the two chromophores are shown in Figure 1.3. The folate class includes enzymes from *Escherichia coli, Neurospora crassa, Bucillus firmus,* and *Saccharomyces cerevisiae*, and the deazaflavin class includes photolyases from *Anacystis nidulans, Streptomyces griseus,*

Figure 1.2. Basic mechanism of dimer splitting by photolyase. PRE is the photoreactivating enzyme, and pyr<>pyr is a pyrimidine dimer in damaged DNA.

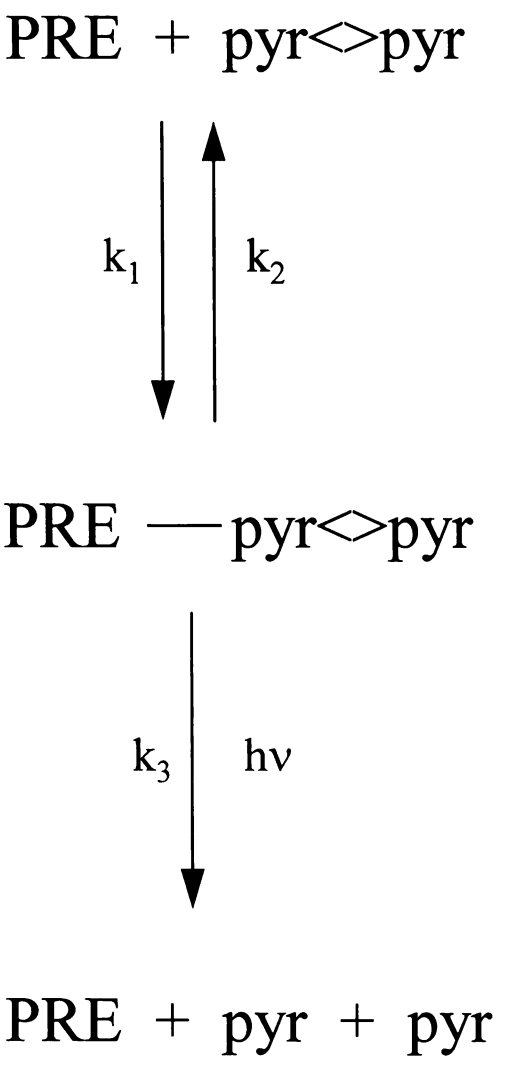
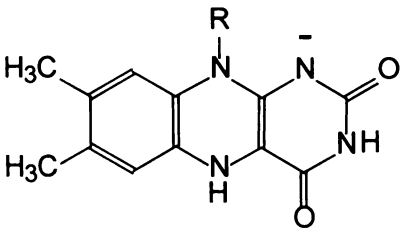
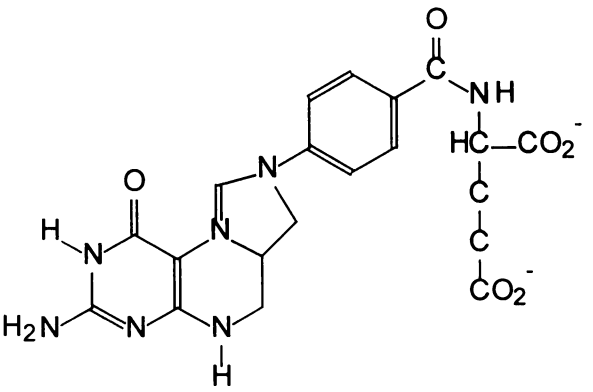


Figure 1.2

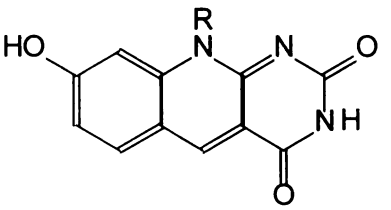
Figure 1.3. Chromophores of DNA photolyase.



a) FADH -



b) 5, 10-MTHF



c) 8-HDF

Kim, S. and Sancar, A. *Photochemistry and Photobiology* **1993**, *57*, 896.

Figure 1.3

Scenedesmus acutus, Halobacterium halobium, and *Methanobacterium thermoautotrophicum.*

In photolyase, the flavin chromophore can by found in all three of its oxidation states: the fully oxidized FAD_{ox}, the semiquinone $FADH^{\cdot}$, and the fully reduced $FADH_2$ ($FADH^-$). The E-FAD_{ox} form is catalytically inert[9] and the E-$FADH^{\cdot}$ form is active, but with low quantum yield. The $FADH^-$ form is the active form of the enzyme with a quantum yield between 0.5 and 0.9.[10] Flavin is required for catalysis because it is the photoactive species in the enzyme. It may also play a structural role, however, as the flavin cofactor may provide extra stabilization to the enzyme by keeping the two helix clusters together; the clusters probably have to move apart for binding of FAD to the apoenzyme.[11]

In *E. coli* the second chromophore, MTHF, functions as a light harvesting molecule. It absorbs a photon of light and transfers this energy to FADH. It is not necessary for substrate binding and does not have to be present for dimer cleavage to occur. The MTHF cofactor is located on the periphery of the enzyme. This explains why enzyme lacking this cofactor retains its natural conformation and thymine dimer binding affinity. Moreover, since the MTHF cofactor is solvent accessible, it readily dissociates from the enzyme during isolation.

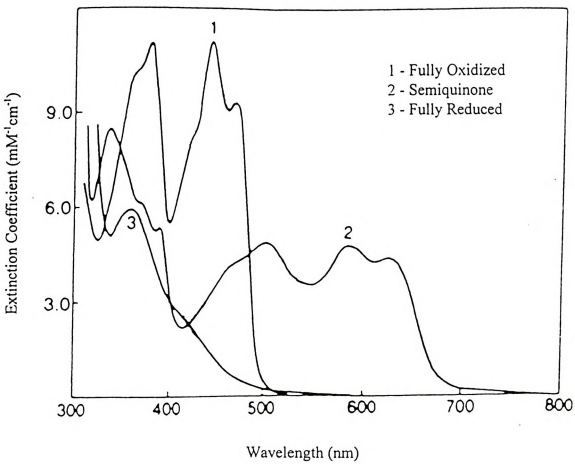
Why does photolyase need two chromophores when flavin is able to absorb light directly and can donate an electron to the dimer? Flavins are ubiquitous as redox-active components of many enzymes. Although flavins can absorb light, they are not very efficient ($\varepsilon = 5{,}000\ M^{-1}cm^{-1}$ at 350nm for $FADH^-$). The folate is much more efficient at

this task (ε = 25,000 $M^{-1}cm^{-1}$ at 390nm).[12] The folate is thus used in an antenna function to absorb light and to transfer it to the flavin, which is used for chemistry. Therefore both chromophores are needed for efficient dimer repair.

The native form of the enzyme contains one mole of MTHF and one mole of FADH per mole of apoenzyme. Its absorption spectrum is characterized by a peak at 280nm due to aromatic amino acid residues and a peak at 384nm due to enzyme bound MTHF. All three oxidation states of the flavin cofactor in the enzyme have characteristic absorption spectra,[13] as can be seen in Figure 1.4. The semiquinone, or one electron reduced form of the enzyme, is blue in color and the enzyme readily turns blue during purification due to oxidation of flavin to the semiquinone form. The absorption spectrum of the semiquinone form has peaks at 480, 580, and 625nm typical of the neutral flavin radical. The oxidized form is bright yellow in color due to its high extinction coefficient at its absorption maxima of 450nm (ε = 11,300 M^{-1} cm^{-1}). Upon full reduction, the enzyme turns a pale yellow and absorbs at 366nm (ε = 5680 M^{-1} cm^{-1}).

In 1995, the crystal structure of photolyase was reported by Park et *al.* The enzyme is made up of a single polypeptide chain of 471 amino acids and the light harvesting and catalytic chromophores, both of which are noncovalently attached chromophores. The polypeptide chain is folded into five β strands, 20 α helices, five short 3_{10} helices, and connecting segments. There are two major domains, an α/β domain which contains the MTHF chromophore, and the helical domain that binds the FADH chromophore. The two chromophores are separated by 16.8 Å; the excitation energy is transferred from MTHF to $FADH^{\cdot}$ with a time constant of about 200ps and 62%

Figure 1.4. Absorption spectra of the oxidized, semiquinone, and reduced oxidation states of DNA photolyase.



Jorns, M.S., Wang, B., Jordan, S.P., and Chanderkar, L.P., *Biochemistry* 1990, 29, 560.

Figure 1.4

efficiency.[14]

Substrate binding depends on both pyr<>pyr structure and the local structure about the dimer in the DNA backbone with pyr<>pyr binding to the flat surface of the enzyme's helical domain. The enzyme binds a T<>T-containing substrate by contacting the first phosphate 3' to T<>T on the damaged strand and the phosphate opposite the T<>T across the minor groove on the undamaged strand of a duplex. There is a depression or "hole" in the helical domain of the enzyme that leads directly to the FAD binding site. This hole has the right dimension to allow a thymine dimer to fit precisely and come within van der Waals contact distance of the FAD. A thymine dimer has asymmetric polarity, which fits well with the asymmetry of the hole as the residues lining the hole are hydrophobic on one side and polar on the other. Binding in this fashion would allow electron transfer between FAD and the substrate to occur over a short distance and with high efficiency. The pyr<>pyr is proposed to bind in this way by flipping out of the helix and into the hole.

