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SP Lyase Recognition and Binding to DNA

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Major Professor's Signature

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SP LYASE RECOGNITION AND BINDING TO DNA

By

Egidijus Zilinskas

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ABSTRACT

SP LYASE RECOGNITION AND BINDING TO DNA

By

Egidijus Zilinskas

UV radiation is a mutagen and can trigger the formation of a series of lesions within DNA. 5-Thyminyl-5,6-dihydrothymine ("spore photoproduct") is generated in *Bacillus* spores upon irradiating with 254 nm UV light. Spore photoproduct lyase (SPL) specifically targets the spore photoproduct and catalyzes its repair to two thymines. SPL is a member of family of enzymes that involve [4Fe-4S] clusters and S-adenosylmethionine (AdoMet) to generate catalytically essential radicals. Investigations of the interactions between SPL and DNA/oligonucleotides that have adjacent thymines, were carried out using agarose gel and gel-shift assays. The results suggest that SPL binds to DNA. DNA repair was done by using ^{3}H-labeled pUC18 DNA and SPL. Various attempts to generate spore photoproduct from thymidine at different conditions and at different UV wavelengths were also made.

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TABLE OF CONTENTS

LIST OF SCHEMES

LIST OF TABLES

LIST OF FIGURES

Images in this thesis are presented in color.

LIST OF ABBREVIATIONS

5'-dAdo..... 5'-deoxyadenosine

Adomet..... *S*-adenosyl-L-methionine

ATP.....adenosine triphosphate

APS.....ammonium persulfate

β-ME.....*β*-mercaptoethanol

BSA.....bovin serum albumin

EDTA.....(Ethylendinitrilo)tetra-acetic acid disodium salt dihydrate

HPLC.....high-performance liquid chromatography

DTT.....dithiotreitol

E.coli.....*Escherichia coli*

EPR.....electron paramagnetic resonance

Hepes.....*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid

IPTG.....isopropyl-*β*-D-thiogalactopyranoside

LAM.....lysine 2,3 –aminomutase

LB.....Luria-Bertani

NMR.....nuclear magnetic resonance

MOPS.....3-(N-morpholino) propanesulfonic acid

PFL.....pyruvate formate-lyase

PFL-AE.....pyruvate formate-lyase activating enzyme

PMSF.....phenylmethylsulfonyl fluoride

SDS-PAGE.....sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SP.....spore photoproduct

SPL.....spore photoproduct lyase

SspC.....small acid soluble protein

T4 PNK.....T4 polynucleotide kinase

TEMED.....N,N,N',N'-Tetra-Methyl-ethylendiamine

TFA.....trifluoro acetic acid

Tris.....tris(hydroxymethyl)aminomethane

CHAPTER I

INTRODUCTION

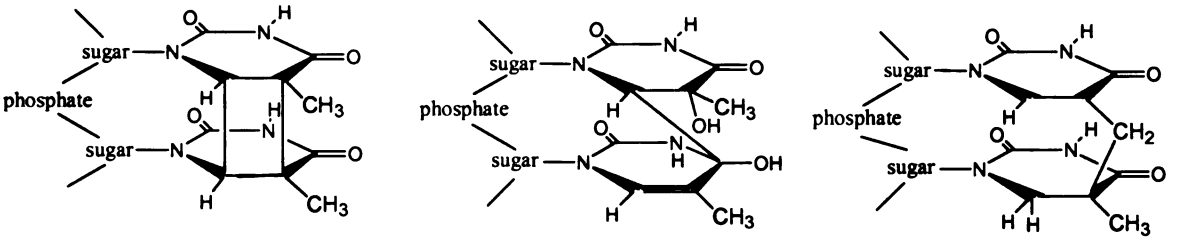
I.1 DNA and spore photoproduct

The chemical stability of DNA is one of the prerequisites of life. Even though DNA is a very stable molecule (more stable than proteins and RNA), this stability is not sufficient for its role as the long-term carrier of genetic information. Alterations in the structure of DNA occur frequently and DNA damage can be classified in two categories (1):

1) Intrinsic DNA damage (oxidative damage, deamination of bases, or mismatches during DNA synthesis)
2) Environmental DNA damage (chemical, for example from cross-linking agents; physical, such as ionizing and UV radiation)

UV radiation is a mutagen and it is the most harmful component of the solar radiation spectrum. UV induces the formation of a series of lesions within DNA. In the UV-C (100-290 nm) and UV-B (290-320 nm) range, damage is triggered by the direct absorption of the incident light by DNA bases (1, 2). Reactions of the resulting excited species lead mostly to the dimerization of pyrimidine bases thymine (T) and cytosine (C). The major DNA photoproducts of UV irradiation are cyclobutane pyrimidine dimers (TT, CT, and CC dimers) and 6,4-photoproducts, while 5-thyminyl-5,6-dihydrothymine is a minor product (Scheme I.1)(3, 4). UV-A (320-400 nm) light also has cytotoxic and mutagenic features, however to a smaller extent than UV-B radiation. DNA is not a

chromophore for a UV-A radiation with the exception of photons around 360 nm. It is likely that UV-A photons are absorbed by the unidentified endogenous photosensitizers that may damage DNA through photooxidation reactions (4). Different cells, however, can respond differently to UV irradiation. The major UV photoproduct formed in DNA of vegetative cells is a *cis,syn*-cyclobutane-type thymine dimer, while in spores the major photoproduct, generated by 254 nm UV irradiation, is 5-thyminyl-5,6-dihydrothymine ("spore photoproduct") (5-7).



A B C

Scheme I.1 a) Cyclobutane pyrimidine dimer, b) pyrimidine-pyrimidone (6-4) photoproduct, c) spore photoproduct, (5-thyminyl-5,6-dihydrothymine)

Dormant spores of the various *Bacillus* and *Clostridium* species, including *Bacillus subtilis*, are 5 to 20 times more resistant than vegetative cells to UV radiation (8). The spore resistance is due to:

a) the UV photochemistry of DNA within spores which generates few if any cyclobutane dimers, but significant quantities of SP, and

b) DNA repair, and particularly SP-specific repair, during spore germination.

The novel UV photochemistry has been proposed to be a result of DNA being in the A conformation rather than the B conformation in bacterial spores (Figure I.1)(9). However other studies have shown that SP formation was not necessarily dependent on DNA conformation, but rather on DNA hydration, with increasing SP and decreasing cyclobutane dimers observed as DNA hydration decreased (10). Since the levels of water in the spore core are low (11), the explanation is that the reduced hydration in the core causes a conformational change in spore DNA resulting in its novel UV photochemistry (9). Spore DNA was suggested to be in an A-like conformation, but during sporulation the spore DNA photochemistry changes well before full spore core dehydration takes place, suggesting that DNA hydration is not the only factor. In addition, while UV irradiation of poorly hydrated DNA in vitro gives SP, significant amount of cyclobutane

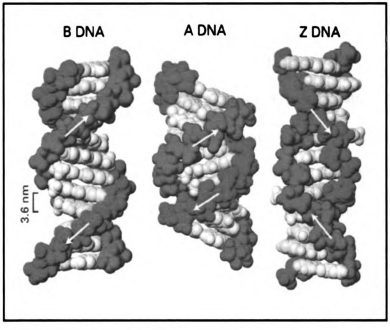


Figure I.1 Different conformations of DNA

dimers is also generated (9). Different hydration levels were tested *in vitro*, but cyclobutane pyrimidine dimer formation was still significant, and so hydration levels alone do not appear to explain the unique photochemistry observed in bacterial spores (10).

I.2. Small acid-soluble proteins and spore photoproduct lyase

The other reason for unique spore photochemistry could be the presence of small acid-soluble proteins (SASP), which appear 3-4 hr after the onset of sporulation in bacteria and constitute up to 20% of the protein in mature spores (8). These proteins range from 60 to 73 amino acids (5-7 kDa) and belong to a multi-gene family that is highly conserved and found in spore-forming bacteria such as *Bacillus* and *Clostridia.* These small proteins exhibit a very high degree of amino acid sequence homology both within and across *Bacillus* species. However, these proteins exhibit no obvious homology to any other proteins in available databases. The α/β-type SASP are encoded by a multigene family, with individual members (termed *ssp* genes) scattered widely on the chromosome as small (200 bp to 300 bp) monocistronic transcription units; the *ssp* genes are expressed in parallel only in the developing spore midway to sporulation (8). During spore germination the α/β-type SASP are rapidly degraded to amino acids, with their degradation initiated by a novel endoprotease termed the germination protease (GPR), which recognizes and cleaves within a pentapeptide sequence conserved in all α/β-type SASP (8). The SASP's presence has been correlated with the marked resistance of *Bacillus* to UV radiation, as deletion mutants of the SASP's lose their UV resistance (12). It also has been shown that SASP's are associated with the spore chromosomal DNA *in*

vivo. It has been proposed that binding of the SASP's to DNA promotes a structural change (13) and a change in the level of hydration of the DNA that results in formation of SP rather than cyclobutane thymine dimers (14). SASP's have been shown to be largely random coil in the absence of DNA, but to fold into a α-helical structure when bound to DNA (15). Spectroscopic evidence also suggests that SASP binding to DNA converts the DNA into what can best be described as A-from (Figure I.1)(13). However, the structure of the SASP-DNA complex has not been determined and it is not known if TT sequences have an impact on conformation that may help explain the formation of SP rather that other photoproducts.

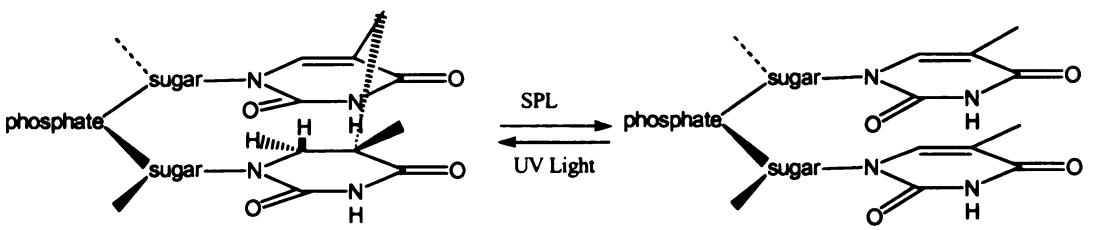
Repair of UV-induced damage helps to prevent of a number of adverse conditions such as a melanoma. Pyrimidine dimers such as SP damage the cells by blocking replication and transcription, which can result mutations if transcription does proceed past the region of the dimer. Repair of these dimers is therefore important in order to avoid mutations and is the key to UV resistance. Pyrimidine dimers can be excised and replaced, but the only example of direct pyrimidine dimer conversion to the starting material is the photoreactivation catalyzed by DNA photolyase. However, this reaction has been shown to be absent in many species, including *Bacillus*, suggesting that alternate means of pyrimidine dimer repair might be found (16).

The enzyme spore photoproduct lyase (SPL) is the first identified pyrimidine dimer lyase that does not require photoactivation to repair spore damaged DNA (17). Together with the SASP's, SPL confers on *Bacillus* spores their unusual resistance to UV radiation (18). Bacterial spores can survive harsh conditions for extended periods of time (11). During dormancy these spores are unable to repair damage to their DNA from UV

exposure, which is caused by accumulation of SP. Spores can, however, undergo DNA repair in the very early germination cycle through two repair mechanisms (11, 16):

- the nucleotide excision repair pathway (NER), and
- the reversal of SP to thymine catalyzed by the enzyme spore photoproduct lyase.