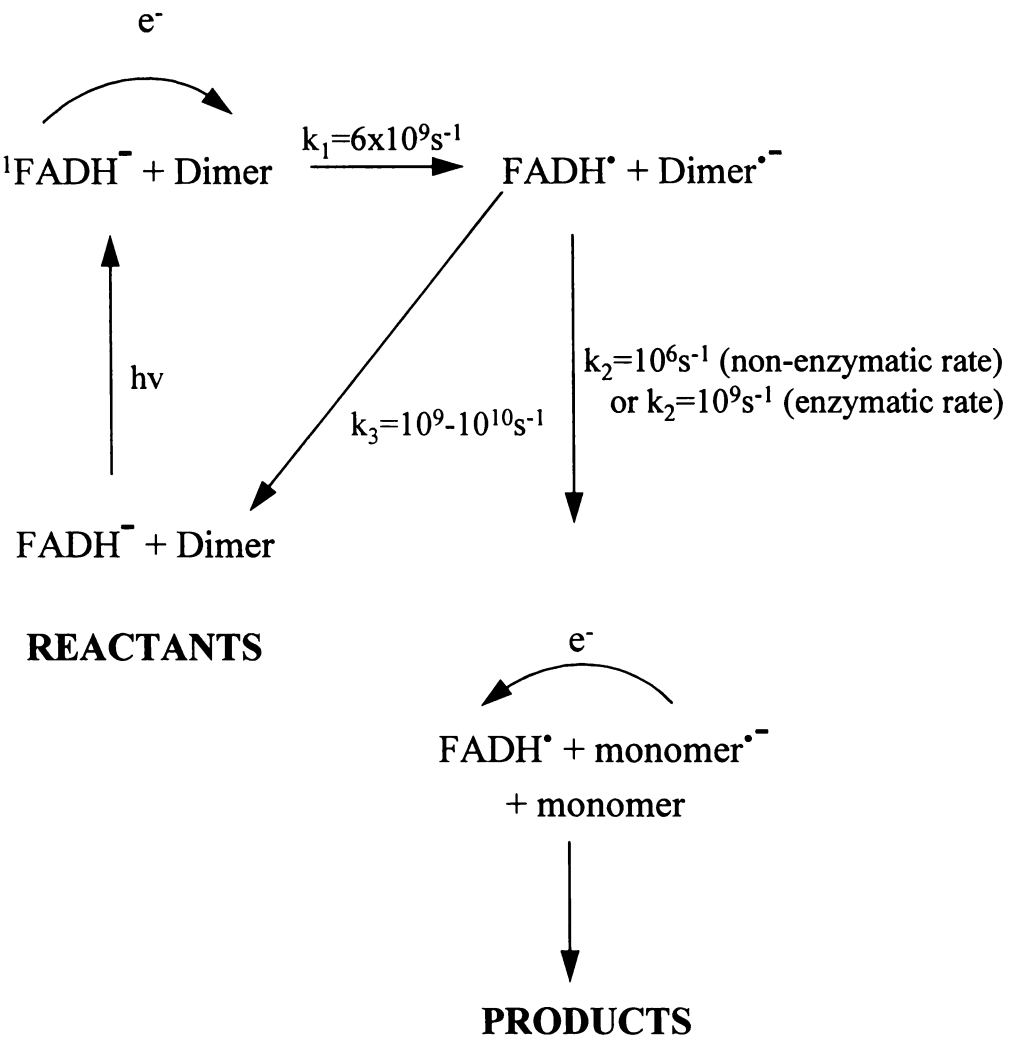
Photolyase is a "structure specific" protein. This means that its catalytic specificity is determined by the overall structure of the DNA in contrast to a "sequence specific" protein, which relies on the sequence of the DNA for binding. The natural substrate for the enzyme is a pyr<>pyr duplex. A minimum length of DNA is required and the substrate must be at least a 5-mer of the form NpT<>TpNpNp.[15] Most of the binding free energy comes from the DNA backbone where the enzyme makes close contact with the pentose part of the backbone. The base composition of the dimer is also important with the T<>T composition giving the highest affinity for binding. The

secondary structure of the DNA strand also plays a role in binding. Photolyase can bind to either UV-irradiated single- or double-stranded DNA with similar affinity.[16] The tertiary structure has no effect on activity and photolyase can repair relaxed and supercoiled DNA with equal efficiency. The T<c,s>T is the natural substrate for the enzyme.

The proposed repair mechanism of pyrimidine dimers formed in DNA is shown in Figure 1.5. Photolyase binds to the dimer formed in the DNA strand independent of light. The folate chromophore absorbs a 350-450nm photon and transfers the excitation energy to the flavin chromophore. The photoexcited flavin then transfers an electron to pyr<>pyr and the 5-5 and 6-6 bonds of the cyclobutane ring are broken leaving a pyr and a $pyr^{\cdot}$. The $pyr^{\cdot}$ donates an electron back to the one-electron oxidized flavin chromophore to regenerate $FADH^{\cdot}$ along with two neutral pyrimidine molecules. The enzyme then dissociates from the DNA fully intact and able to repair another dimer.[17, 18, 19]

In addition to the light-induced electron transfer process that underlies dimer repair, a second photoinduced electron transfer can be observed. When DNA photolyase is isolated, one electron oxidation of the fully reduced active form of the enzyme may occur. This semiquinone form is dark stable and catalytically inactive. Studies have shown that in vitro photoreactivation proceeds by photoreduction of the flavin radical before dimer repair can take place.[20, 21] Excitation of the flavin radical results in intramolecular electron or hydrogen atom transfer from an amino acid residue to the radical. Heelis, et al. studied the exited-state properties of photolyase in the semiquinone form to investigate the identity of this amino acid.[22] They acquired a flash-induced

Figure 1.5. Proposed mechanism for dimer repair.



Heelis, P.F., Hartman, R.F., and Rose, S.D., *Chemical Society Reviews* **1995**, 24, 295.

Figure 1.5

difference spectrum of photolyase and compared it to spectra of the only three amino acids with absorptions above 350nm: tryptophan, tyrosine, and histidine. Only the addition of the tryptophan spectrum to the enzyme spectrum coincided with the spectrum observed following laser excitation of the enzyme. These results suggested that tryptophan is the amino acid donor in the photolyase enzyme system.

Li et al. carried these results one step further to determine the specific tryptophan in the enzyme that was responsible for donating the electron or hydrogen atom.[23] This study involved the use of site-directed mutagenesis to replace all fifteen tryptophan residues individually by phenylalanine. Using flash photolysis they found the trp-306 mutation caused photoreduction of $FADH^{\cdot}$ to cease without effecting the excited state properties of $FADH^{\cdot}$ or the substrate binding. This led to the conclusion that trp-306 was the photoreductant. Kim et al. confirmed this assignment by the use of time-resolved EPR studies.[24] They obtained a transient EPR spectrum of Enz-$FADH^{\cdot}$ induced by a flash of light. The spectrum showed emissive and absorptive patterns, typical of spin-polarized states, consisting of three components split by about 15G. Superimposed on each of the three components is partially resolved fine structure with splittings of about 5G. Photolyase grown in poor medium containing isotopically labeled tryptophan was used to test the identity of the amino acid donor. Upon deuteration the fine structure collapses, but the three major components are still observable, suggesting that the radical did arise from a tryptophan molecule. To identify the particular tryptophan in photolyase that photoreduces the flavin, site directed mutagenesis was performed, as above, with the exception that EPR was used for analysis. The transient spectrum disappeared when trp-

306 was mutated, while for all other tryptophans no change in the transient could be observed. From the results of these studies, the conclusion was made that the donor was indeed a tryptophan molecule and further that it was trp-306.

These experiments led to the proposal that trp-306 is the amino acid donor in the reactivation process that reduces the flavin semiquinone to its active fully reduced form. In this mechanism, the trp-306 can either donate an electron or a hydrogen atom to the photoexcited flavin, thereby producing the active form of the enzyme. These two possibilities raise the issue of the ionization state of the reduced flavin cofactor in the active enzyme and a number of studies have been carried out to determine whether the neutral or anionic flavin hydroquinone is physiologically the more important. Hartman and Rose studied reduced flavin model compounds and concluded that the anionic, single protonated form is more active in dimer repair than the doubly protonated form.[25] Photolysis of $1.2x10^{-5}$M flavin and 3.4mM N(1), N(3)-dimethyluracil *cis-syn*-cyclobutane dimer in solutions ranging from pH 5.5 to 9.1 was carried out. A titration curve of the splitting efficiency, centered at pH 7.7, clearly showed the flavin anion was approximately eight times more active in dimer repair than the neutral form. In another study, Ghisla et al. showed that the absorption spectra of reduced flavins are influenced by the degree of planarity of the flavin molecule.[26] Neutral reduced flavins posses maximum planarity with a typical λ_{max}~390nm. Ionization at N1 allows flexing along the N5-N10 axis which results in a blue shift to 350±10nm. The reduced flavin in the active form of photolyase has λ_{max}=366nm[27] which may correspond to this flexing upon ionization.

Isotopic labeling is a third route used in identifying whether the donated species is an electron or a hydrogen atom. Isotopic labeling studies have been conducted on the tryptophan responsible for the donation of the electron or hydrogen atom. The neutral and cationic forms of the tryptophan radical differ in unpaired electron spin densities. The tryptophan cation radical has high spin density at the C(2) α-proton and at the C(3) β-methylene carbon position while the neutral tryptophan radical has significant spin density at the N(1) nitrogen and C(3) β-methylene carbon position.[28] Therefore the two forms can be discriminated by EPR spectroscopy when the tryptophan has been isotopically labeled at these positions. A sample was specifically labeled at the α-ring proton positions causing the smaller hyperfine couplings (~5G) to disappear. These results suggest that the transient EPR spectrum arises from a cation rather than a neutral tryptophan radical. Although these labeling studies were conducted on the FAD_{ox}-trp radical pair, the results may be carried over to the $FADH^{-}$ -trp system because the tryptophan radical, not the flavin, of the radical pair is believed to give the transient EPR signal.[29] The observation that the active reduced flavin is present in its anionic form in photolyase coincides with the conclusions that most photoexcited flavins reacting with a tryptophan molecule are primarily electron transfer types of reactions.[30]

Now that an electron rather than a hydrogen atom transfer mechanism has been proposed for photo-repair a question arises as to the direction of the electron transfer, from reduced flavin to dimer or the reverse? When $FADH^{-}$ absorbs a photon, an electron is promoted from the HOMO (π-orbital) to the LUMO (π^{*} orbital). Therefore $^{1}FADH^{-}$ becomes a good electron donor and an electron can be transferred from the π* orbital of

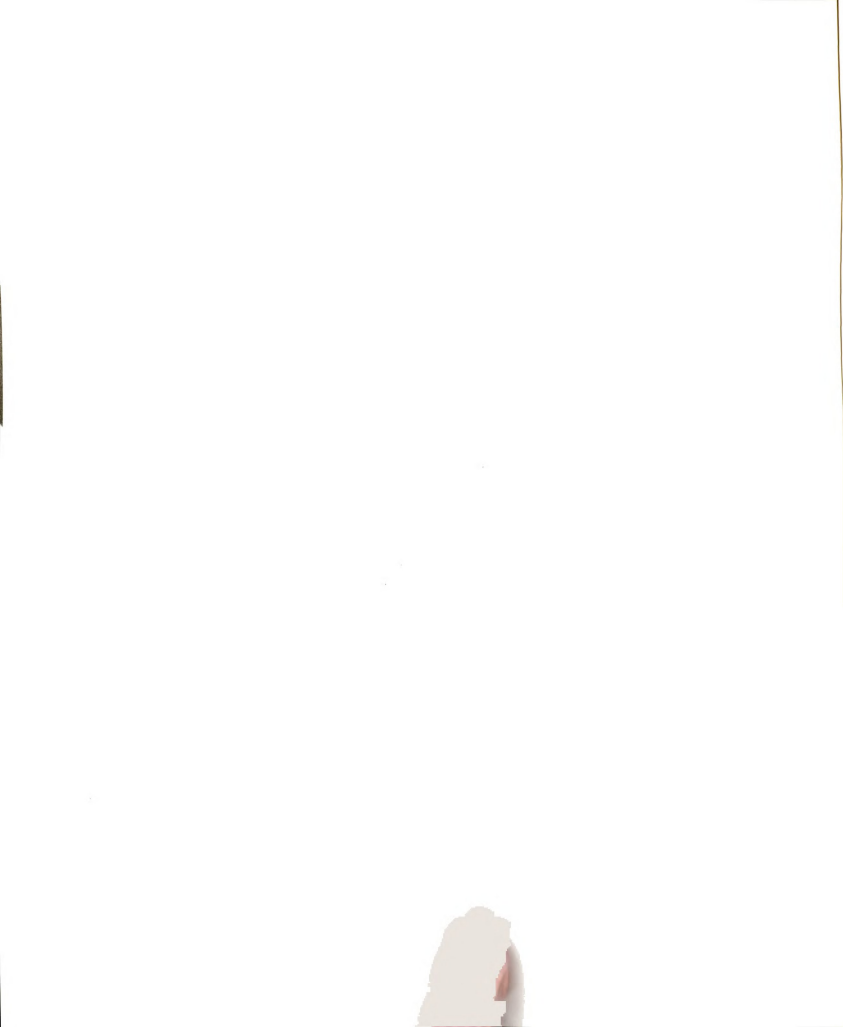
$^1FADH^-$ to the LUMO of the dimer. The reverse case, electron transfer from dimer to $FADH^-$, would be unlikely because reduced flavins are much better donors than acceptors.[31]

Another way to determine the direction of electron transfer is to calculate the free energy change for both reactions. The equation used is as follows:

$$\Delta G(kJ\ mol^{-1}) = 96.5\ [E^\circ\ (D^{\cdot+}\ D) - E^\circ\ (A\ A^{\cdot-}) - 0.026] - E_{o,o}$$

where $E^\circ\ (D^{\cdot+}/D)$ and $E^\circ(A/A^{\cdot-})$ represent the reduction potentials of the donor and acceptor, and $E_{o,o}$ is the energy of the excited state of the flavin chromophore. The free energy change for electron transfer from flavin to dimer is calculated to be $\Delta G = -125$ kJ mol^{-1} and from dimer to flavin is $\Delta G = +180$ kJ mol^{-1}. Therefore electron transfer from flavin to dimer is energetically more favorable[32].

The purpose of the project described in this thesis is investigation of the spin polarized EPR signal of photolyase and the mechanism of electron transfer in dimer repair. A new isolation procedure facilitated this research by providing a more stable sample with which to work. This improved procedure has cut the time needed for isolation of the enzyme by over two-thirds. In addition, a new buffer system has been employed to improve the stability of the enzyme. Due to shorter preparation time and increased stabilization, one can consistently expect to get a purified product in the one electron reduced semiquinone form. Consistency of the end product and optical spectroscopy allows for accurate characterization of the samples being used for experimentation.



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The identification of the spin polarized species giving the transient signal has also been investigated by using deuterium labeled samples and EPR spectroscopy. The spin polarized EPR signal observed may originate from either or both of the species making up the spin polarized pair. Using labeled tryptophan species, we have observed that only the trp radical contributes to the transient EPR spectrum as shown in chapter 4. These results can contribute to the better understanding of this photobiochemical reaction that perserves the integrity of the DNA strand. The dimer repair process performed by photolyase is becoming especially crucial as the result of the increased incidence of UV light reaching the earth's surface over the past several years.

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Chapter 2: Isolation and Characterization of Photolyase

Exposure of DNA to ultraviolet radiation causes cyclobutane pyrimidine dimers to form on the DNA strand. If left unrepaired, pyrimidine dimers can cause mutation, cancer, and death.[1] Photoreactivation is mediated by DNA photolyase which binds to DNA-containing pyrimidine dimers independent of light and splits the dimers upon exposure to near-UV light.[2] To be able to study effectively the mechanism of action of photolyase, large quantities of the enzyme are needed. Since very low concentrations of photolyase are found in cells (10 to 20 molecules per cell in E. coli) the study of this enzyme was difficult until 1978 when the photolyase gene was cloned[3] and a photolyase over-producing strain of E. coli was constructed in 1983.[4] Sancar *et al.* were successful in constructing a photolyase over-producing plasmid by joining a *tac* promoter to the photolyase apoenzyme gene, *phr*. In this strain, photolyase constituted 15% of total cellular proteins and the *tac-phr* plasmid amplified E. coli DNA photolyase approximately 15,000-fold in the cell. By using this strain carrying the over-producing plasmid, several milligrams of enzyme could be purified per gram of cells.

The buffer conditions (50mM Tris-HCl at pH 7.4) used in the lab of A. Sancar in the purification procedure left the enzyme highly susceptible to air oxidation and denaturation. The isolation procedure involved sonication, centrifugation, three dialysis steps, an affinity column purification, gel filtration, and an hydroxyapatite column, all of which lengthened the isolation procedure to several days.[5] Owing to the time required for this procedure, the enzyme had a greater chance of reacting with oxygen, causing the oxidation state of the final product to be a mixture of semiquinone and oxidized states.

Since knowing the oxidation state of the enzyme is crucial to studying the mechanism of repair, this isolation procedure was unsatisfactory and improvement was necessary.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Escherichia coli CSR603 was obtained from the *E. coli* Genetic Stock Center, Yale University. MS09 is identical to CSR603 with the exception that it is carrying the *tac-phr* plasmid pMS969, the DNA photolyase strain. MS09 was obtained from A. Sancar at the North Carolina School of Medicine, Chapel Hill.

Chemicals

Isopropyl-β-D-thiogalactopyranoside (IPTG) used to induce the cells was obtained from Boehringer Mannheim GmbH. The RNase and DNase used to cleave the RNA and DNA strands were obtained from Sigma. The column materials used in the purification steps of the isolation were Blue Sepharose CL-6B and Heparin Sepharose CL6B obtained from Pharmacia LKB Biotechnology. The desalting columns packed with P-6DG polyacrylamide gel (exclusion size of 6000 D) were obtained from Bio-Rad. The SDS-PAGE molecular weight standard was from Bio-Rad.

Buffers

The lysis buffer was made up of 50 mM Hepes, pH 7.0 with 100 mM NaCl, 10%(w/w) sucrose, and 10 mM 2-mercaptoethanol. Buffer A consisted of 50 mM Hepes, pH 7.0, 10%(v/v) glycerol, and 10 mM 2-mercaptoethanol. Buffer B and Buffer C were identical

to Buffer A with the addition of 0.1 M KCl to Buffer B and the addition of 2.0 M KCl to Buffer C.

Equipment

The French press used to break the cell walls in the initial step was obtained from SLM Aminco, Urbana, IL. The optical spectra were obtained by using an AVIV Spectrophotometer, Model 14DS UV-VIS, Lakewood, NJ. The centrifugations were accomplished with the use of a Dupont Sorvall RC-5B Refrigerated Superspeed Centrifuge, Hoffman Estates, IL. The SDS-polyacrylamide gels were produced by utilizing a Bio-Rad Mini-PROTEAN II Electrophoresis Cell, Hercules, CA.

Assay of DNA Photolyase

The activity assay was performed as described in Jorns *et al.*[6] by H. Sackett in the Chemistry Department at Michigan State University. The substrate was poly dT_{10} DNA obtained from the Biochemistry Department at Michigan State University and the host was a concentrated stock solution of photolyase enzyme. The DNA was damaged and repaired by using a UVP model UVGL-58 (VWR Scientific) combination longwave/shortwave UV lamp. Photolyase was then added in the inactive semiquinone form and was activated to the fully reduced form by being exposed to 366nm light.

RESULTS

Cell Growth

The cells were grown in Luria broth, which consists of 25g of Luria Broth powder (10 grams of tryptone, 10 grams of NaCl, and 5 grams of Yeast extract) per one liter of water.

The flasks were shaken at a speed of 250 rpm at 38°C. After the cells reached an optical density of A_{600} = 0.65 to 0.8 (after about 6 to 8 hours), 2mM isopropylthio-b-D-galactoside was added to induce the cells thereby causing the repressor on the DNA strand to dissociate. This allowed the *lac* operon genes to be expressed and photolyase concentration to be increased in the cell. The cells then continued to grow to reach log phase (16 to 18 hours). The cells were harvested by centrifugation for 15 minutes at 4500rpm in a GSA rotor at 4°C. The cells were resuspended on ice in lysis buffer with approximately 3ml of buffer per liter of culture. The suspension was homogenized and stored at -80°C in a Sarstedt tube. Each liter of culture yielded about 3.5 grams of cells (wet weight).

Isolation

The Sarstedt tube containing approximately 12 ml of suspended cells was thawed in room temperature water, which took approximately 15 minutes. The cells were broken by passing them through a French pressure cell two times at 20,000 psi and 4°C. To remove any unbroken cells and cell fragments the lysed cells were centrifuged for 30 minutes at 19,000 rpm in an SS-34 rotor at 4°C. The clear brown supernatant containing the photolyase was decanted from the pellet and its volume measured.

Three crystals each of DNase and RNase were added to the supernatant to cleave the unwanted DNA and RNA strands further. This incubation was carried out for half an hour with gentle stirring at 4°C. To precipitate the enzyme, ammonium sulfate (0.43g per ml of solution) was slowly added with stirring. After all of the ammonium sulfate had dissolved, the suspension was centrifuged for 20 minutes at 19,000 rpm in an SS-34 rotor

at 4°C. The pellet was resuspended in approximately 30ml of buffer A. Desalting columns obtained from Bio-Rad were used to remove the ammonium sulfate from the protein solution. The desalting columns were equilibrated with buffer A and then 3ml of protein solution was added to each of ten desalting columns. To wash the protein from the columns, 4ml of buffer A was added to each column and the brown eluent was collected.

A blue sepharose column (3 cm by 10 cm) was equilibrated with buffer A and the eluent, diluted three-fold with buffer A, was loaded on to the column. The column was rinsed with buffer A until two or three clear tubes had been collected. The enzyme was then eluted with buffer C and the tubes with a blue color were combined. The protein was then loaded onto desalting columns to remove the KCl in the eluting buffer. The salt free solution was loaded onto a heparin column (2 cm by 10 cm) equilibrated with buffer A. The column was rinsed with three column volumes of buffer A and the protein eluted with a linear gradient composed of 100 ml of buffer B and 100 ml of buffer C. The blue fractions were combined and concentrated with the Amicon Ultrafiltration device with a PM-10 membrane. Glycerol was added to give a final concentration of 30%(v/v) and the protein was divided into 1ml aliquots and stored at -80^{0}C in Eppendorf tubes. A flow chart of this isolation procedure is shown in Figure 2.1. Photolyase is isolated with greater than 95% purity as can be seen from the SDS-PAGE shown in Figure 2.2. An absorbtion spectrum of the isolated enzyme can be seen in Figure 2.3 where absorbance at 580nm can be used to determine the final concentration of photolyase isolated (ε_{580}=4800$M^{-1}cm^{-1}$).

Figure 2.1. Flow chart of procedure for isolating photolyase.