NER detects and removes lesions from the DNA including SP and cyclobutane-type pyrimidine dimers (1). SP lyase specifically targets the SP and cleaves into two thymines by an unknown but light-independent mechanism (19) (Scheme I.2).

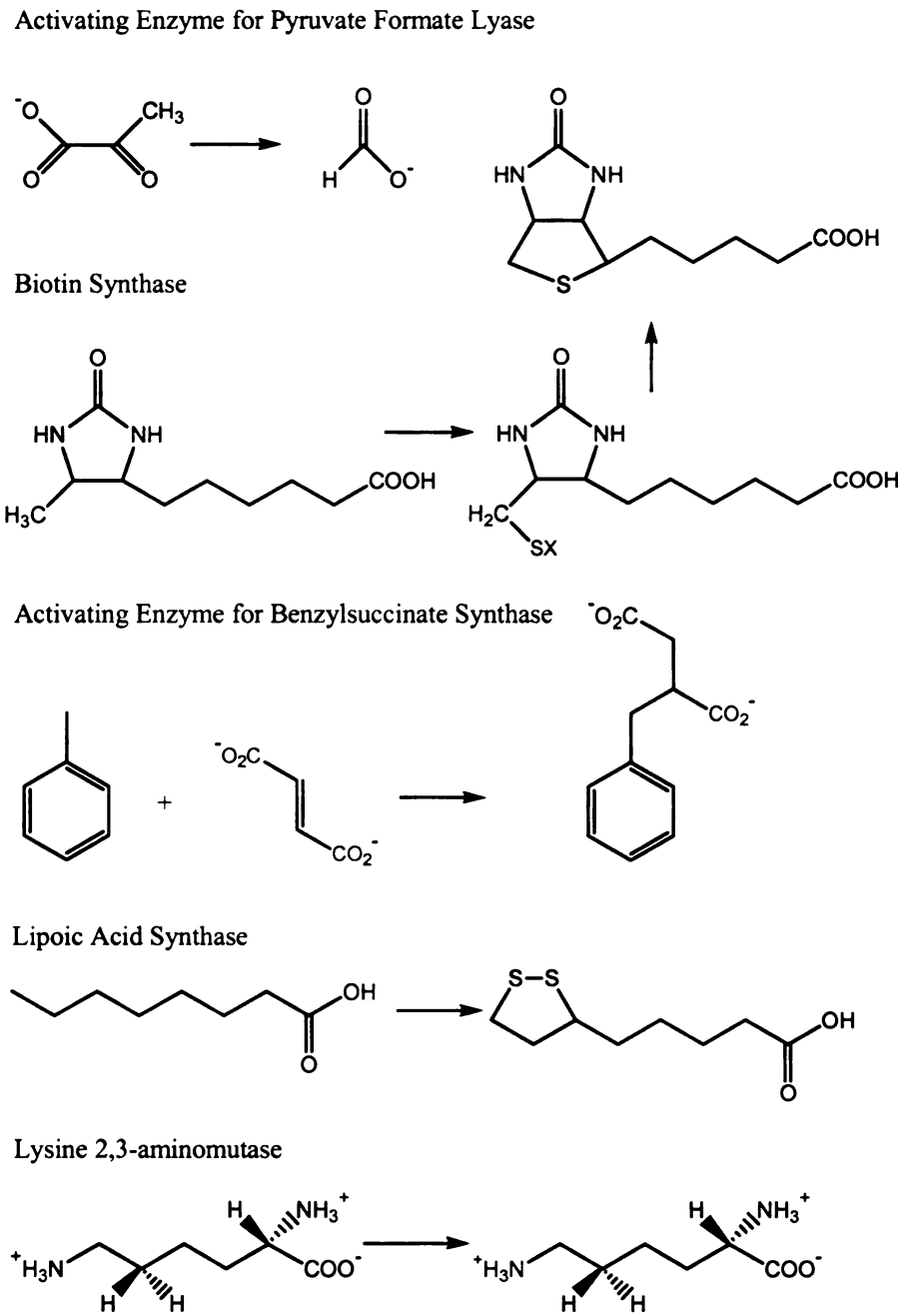


Scheme I.2 The repair of spore photoproduct by SPL to thymines

I.3 Adenosylmethionine-dependent iron-sulfur enzymes

Iron-sulfur clusters are widespread in biological systems and participate in electron transport, as well as mediating redox catalysis and non-redox catalysis (20). Aconitase is one of the most studied enzymes that mediate non-redox catalysis, in which a $[4Fe\text{-}4S]^{2+}$ cluster serves as a Lewis acid to catalyze the interconversion between citrate and isocitrate (20). Fe-S clusters are also involved in regulation of gene expression, for example in the iron-responsive protein (IRP) and in SoxR, which respond to changes in levels of iron and superoxide, respectively (20). Another role of Fe-S clusters is that some enzymes use Fe-S cluster and AdoMet to initiate radical catalysis. This Fe-S cluster-

mediated radical catalysis includes glycyl radical formation, rearrangement reactions, cofactor biosynthesis, and repair of DNA damage (Scheme I.3)(21, 22).



Scheme I.3 Enzymatic reactions of the Radical-SAM superfamily

More that 600 related enzymes that involve AdoMet-derived radical biochemistry have been recently identified (21). The activating enzymes include two *E. coli* enzymes that generate catalytically essential radicals on enzymes required during anaerobiosis, the anaerobic ribonucleotide reductase-activating enzyme (aRNR-AE) and the pyruvate formate-lyase activating enzyme (PFL-AE)(23). Lysine 2,3-aminomutase (LAM) catalyzes a rearrangement reaction analogous to those catalyzed by adenosylcobalamin-dependent enzymes, except that no cobalamin cofactor is required (24). Biotin synthase and lipoic acid synthase catalyze sulfur insertion reactions (25, 26), presumably by a radical mechanism (27, 28). For both PFL-AE and LAM, label transfer studies have provided evidence for an adenosyl radical intermediate derived from the required AdoMet cofactor (22). The information available on these enzymes suggests that all catalyze radical chemistry mediated by S-adenosylmethionine, although the mechanistic details have yet to elucidated (20).