Isolation Procedure

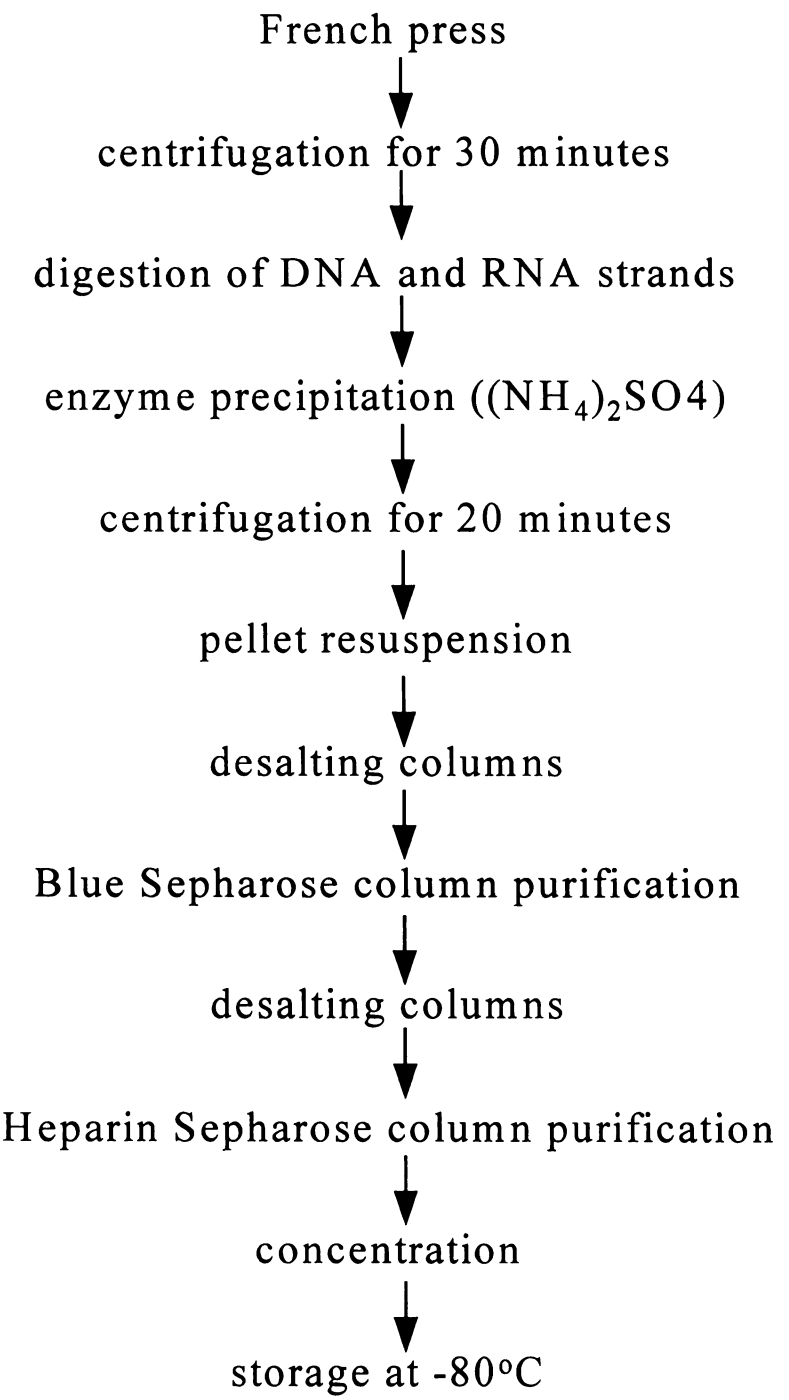

Figure 2.1

Figure 2.2. A Coomassie Blue-stained SDS-PAGE gel of photolyase. *Lanes 1* and *8*, molecular mass standards (from top to bottom): ovalbumin (45 kDa), trypsin anhydrase (21.5 kDa), aprotinin (6.5 kDa); *lane 2*, unbroken *E. Coli* cells; *lane 3*, after French press; *lane 4*, after DNase and RNase incubation; *lane 5*, after salt precipitation; *lane 6*, after blue Sepharose column; *lane 7*, after white Sepharose column. Each lane contained 15μL of protein.

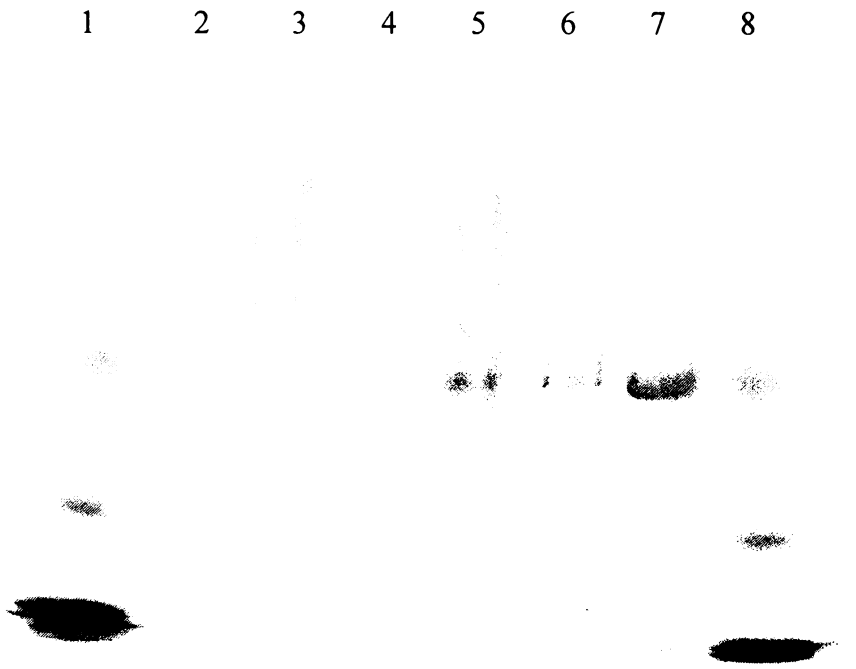

Figure 2.2

Figure 2.3. Absorption spectrum of photolyase in its semiquinone form.

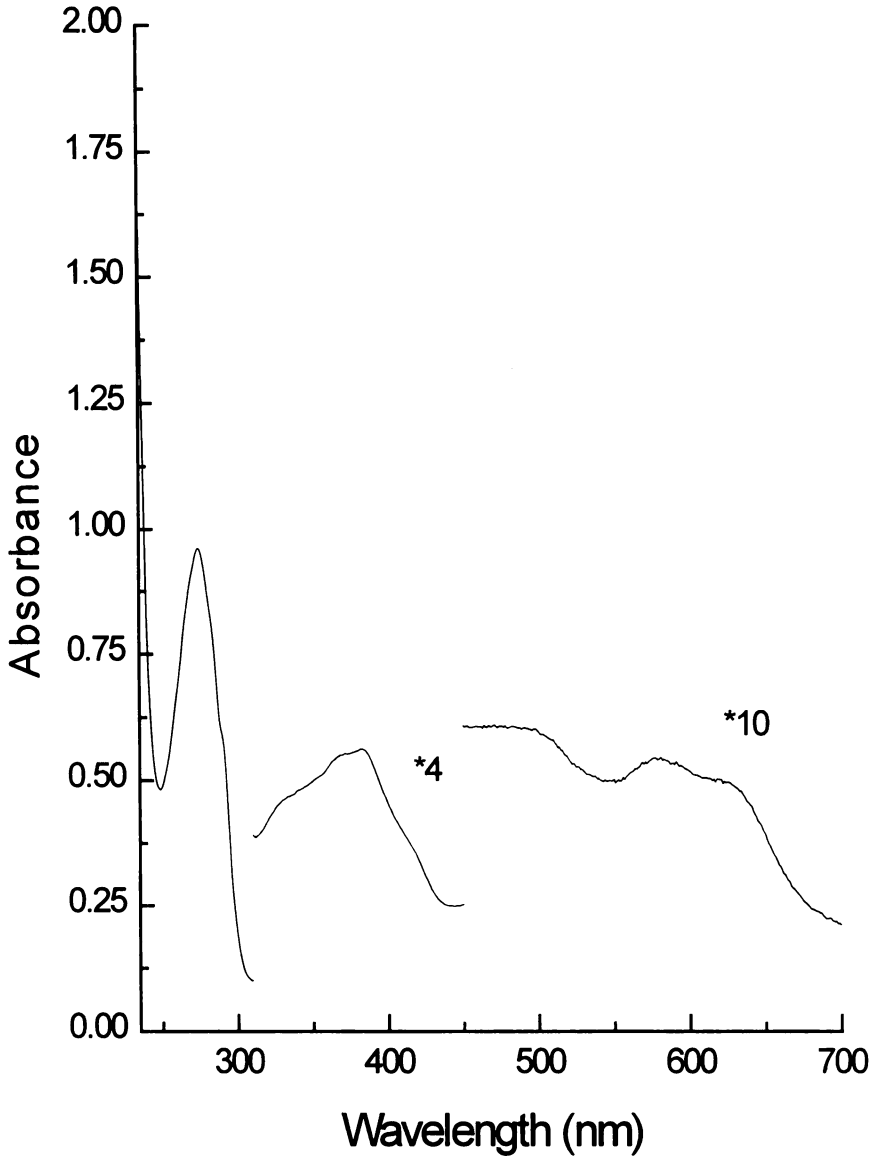
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Absorbance
*4
*10
300
400
500
600
700
Wavelength (nm)

Figure 2.3

Activity of the Purified Enzyme

The isolation procedure described in this chapter was made significantly more time efficient by reducing the number of steps involved in the isolation relative to the number of steps in the Sancar procedure. The buffer conditions were also altered in order to make the enzyme more stable and therefore less susceptible to air oxidation. An activity assay was conducted to ensure that the new buffer conditions did not impair the activity of the enzyme. A stock solution of poly(dT) ($2x10^{-5}$M) in 50mM Hepes, pH 7.0, 100mM NaCl, 10mM 2-mercaptoethanol, and 10%(w/w) glycerol was irradiated in a stoppered quartz semimicro cuvette (500 μL/cuvette). The cuvette was laid horizontally and irradiated at a distance of 4cm from above by a short (λ=254nm) UV lamp for one minute intervals at 4°C. The extent of dimer formation was monitored by the decrease in absorbance at 260 and 290nm after each minute. When there was no longer any change in absorbance, illumination was stopped.

The DNA stock solution was diluted to approximately $4.2x10^{-5}$M in thymine concentration. The enzyme ($1x10^{-7}$M) was added and the mixture was placed in a stoppered semimicro cuvette with a final volume of 1ml. The cuvette was illuminated in a horizontal position with the long (λ=366nm) wavelength lamp (covered with a watchglass to filter out radiation below 300nm) at 25°C. After each interval the absorption at 260, 290, and 400nm was noted until the absorption readings remained unchanged. The procedure was repeated with 50mM Tris-HCl at pH 7.2. As Figure 2.4 demonstrates, the activity of the enzyme was comparable in HEPES and Tris buffers.

Figure 2.4. Activity assay of photolyase in HEPES buffer and in Tris buffer at 25°C.

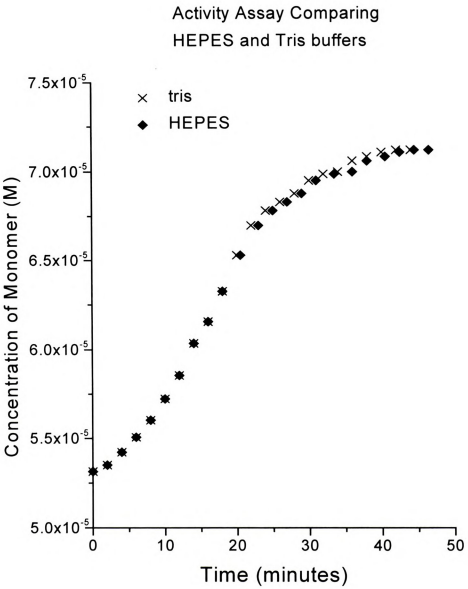
Activity Assay Comparing
HEPES and Tris buffers
tris
HEPES
Concentration of Monomer (M)
7.5x10-5
7.0x10-5
6.5x10-5
6.0x10-5
5.5x10-5
5.0x10-5
0
10
20
30
40
50
Time (minutes)

Figure 2.4

DISCUSSION

By using the isolation procedure described here, photolyase enzyme could be obtained with comparable purity to the procedure developed in the lab of A. Sancar. In addition to obtaining a product that was 95% pure, this procedure was more time efficient thereby allowing for a more accurate characterization of the enzyme and also giving a greater product yield. The isolation procedure was made more time efficient by utilizing techniques that accomplished the same task as in previous protocols, but in a shorter time period. The number of steps required for purification of the enzyme was also reduced, which decreased product loss and increased the yield by 92%. The breaking of the cells was accomplished by utilizing a French press in place of sonication, which allowed a greater percentage of cells to be broken effectively. Desalting columns were substituted for dialysis, which resulted in less time needed to remove the salt from the eluting buffer. Two affinity chromatography columns, which bound the photolyase while allowing the impurities to pass through the column, were used in the final purification steps of the new procedure. Photolyase was then eluted with a salt gradient. Because this procedure was much quicker and was able to be performed at 4°C, the enzyme was much more stable in that it was not as easily oxidized, which allowed us to isolate the protein in its blue semiquinone form. The catalytic and spectroscopic properties of the enzyme isolated by the procedure described here are comparable to literature values.

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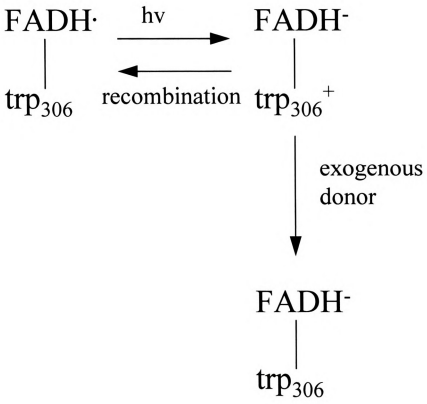
Chapter 3: Identification of the transient, spin polarized EPR signal of photolyase

When DNA photolyase is isolated *in vitro,* one electron oxidation of the fully reduced active form to the inactive semiquinone form occurs. It has been proposed that the flavin is reduced to its active form, $FADH^{\cdot}$, by trp-306 that is located more than 13 Å away from the flavin.[1] The oxidized tryptophan is then reduced by an exogenous reducing agent. When there is no external reducing agent present, the flavin transfers the electron back to the tryptophan within 10ms.[2] A proposed mechanism for activation of photolyase is shown in Figure 3.1.[3] The fully reduced active form of the enzyme catalyzes the repair of cyclobutadipyrimidines, which are created by exposure to ultraviolet radiation (200 to 300nm), by using a light-induced electron transfer mechanism.[4,5]

A mechanism of dimer cleavage by photolyase has been suggested from the crystal structure solved by Deisenhofer *et al.*[6] From this crystal structure, it was proposed that the pyrimidine dimer flips out of the DNA helix into a hole that leads to the binding site found in the flat surface of photolyase. Once the dimer is bound to the enzyme, the absorption of a photon initiates the repair mechanism. The folate molecule absorbs an incident photon and subsequently the energy is transferred to the fully reduced flavin molecule. The flavin, now in its excited state, donates an electron to the pyrimidine dimer to generate a pyrimidine radical anion and $FADH^{\cdot}$. The radical anion then splits into monomers while transferring an electron back to the flavin.

Understanding the electron transfer processes described above is essential to studying the mechanism of DNA repair. In order to study effectively these electron

Figure 3.1. Activation of photolyase by electron transfer from trp-306.



Heelis, P.F., Okamura, T. and Sancar, A. *Biochemistry* **1990**, 29, 5694.

Figure 3.1

transfer processes, the oxidation states of the flavin must be known and controlled. Each oxidation state has distinct absorption spectra as discussed in Chapter 1 and therefore can be used to identify the oxidation state of the sample. In this study, the identity of the species responsible for an observed transient EPR signal is investigated. This species was previously reported as originating from the semiquinone form of the enzyme, but the better understanding and control of the enzyme that we have obtained through the biochemical approaches described in Chapter 2 has led to a different conclusion that is discussed below.

Materials and Methods

Isolation of photolyase was performed as described in Chapter 2. Experiments were carried out with protein samples that contained 50mM HEPES pH 7.0, 0.10M NaCl, 50% glycerol, 700μL/L BME and 3mM ferricyanide. The isolated enzyme in its semiquinone form is relatively resistant to air oxidation and therefore requires the addition of ferricyanide to oxidize the flavin cofactor. Prior to each experiment an absorption spectrum was taken of the enzyme to verify the oxidation state by using an Aviv 14DS UV-VIS Spectrophotometer (Lakewood, NJ). EPR measurements at 0°C were performed with a TM011 cavity in a Varian E-4 that was interfaced to a personal computer. The sample was held in the cavity by a TM quartz flat cell (Wilmad, Buena, NJ). The frequency of the cavity was monitored with an EIP Microwave, Inc. (San Jose, CA) frequency counter.

Photoexcitation of the sample was accomplished by using a Xenon flashlamp with a 17μs pulse and a 50J lamp energy or a Quanta-Ray DCR-11 pulsed Nd-YAG laser (Spectra-Physics, Mountain View, CA) equipped to provide 532 or 355nm light pulses with a width of 10ns. Steady state EPR spectra were collected with a Metrabyte WAAG data acquisition board to digitize the analog signal from the spectrometer. Kinetic traces were collected by the use of computer software with data acquisition and timing controlled by the Metrabyte WAAG board and a Metrabyte CTM-05 timing board.[7] The EPR instrument had a 100KHz modulated detection system that provided an instrument risetime of about 30μs . Transient EPR spectra were obtained by using an internally triggered Stanford Research Systems SR 250 gated integrator and boxcar averager (Palo Alto, CA).

Results

Figure 3.2 shows a steady state EPR spectrum of photolyase in its semiquinone inactive form. The spectrum shows no well-resolved hyperfine couplings and a peak to peak width of ~20G. It has an apparent g-value of 2.0039, which is consistent with reports of flavin radicals that occur in proteins (2.0030-2.0040).[8] Flash photolysis with the Xenon flashlamp was conducted on the sample and produced an EPR transient spectrum as shown in Figure 3.3. The transient spectrum was collected for 48μs following a delay of 4μs after the lamp was triggered. The spectrum shows three major peaks in a 1:2:1 intensity ratio each split by 15G. Superimposed on these 15G splittings are smaller ones with 5G splitting. The kinetic trace that corresponds to the transient spectrum is shown in Figure 3.4. The rise and fall of the light-induced radical occurs

Figure 3.2. Steady state spectrum of photolyase in its semiquinone form at a concentration of 62.5μM. Experimental conditions were: 2.0 mW microwave power, 9.22606 GHz microwave frequency, 30 μs time constant, 3290 G center field, 100 G sweep width, 2.5 G modulation amplitude at 25°C.

Figure 3.2. Steady state spectrum of photolyase in its semiquinone form at a concentration of 62.5μM. Experimental conditions were: 2.0 mW microwave power, 9.22606 GHz microwave frequency, 30 μs time constant, 3290 G center field, 100 G sweep width, 2.5 G modulation amplitude at 25°C.


80
60
40
20
0
-20
-40
-60
-80
dX/dH
3240
3260
3280
3300
3320
3340
Field (G)

Figure 3.2

Figure 3.3. Transient EPR spectrum of photolyase in its oxidized form at a concentration of 73.5 μM. Experimental conditions were as follows: 6.3 mW microwave power, 9.39908 GHz microwave frequency, 2.5 G modulation amplitude, 30 μs time constant, and 100 G sweep. Boxcar conditions were: 40 μs delay, 435 μs window, 15 one hour scans, and a flash interval of 600 ms at a temperature of 4°C.

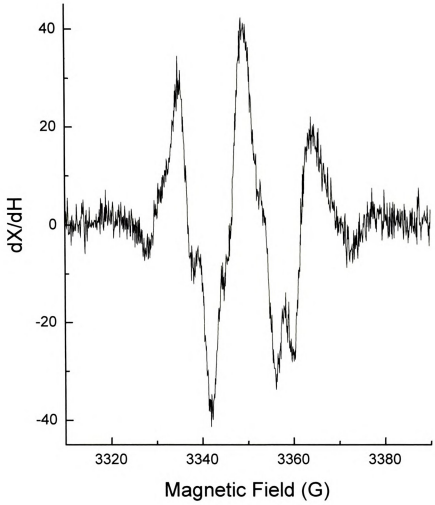


Figure 3.3

Figure 3.4. Kinetic trace of the transient radical with photolyase in its oxidized state at a concentration of 84.8μM and 200 flashes averaged. Experimental conditions were: 6.3 mW microwave power, 9.411315 GHz microwave frequency, 3350 G microwave field, 30 μs time constant and 2.5 G modulation amplitude at a temperature of 25°C.