Previous work suggested that SP lyase utilized S-adenosylmethionine (AdoMet) and contained an iron-sulfur cluster, suggesting that it was a member of the Fe-S/AdoMet family of enzymes (29). As shown in Figure I.2, the Fe-S/AdoMet enzymes have in common a three-cysteine motif, and these three cysteines have been shown for several of the enzymes to be responsible for coordination of the catalytically essential iron-sulfur cluster (20, 22). SP lyase also contains three cysteines in the CX_3CX_2C motif unique to the Fe-S/AdoMet enzymes, and significant sequence homology to the Fe-S/AdoMet enzymes is observed between residues 80 and 115 of SP lyase (20).

•SP Lyase	86	IPFATG**C**MGH**C**HY**C**YLQTT 104
•PFL-AE	24	ITFFQG**C**LMR**C**LY**C**HNRDT 42
•aRNR-AE	20	VLFVTG**C**LHK**C**EG**C**YNRST 38
•Biotin Syn	47	SIKTGA**C**PQD**C**KY**C**PQTSR 65
•Lipoate Syn	48	MILGAI**C**TRR**C**PF**C**DVAHG 66
•LAM	132	LLITDM**C**SMY**C**RH**C**TRRRF 150

Figure I.2 Sequence of SPL and the radical SAM superfamily enzymes. SP Lyase, spore photoproduct lyase; PFL –AE, pyruvate formate-lyase activating enzyme; aRNR-AE, anaerobic ribonucleotide reductase activating enzyme; Biotin syn, biotin synthase; Lipoate syn, lipoate synthase; LAM, lysine 2,3-aminomutase.

Purified SP lyase has been shown to contain iron and acid-labile sulfide, and the UV-visible spectrum is suggestive of the presence of an iron-sulfur cluster (29, 30). Addition of AdoMet to the in vitro activity assay was found to stimulate SP repair activity of SP lyase (29). This and other early studies of SP lyase, however, were hampered by the inability to isolate active enzyme in sufficient quantities for physicochemical studies (30). The limited spectroscopic and mechanistic studies that have been done on SP lyase were therefore done on inactive enzyme (29, 30), leaving open numerous questions, including the relevance of the purported Fe/S cluster and the AdoMet dependence.

SP lyase also shares some sequence homology with the members of the DNA photolyase/(6-4) photolyase/blue light photoreceptor protein family (18). The conserved residues are in the general region believed to be involved in substrate binding by DNA photolyase. Both SP lyase and photolyase perform the same type of chemistry, which is

the cleavage of nucleotide dimers to monomers, however there are some major differences:

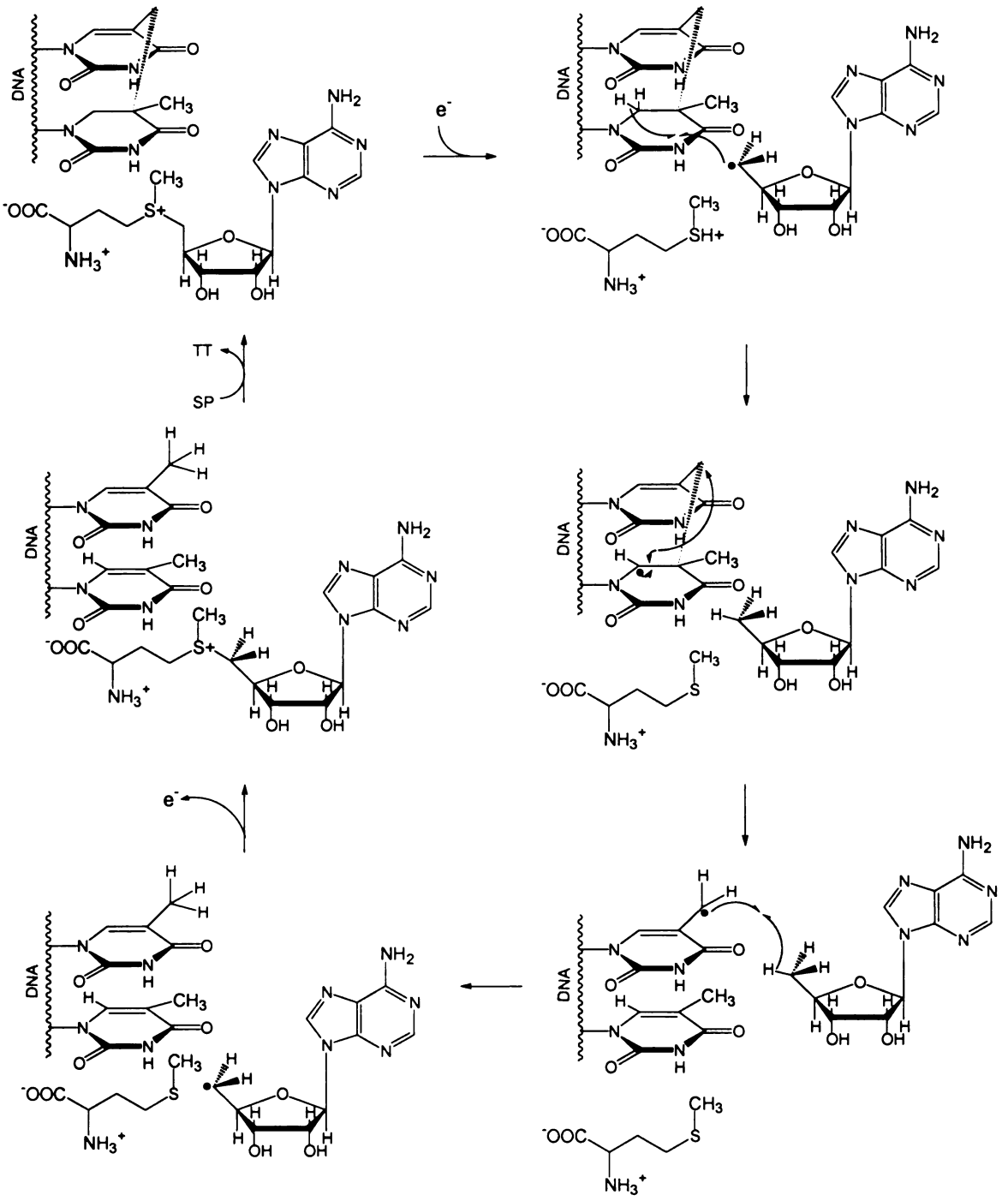
1) the DNA photolyases, but not SP lyase, are activated by visible light (31),
2) the DNA photolyases contain a flavin (FAD) and either folate or a deazaflavin (32), while SP lyase contains an Fe-S cluster (29), and
3) the DNA photolyases cleave cyclobutane pyrimidine dimers to monomers (33) while SP lyase cleaves methylene-bridged thymine dimers to monomers (18).