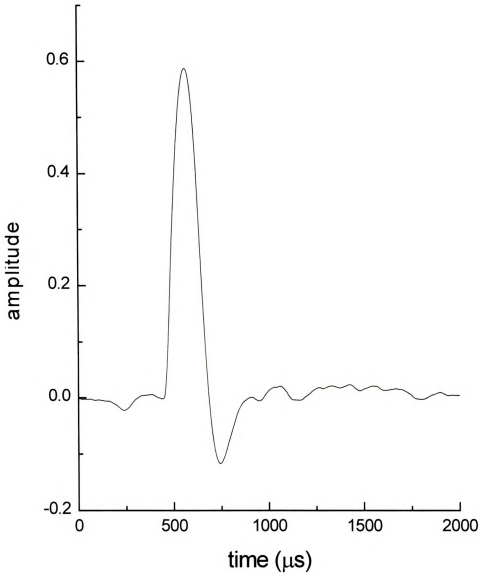


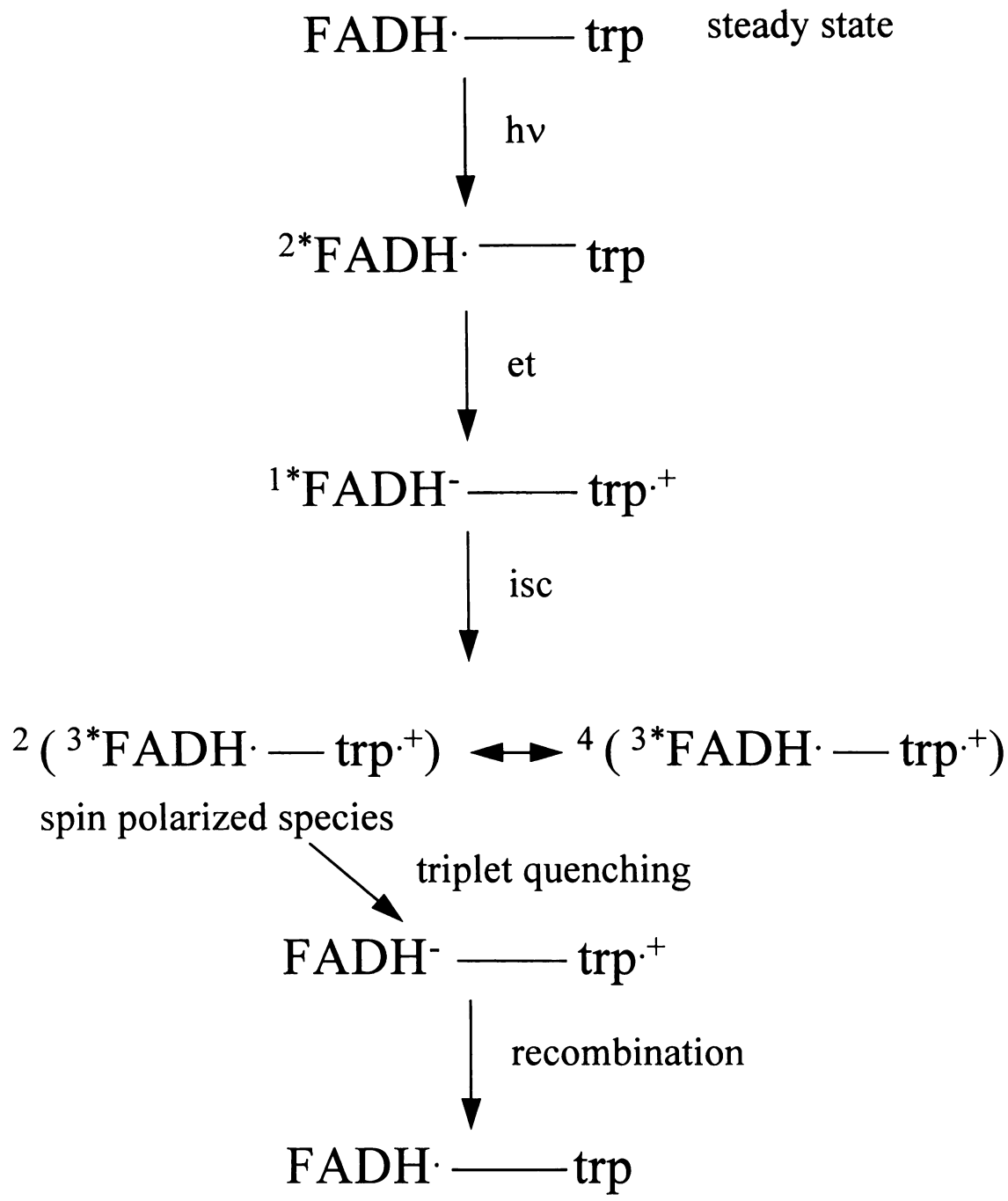
Figure 3.4

with a half time of approximately 35μs. This decay time, however, does not accurately represent the lifetime of the radical due to limited instrument response time.

Previous reports stated that the steady state and transient signals arose from the semiquinone form of the enzyme.[9,10] Figure 3.5 shows the mechanism for photoreduction with the spin polarized species proposed to give the transient spectrum being $^{3*}FADH^{\cdot}$ --- $trp^{+}\cdot$.[11] The enzyme samples used in those experiments were obtained from A. Sancar and were a mixture of semiquinone and oxidized protein in ratios that varied from sample to sample. Due to this mix of states, the assignment of the transient EPR signal to the semiquinone form alone is difficult because either state, oxidized or semiquinone, may contribute to this signal. The possible contribution from the oxidized form of the enzyme was not considered in these experiments and therefore an incorrect assignment was made. Evidence for this statement will be given in the following paragraphs.

By using samples obtained via the isolation procedure described in Chapter 2, a more accurate assignment of the species giving the EPR spectra could be made because there was no longer a mixture of oxidation states. The samples used in the following experiment were blue in color and made up of mainly the semiquinone form of the enzyme, as determined by its absorption spectrum (see Figure 3.6). The semiquinone form of photolyase has absorption maxima at 480, 580, and 625nm. The concentration of the sample was determined by its absorbance at 580nm (ε=5000$M^{-1}cm^{-1}$) and found to be 13.5μM. By using this sample, an intense steady state EPR signal could be detected as shown in Figure 3.7. This signal was identical to the signal reported by Essenmacher *et*

Figure 3.5 Proposed reaction mechanism for photoreduction that includes the spin polarized species thought to give the transient EPR signal.



Essenmacher, C. PhD thesis **1995**, Michigan State University.
Figure 3.5

Figure 3.6. Absorption spectrum of photolyase in its semiquinone form. The concentration of the sample was 13.5μM. The spectrum was obtained by using an AVIV Spectrophotometer, Model 14DS UV-VIS at 25°C.

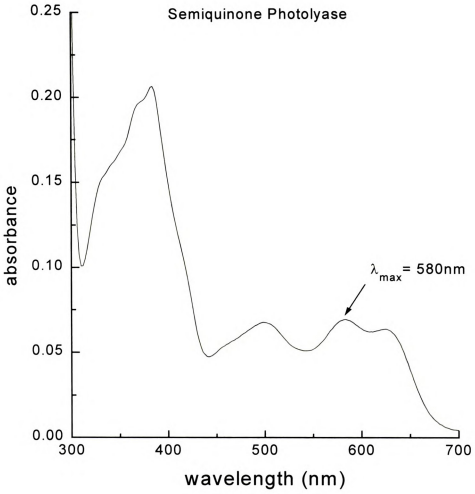

Semiquinone Photolyase
absorbance
wavelength (nm)
0.25
0.20
0.15
0.10
0.05
0.00
300
400
500
600
700
λmax = 580nm

Figure 3.6

al.[8,9] They also reported a transient signal and concluded that this signal originated from the semiquinone state of the enzyme. We used the same sample that we had used for the steady state experiment above and used similiar instrument parameters as Essenmacher, *et al.* to try to reproduce their results, but we were not able to detect such a transient signal with our sample.

A sample of yellow, oxidized protein was then used. The sample was oxidized via the addition of 3mM ferricyanide to enzyme in the semiquinone form, which was then air oxidized for twenty four hours. Before the addition of ferricyanide the ratio of semiquinone to quinone was 1.37 and after oxidation was 0.157, indicating that most of the sample was now in its oxidized state, as can be seen by its absorption spectrum shown in Figure 3.7. The oxidized form of photolyase has an absorption maximum at 450nm from which its concentration can be determined (ε=11,300 $M^{-1}cm^{-1}$). Upon exposure to flashes of light, the kinetic trace could be generated with this oxidized protein sample. From this kinetic trace, a transient EPR signal was obtained identical to the transient signal reported by Essenmacher *et al.* [8,9], the only difference being that our sample was fully oxidized while their sample contained both semiquinone and oxidized photolyase. Considering the mixed oxidation state of the sample used in the previous study by Essenmacher *et al.*, a conclusion can be made as to why the results differ from the results presented here. The portion of sample in the semiquinone form gives the steady state spectrum while the oxidized portion gives the transient signal. The transient signal is only generated when light with wavelengths in the range of 400-450nm is used. No signal could be observed when wavelengths above 500nm were used. Wavelengths

below 500 nm were filtered out by using a 500nm high pass filter with the Xenon flashlamp. Since a transient signal is only observed when using wavelengths near the absorption maximum of the oxidized protein and not the absorption maximum of the semiquinone, the conclusion can be made that the transient signal arises from the oxidized form of the enzyme.

Another experiment was conducted to observe the relationship between the amplitude of the steady state and transient signals.[12] If both signals originate from the same species then their amplitudes should increase or decrease proportionally with the change in semiquinone concentration. To perform this experiment, ferricyanide was added to a 55μM solution of photolyase to a final concentration of 3mM. A steady state spectrum was taken and the amplitude of the signal noted. Then a kinetic trace was obtained immediately after obtaining the steady state spectrum by giving the sample 500 flashes at 3349G and its amplitude noted. Every fifteen minutes this procedure was repeated. As can be seen in Figure 3.8, the steady state signal decreases over time, indicating that the sample is being oxidized, as the kinetic trace amplitude increases. Therefore the two EPR signals, steady state and transient, cannot originate from the same species. From these results and the experiments outlined above, it can be concluded that the transient EPR spectrum originates from the oxidized, not the semiquinone form of photolyase. A mechanism can be proposed, shown in Figure 3.9, with the fully oxidized flavin as a member of the spin polarized pair. The oxidized flavin is excited and intersystem crosses to the triplet state. The flavin triplet then abstracts an electron from tryptophan. A correlated radical pair is formed consisting of a flavin doublet and a trp

Figure 3.7. Absorption spectrum of photolyase in its oxidized form. The concentration of the sample was 115μM. The spectrum was obtained by using an AVIV Spectrophotometer, Model 14DS UV-VIS at 25°C.