The commonly accepted mechanism for photorepair by DNA photolyase involves dimer splitting as a consequence of a single electron transfer from the enzyme to the dimer, a mechanism recently supported by quantum chemical calculations (34). The functional similarity between SP lyase and the Fe-S/AdoMet enzymes implies that cleavage of the SP dimer also occurs through an iron-sulfur cluster-mediated radical mechanism.

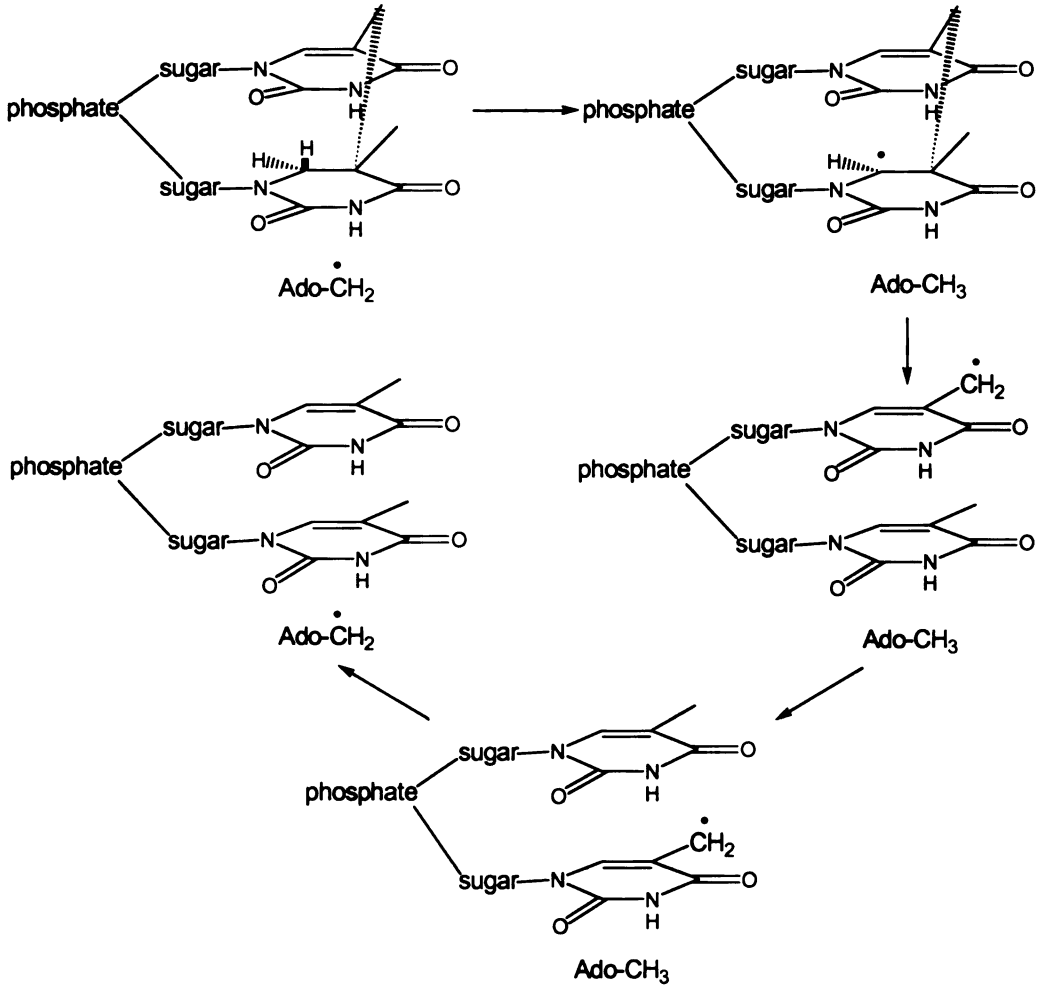
The putative involvement of an Fe-S cluster in DNA repair suggests that the cluster could serve as a spectroscopic handle for detailed mechanistic studies of thymine dimer repair. The preliminary observations on SP lyase point to a novel mechanism of repair of UV-induced DNA damage, which involves a Fe-S cluster and AdoMet (29). This mechanism was initially proposed by Mehl and Begley (35), and further supported by Cheek and Broderick (36). The latter workers showed that 5'dAdo radical is involved in the SP lyase enzymatic mechanism (Scheme I.4). During the repair ^{3}H label was transferred from C-6-tritiated spore photoproduct to AdoMet (36). This label transfer suggests that SP repair is initiated by C-6 H atom abstraction, with the resulting substrate radical presumably undergoing β-scission to provide the monomeric products (36).

AdoMet also acts as a catalytic cofactor during repair to reversibly generate the putative adenosyl radical intermediate (Scheme I.4)(36). The quantum chemical calculations, which employed hybrid Hartree-Fock/density functional theory, proposed that an inter-thymine hydrogen atom transfer step takes place before the back-transfer of the hydrogen atom from the adenosine cofactor (37). The last step is shown to be the rate-determining step in the reactions (Scheme I.5)(37).

It is known that SPL is one of the radical SAM-superfamily enzymes and it repairs SP (29). It was also shown that SPL is similar to DNA photolyases (18), but there is little knowledge on how SPL recognizes damaged DNA and how much of SP can be repaired, how repair depends on time and other factors. In our research we focused on creating SP in pUC18 DNA and doing SP repair assays. We also tested how SPL binds on pUC18 DNA and short nucleotides. More research, however, has to be done to investigate if SPL binding is specific and how SPL recognizes thymine-thymine dimer and/or SP. Various attempts to generate spore photoproduct from thymidine at different conditions and at different UV wavelengths were also made.



Scheme I.4 The proposed mechanism of SP repair by spore photoproduct lyase (36)



Scheme I.5 Modified Reaction Mechanism of SPL (Adapted from Ref. (37))

CHAPTER II

OVEREXPRESSION AND CHARACTERIZATION OF SPORE PHOTOPRODUCT LYASE

II.1 Introduction

The enzyme SP lyase is the first identified non-photoactivatable pyrimidine dimer lyase, and, together with the SASP's, is responsible for *Bacillus* spores unusual resistance to UV radiation. The SP lyase was previously characterized using both analytical and spectroscopic methods (30). It was found the enzyme contains iron (1.8 mol/mol protein) and acid-labile sulfide (1.8 mol/mol protein), consistent with the presence of an iron-sulfur cluster. The UV-visible spectrum of the purified protein exhibits a broad shoulder with maxima at 410 nm (4.75 $mM^{-1}cm^{-1}$) and 450 nm (4.03 $mM^{-1}cm^{-1}$) (FigureII.1).

The electron paramagnetic resonance (EPR) spectrum of the isolated enzyme exhibits a sharp nearly isotropic resonance that has g values, anisotropy, and relaxation properties consistent with its assignment to a $[3Fe\text{-}4S]^{1+}$ cluster (Figure II.2) (30). This EPR signal typically accounts for 30-40% of the iron in the sample, and therefore a significant proportion of the iron is present in an EPR-silent state. The data from Mössbauer spectroscopy confirms the presence of approximately 40% $[3Fe\text{-}4S]^{1+}$ cluster, and the remaining iron is present as a $[2Fe\text{-}2S]^{2+}$ cluster (Figure II.3) (30).

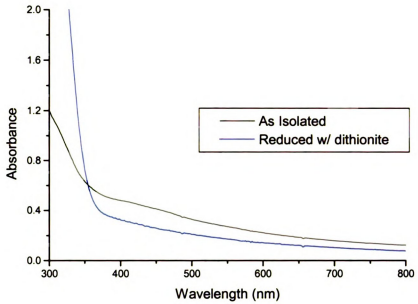


Figure II.1 Titration of SPL with sodium dithionite, monitored by UV-visible spectroscopy. The decrease in the visible absorption is consistent with reduction of the cluster by sodium dithionite (30).

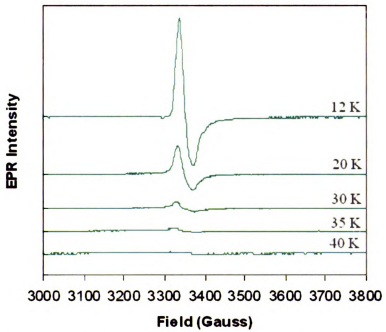


Figure II.2 Temperature dependent EPR signal of the as isolated SPL (137μM) in 50mM Hepes, 300mM NaCl, 10mM BME, 150mM imidazole, 5% glycerol, pH=7.2 (30).