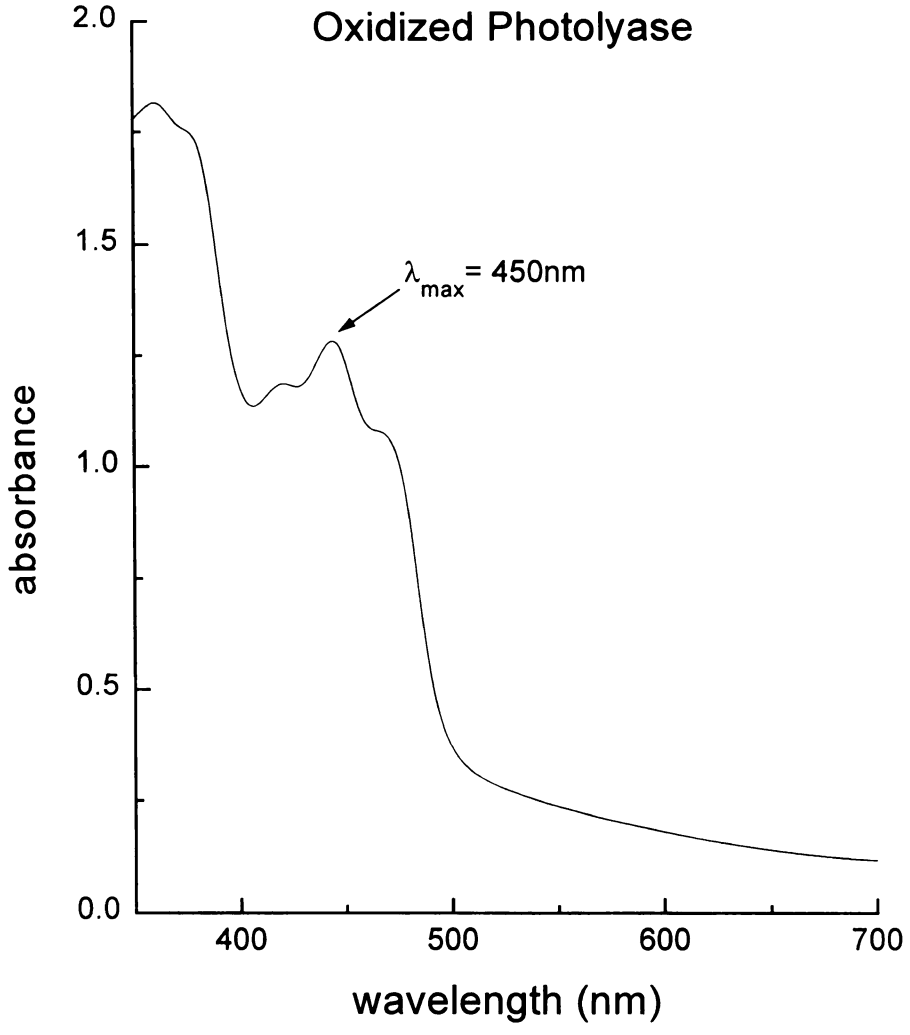

Oxidized Photolyase
2.0
1.5
1.0
0.5
0.0
absorbance
λmax = 450nm
400
500
600
700
wavelength (nm)

Figure 3.7

Figure 3.8. Amplitude of transient signal versus amplitude of steady state signal (see text for details).

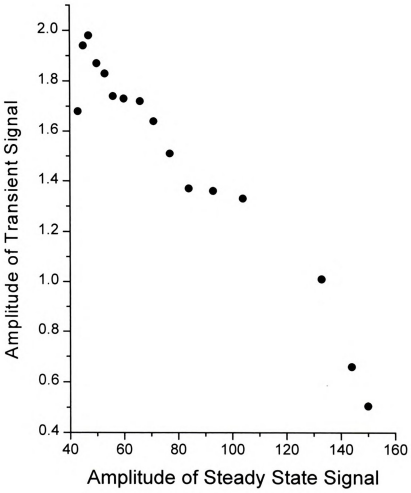

Amplitude of Transient Signal
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
40
60
80
100
120
140
160
Amplitude of Steady State Signal

Figure 3.8

Figure 3.9. Proposed reaction mechanism with the fully oxidized flavin as a member of the spin polarized pair.

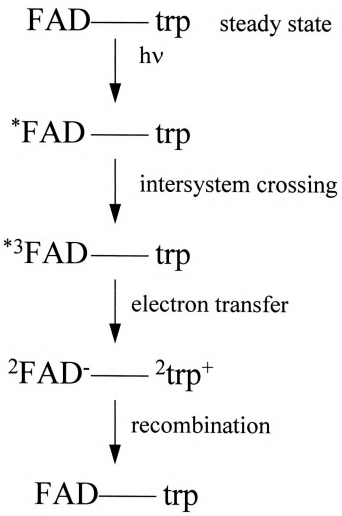

FAD—— trp
steady state
hν
*FAD —— trp
intersystem crossing
*3FAD —— trp
electron transfer
2FAD- —— 2trp+
recombination
FAD—— trp

Figure 3.9

doublet producing the transient spin polarized signal. Recombination can then occur to regenerate the original species.

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Chapter 4: Isotopic labeling studies performed on photolyase

When DNA photolyase is isolated, it is oxidized to the inactive $FADH^{\cdot}$ form of the enzyme, as discussed in Chapter 1. Reactivation to the fully reduced form occurs when photolyase is irradiated with visible light and an electron is donated from a nearby amino acid.[1] In Kim *et al.*[2], it was proposed that trp-306 is the immediate internal photoreductant of $FADH^{\cdot}$.

Using EPR spectrometry, a transient spectrum can be observed upon illumination of photolyase, as discussed in Chapter 3. The amplitude and sense of the kinetic signal observed in a time-resolved experiment at fixed field changes with applied magnetic field. The overall spectrum consists of three components in a 1:2:1 ratio in an emission/absorption line shape. Each of the three components is split by approximately 15G and superimposed upon them are smaller splittings of 5G. The emissive and absorptive transitions suggest that spin polarization occurs between the flavin chromophore and the tryptophan radical.[3] Preceding the spin polarization process, flavin, in its fully oxidized state, is photoexcited and then intersystem crosses to the triplet state. The flavin triplet then abstracts an electron from trp-306. This electron transfer produces a flavin doublet and a trp doublet that act as a correlated radical pair to produce the transient spin polarized signal.

To confirm that tryptophan is the transient amino acid radical involved in the photoreduction of the flavin molecule, the indole ring of tryptophan was deuterated.[2] When deuterium is substituted for hydrogen, the hyperfine couplings due to deuterium are one-sixth those of hydrogen.[4] Therefore, isotopic labeling can be used to determine

which species is responsible for the transient EPR spectrum that is seen with photolyase. Hyperfine couplings will not be resolved in the spectra in which an isotopically labeled sample is used because the couplings due to dueterium would be too small compared to the line width of the EPR transitions of photolyase. When the tryptophan residue was deuterated at positions 2, 4, 5, 6 and 7 (see figure 4.1), the hyperfine structure collapsed, but the three major components were still observable.[5] These results suggested that the smaller 5G couplings were due to coupling to α-protons on the tryptophan ring. The conclusion has been made that the hyperfine coupling specifically originates from the C(2) α-proton and the N(1) nitrogen on the indole ring of trp-306.[2]

The next question that arises is the origin of the major three line pattern of the transient EPR spectrum. Molecular orbital (MO) calculations by the Hückel-McLachlan technique predict a three line pattern arising from coupling to the C(3) β-methylene protons of a tryptophan cation radical.[6] To test this prediction and to rule out any contribution from the flavin radical, specifically labeled tryptophan at the β-methylene position can be used. If the three line pattern does indeed arise from coupling to these β-methylene protons, then the structure will collapse upon deuteration, as in the case of the 5G splittings. In theory, both radicals could contribute to the spin polarized signal, but evidence shows that only the tryptophan contributes to the signal observed.[4] This observation is troublesome because a contribution from the flavin doublet should also be seen in the spectrum. Experiments performed on enzyme samples that have deuterated tryptophan at the β-methylene positions would help to investigate this issue.

Figure 4.1. Numbering scheme for the tryptophan molecule.

The goal of the work described in this chapter has been to learn to grow photolyase on well-defined growth medium whereby the labeled tryptophan could be incorporated into the enzyme, and ultimately to perform EPR experiments on these samples. At present, photolyase has been successfully grown on the M-9 medium constitution (see materials and methods for composition) while maintaining its EPR characteristics, as can be shown by the work done with the control sample described below. Future work will involve growing photolyase on the M-9 medium containing labeled tryptophan at the β-methylene position. Using this labeled sample, EPR experiments can then be performed to test if the large 1:2:1 splittings originate from these β-methylene protons of the tryptophan residue.

Materials and Methods

Escherichia coli (strain PLK983/pMS969) was grown in M-9 medium containing labeled tryptophan. M-9 medium consists of 200ml M-9 salts (64g sodium phosphate, 15g potassium phosphate, 2.5g sodium chloride, and 5g ammonium chloride diluted to 1L), 50g/L casein acids (Sigma, product number 65072-006), 0.1M calcium chloride, 1M magnesium sulfate and 600mL Milli-Q water. This strain is unable to produce its own tryptophan, which allows for labeled tryptophan to be incorporated into the enzyme. M-9 medium is a minimal medium, which means that it contains the minimal amount of components needed for the cells to grow. In addition, the minimal medium differs from Luria broth in that it does not contain yeast extracts, which would be a source of unlabeled tryptophan. When the M-9 medium is used, the cell growth is retarded by a factor of three owing to the decreased concentration of nutrients.

A control sample was produced by growing cells in M-9 medium that contained unlabeled tryptophan. Isolation of the enzyme was performed as described in Chapter 2. Experiments were carried out with protein samples that contained 50mM HEPES pH 7.0, 0.10M NaCl, 50% glycerol and 700μL 2-mercaptoethanol. The enzyme was isolated in its fully oxidized form; therefore, no addition of ferricyanide was required. Prior to each experiment, an absorption spectrum of the enzyme was recorded to verify the oxidation state of the flavin by using an Aviv 14DS UV-VIS Spectrophotometer (Lakewood, NJ).

The deuterated sample was grown on M-9 medium containing labeled tryptophan at the β-methylene position. The photolyase strain used in this study is unable to produce its own tryptophan, so it relies upon the medium to supply the tryptophan that it needs to survive. Since the tryptophan in the medium is labeled at specific positions, the deuterated tryptophan is incorporated into the enzyme. Mass spectrometry can be utilized to confirm the incorporation of the labeled tryptophan. The labeled enzyme was isolated and prepared under the same conditions as the control enzyme described above.

EPR experiments were performed on a Bruker ESP 300E spectrometer by using a TM011 cavity at 0°C. The sample was held in the cavity by a TM quartz flat cell (Wilmad, Buena, NJ). The control and labeled samples were excited with a 17μs pulse from a xenon flashlamp that was operated at 50J lamp energy. Kinetic traces were collected by using computer software with data acquisition and timing controlled by a Metrabyte WAAG board and a Metrabyte CTM-05 timing board.[7] The EPR instrument had a 100kHz modulation detection system that provided an instrument risetime of approximately 30μs. An automation routine was written that would allow a transient

EPR spectrum to be obtained with the Bruker ESP 300E. This routine was designed so that the magnetic field could be fixed while signal averaging. The automation routine written and stored using ESP 300E internal software is as follows:

```
1  READP <filename>
2  EMF 3460.0
3  DEL
4  DACQ
5  WRITE <filename>_exp_0
6  TP inc 1
7  PG inc 1
8  EMF inc 20.0
9  DEL
10 DACQ
11 WRITE <filename>_exp_0
12 TP inc 1
13 PG inc 1
14 EMF inc -18.0
15 LOOP 3 20
```

Results

Experiments were performed on the control enzyme grown on minimal M-9 medium. This enzyme sample was used so that any changes in the EPR spectrum of the labeled species could be attributed to the effects of labeling and not of the growth medium. As can be seen in Figure 4.2, the absorption spectrum of the control enzyme is similar to the fully oxidized enzyme from cells grown on rich medium. The peak that is indicative of the fully oxidized enzyme is present, but is unexplainably red-shifted to 480nm. Peaks at 580nm and 624nm are also present, indicating that the sample is slightly contaminated with flavin in its semiquinone state. Even though the absorption peak of the oxidized flavin is slightly shifted, this absorption spectrum provides evidence that the control enzyme is not affected significantly by growth on the M-9 medium.