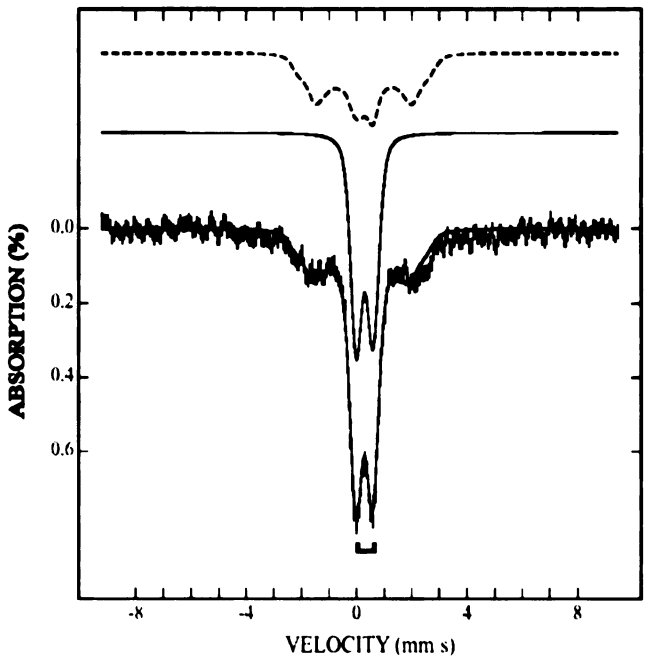


Figure II.3 Mössbauer spectrum of anaerobically isolated ^{57}Fe-enriched SPL in 50mM Hepes, 300mM NaCl, 10mM BME, 150mM imidazole, 5% glycerol, pH=7.2. The spectrum was recorded at 4.2 K in a magnetic field of 50 mT applied parallel to the γ-rays. The solid line plotted above the data is a theoretical simulation of a $[2Fe\text{-}2S]^{2+}$ cluster. This component accounts for 47% of the total Fe absorption. The dashed line is a theoretical simulation of a $[3Fe\text{-}4S]^{1+}$ cluster. The $[3Fe\text{-}4S]^{1+}$ signal accounts for 43% of total intensity. The solid line overlaid with the experimental data is the sum of the contributions of the $[2Fe\text{-}2S]^{2+}$ and $[3Fe\text{-}4S]^{1+}$ clusters (30).

SP lyase can be reduced by titration with sodium dithionite, as is evidenced by the decrease in visible absorption that is characteristic of reducing an iron-sulfur cluster (Figure II.1) (30). An alternative method of reducing SP lyase is by photoreduction in the presence of 5- deazariboflavin. The reduction of SP lyase results in a dramatic change in

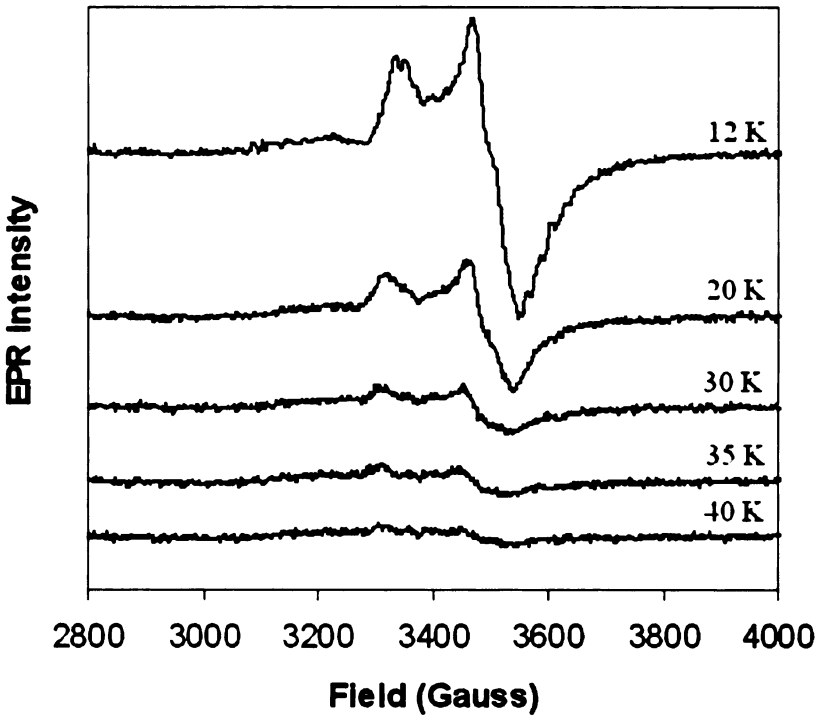


Figure II.4 Temperature dependent EPR spectrum SP lyase-His_6 after the reduction with photoreduced 5-deazariboflavin. The axial signal with g values of 2.04, 1.94 are characteristic of a $[4Fe\text{-}4S]^{1+}$ cluster. Spin quantitation reveals that this signal accounts for approximately 41% of the total iron (30).

the EPR spectral properties (Figure II.4). Rather that the fairly intense, nearly isotropic signal observed for the as-isolated enzyme (Figure II.2), the reduced enzyme has a weak, nearly axial signal that is characteristic of a $[4Fe\text{-}4S]^{1+}$ cluster(Figure II.4). Integration of this signal shows that it accounts for approximately 40% of the total iron in the protein. The remaining iron is present in an EPR-silent state which is believed to be the $[4Fe\text{-}4S]^{2+}$ state based on preliminary Mössbauer studies (30). Therefore reduction appears to convert the $[3Fe\text{-}4S]^{1+}$ and $[2Fe\text{-}2S]^{2+}$ clusters to $[4Fe\text{-}4S]^{2+/1+}$ clusters (30). Such $[3Fe\text{-}4S]^{1+} \rightarrow [4Fe\text{-}4S]^{2+/1+}$ reductive cluster conversions are believed to result from "cannibalization" of the $[3Fe\text{-}4S]^{2+/1+}$ clusters to form the $[4Fe\text{-}4S]^{1+}$ clusters, which are

more thermodynamically stable under reducing conditions (30). Whether these cluster conversions have any physiological relevance for any of the Fe-S/AdoMet enzymes has not be determined yet.

II.2. Experimental methods

II.2.1. Growth and expression of SPL

The gene for spore photoproduct lyase *splB* was previously cloned into the pET-14b (Novagen) vector in order to attach an N-terminal 6 histidine (His_6) tag generating SP lyase-His_6 (36). The plasmid pEt-14b-SPL17 was purified and was transformed into the bacterial competent cells, TUNER(DE3)pLysS *Escherichia coli* (Stratagene). The procedure for this transformation involves the following method. TUNER(DE3)pLysS competent cells (50 μl) were thawed on ice and 1 mL of a pET14b-SPL17 plasmid solution was added. Cells were incubated on ice for 10 minutes and then immersed in a 42 °C water bath for 30 seconds followed by incubation for 2 minutes on ice. SOC media (100 μl) was added and the cells are shaken at 37 °C for 30 minutes. Then cells were removed from the shaker and plated on agar plates containing Luria Base Broth media and ampicillin (50 μg/mL). The plates were incubated overnight for 16 hours at 37 ^{0}C. Small white colonies were seen after incubation.