Figure 4.2. Absorption spectrum of the enzyme grown on M-9 medium with unlabeled tryptophan. The concentration of the sample was 133μM. The spectrum was obtained by using an AVIV Spectrophotometer, Model 14DS UV-VIS at 25°C.

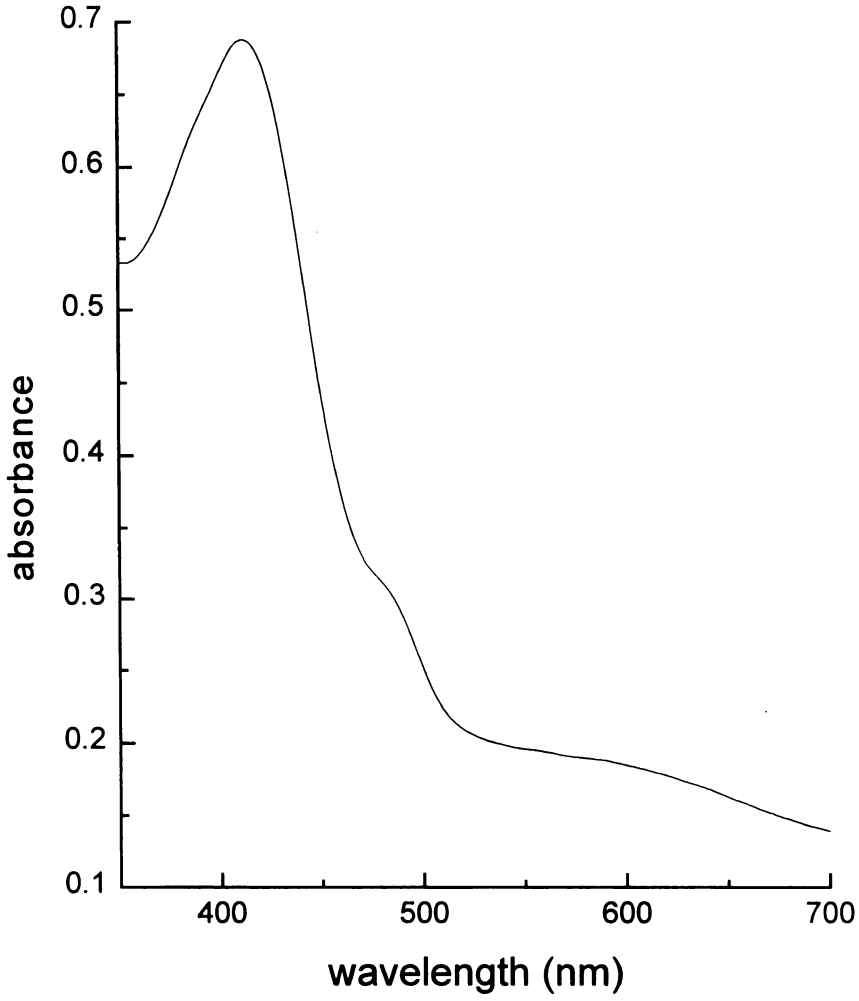

Figure 4.2

Figure 4.3. Kinetic trace of the transient radical obtained with the control enzyme sample in its oxidized state at a concentration of 0.133mM and 300 flashes averaged. Experimental conditions were: 6.3mW microwave power, 9.78 GHz microwave frequency, 3475 G microwave field, 30 μs time constant and 2.5 G modulation amplitude at a temperature of 0°C.

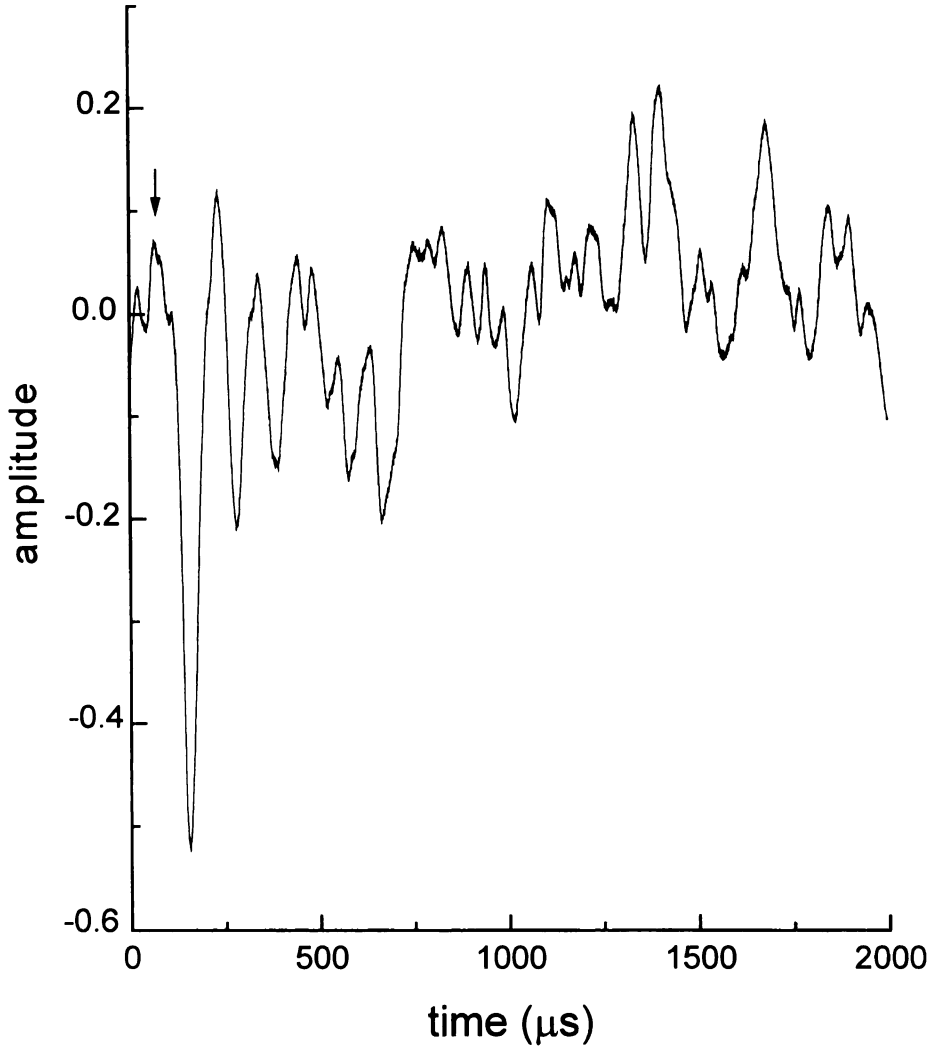

Figure 4.3

Figure 4.4. Kinetic trace of the transient radical obtained with the control enzyme sample in its oxidized state at a concentration of 0.133mM and 500 flashes averaged. Experimental conditions were: 6.3mW microwave power, 9.78 GHz microwave frequency, 3485 G microwave field, 30 μs time constant and 2.5 G modulation amplitude at a temperature of 0°C.

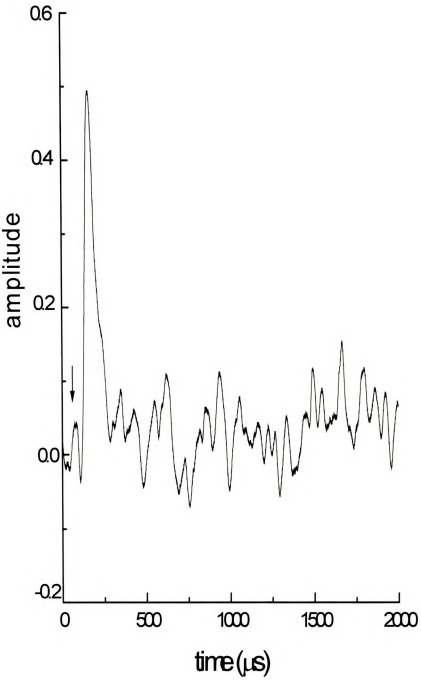

Figure 4.4

Nonetheless, the absorption spectrum provides an indication that the chromophore complement of enzyme from *E. coli* grown on minimal medium is not identical to wild-type enzyme grown on Luria broth.

EPR experiments were performed with this control enzyme sample. Figures 4.3 and 4.4 show kinetic traces of the control enzyme. The transient signal changes sign when the applied magnetic field setting is varied. These results are similar to the results obtained with the fully oxidized wild type enzyme as described in Chapter 3. Since the kinetic traces from the control and original samples behave identically, the conclusion can be made that the growth medium does not affect the transient EPR experimental results. Therefore any changes in the EPR spectra that are observed with the labeled species can be attributed to isotope substitution effects and not to a change in growth medium.

Future Work

In order to prove further the integrity of the control sample, a transient EPR spectrum must be acquired. In order to perform such an experiment with the Bruker ESP 300E, the automation routine described above can be used. This automation routine allows a transient EPR spectrum to be attained by utilizing controlled increments of the magnetic field, while photoexciting the sample and averaging several scans. Once this transient spectrum is obtained by using the control sample, it can be compared to the transient spectrum of the wild type enzyme described in Chapter 3. Since the single field kinetic traces of the wild type and control enzymes were nearly identical in behavior, the transient spectrum is be expected to also be similar. The transient spectrum will allow us

to test conclusively the hypothesis that the growth medium does not affect the spectroscopic characteristics of the enzyme samples.

After successful deuteration of the sample has taken place by following procedures outlined above, the sample can be used for EPR experimentation. The experiments will be performed on the Bruker ESP 300E spectrometer with the automation program described to capture a transient EPR spectrum with the deuterated sample. If the major couplings that are split by 15G in the natural abundance spectrum can no longer be detected, the conclusion can then be made that deuteration of the β-methylene protons has caused these 1:2:1 splittings to collapse, since the labeling of this sample is the only variance from the control sample. These results can be used to test the prediction that the three major peaks observed in the wild type, natural abundance EPR spectrum are due to coupling to the β-methylene protons.

In summary, the growth and characterization of the control enzyme has been conducted and the results analyzed. Now the same procedures must be carried out on the deuterated photolyase sample to determine the origin of the hyperfine coupling responsible for the major splittings of the transient EPR spectrum.

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