A single colony was used to inoculate a 50 mL solution of LB media containing 50 μg/mL ampicillin and grown overnight for 16 hours at 37 °C in the shaker at 250rpm. This overnight culture was used to inoculate 6 Bellco flasks containing a minimal media (950 mL per flask). Minimal media included (per 6 L): 66 g of casamino acids, 50.4 g MOPS, 17.32 g NaCl, 4.6 g Tricine, 3.06 NH_4Cl, 4.6 g KOH and 5.8 L MQ Water, 80

mL "O" solution, 80 mL "P" solution (1M KH_3PO_4), 40 mL "S" solution (276 mM K_2SO_4), 160 mL of glucose (40 g in 200 mL), 200 mL 0.1 M $CaCl_2$. The "O" solution consists of 0.1 g $FeCl_2xH_2O$, 10 mL conc. HCl, 1mL "T" solution, 2.68 g $MgCl_2x6H_2O$ in 50 mL of water. The "T" solutions consists 8 mL conc. HCl, 18.4 mg $CaCl_2xH_2O$, 64 mg H_3BO_3, 40 mg $MnCl_2x4H_2O$, 18 mg $CoCl_2x6H_2O$, 4 mg $CuCl_2x2H_2O$, 340 mg $ZnCl_2$, 605 mg $Na_2MoO_4x2H_2O$ in 100 mL with MQ water. Ampicillin 6 mL of 50 mg/mL and 10 mg each of pyrodoxine, riboflavin, niacinamide, biotin, vitamin B_{12}, pantothenic acid, thioctic acid, folic acid, thiamine (vitamin B_1) were added to 6 L of culture right before inoculation. The flasks were placed in a New Brunswick Scientific shaker at 175 rpm (37 °C) and allowed to grow to an optical density of 0.6 at 600 nm. After the optical density at 600 nm reached 0.6, 1 mL of isopropyl-β-D-thiogalactopyranoside (IPTG) was added with 300mg of $Fe(NH_4)_2(SO_4)_2$ to each flask. The cell culture is grown for additional 3 hours before harvesting by centrifugation at 10,816xg (8000 rpm, Sorvall GS3 rotor). The supernatant was decanted and the cells stored at –80 °C. A typical yield of cells is 10-20 g for 6 L of media.

II.2.3 Purification of SPL

SPL was purified from these cultured Tuner(DE3)pLysS cells transformed with the vector pET-14b-SPL17. All steps of purification were carried out in a single day under anaerobic conditions at 4 °C in a Coy anaerobic chamber (Coy laboratories, Grass Lake, MI). All buffers were degassed prior to use by purging with N_2 for over 1 hour or by repeated pump/purge cycles on the Schlenk line. Approximately 10 g of the cells were brought into the anaerobic chamber and resuspended in 20 mL of a lysis buffer

containing 50 mM Hepes, 200 mM NaCl, 1% Triton X-100, 5% glycerol, 10 mM $MgCl_2$, 5 mM βME, 1 mM PMSF, 1mg lysozyme and trace amounts of (approximately 0.1 mg each) Rnase A and Dnase I (pH 7.5). The cell suspension was agitated for one our and centrifuged at 27,000 rpm (rotor SS34) for 30 minutes at 4 °C. A resulting greenish crude extract was decanted and loaded onto Ni-NTA affinity column (1.6 x 10 cm, Qiagen) equilibrated with Buffer A (50 mM Hepes, 300 mM NaCl, 5% Glycerol, 5 mM BME, 10mM imidazole, pH 7.5). After loading, the column was washed with 40mL of Buffer A followed by a linear gradient from Buffer A to Buffer B (50 mM Hepes, 300 mM NaCl, 5% Glycerol, 5 mM BME, 250 mM imidazol, pH 7.5).

Aliquots from several fractions were suspended in SDS-PAGE sample buffer, boiled, and centrifuged and an aliquot of the supernatant was loaded on SDS-PAGE gel (12% Tris-HCl, BioRad). Fractions that were judged to be >95% pure were pooled. Later the pooled protein was concentrated using an Amicon concentrator with a PM10 membrane. Standard Bradford assay techniques (Sec. II.2.4) were employed to determine the concentration of the pooled protein and a maximum concentration of ~10mg/mL of protein was obtained before significant precipitation of protein occurred. A typical yield of protein is ~10 mg per 6L of culture.

II.2.4 Protein assays

Routine determinations of protein concentration were determined by the method of Bradford (38), with bovine serum albumin is a standard.

II.2.5 Iron assays

Iron assays were carried out by using the method of Beinert (39).

I.2.6 Results and Discussion

In order to do SP repair assay, SPL binding assays and other experiments, first we had to overexpress and purify SPL. SPL was purified from Tuner(DE3)pLysS cells harboring the pET-14b-SPL17 plasmid. Inducing at an earlier log phase (OD_{600} of ~0.6) and supplementing the medium with iron after induction with IPTG contributed to better overexpression and iron inclusion of SPL. The cells were lysed by an enzymatic procedure.

SPL was purified by passing through Ni-NTA affinity column under strictly anaerobic conditions (Figure II.5) in the presence of DTT and pooling fractions based not only on SDS-PAGE analysis (Figure II.6), which shows sharp bands at ~42 kDa size (Lanes 4-8), but also on the UV absorbance at 426 nm, which shows ligand-to-metal charge transfer for iron-sulfur cluster, and 280 nm, which shows the absorbance of nucleic acids (Figure II.5).

The purification of SPL resulted in the isolation of a 42 kDa protein that is about 95% pure according to SDS-PAGE results. The color of the protein was reddish-brown and would change to clear when left exposed to oxygen for a period of time. The protein was also sensitive to temperature and would precipitate rather quickly if left to sit at room temperature. The typical yield of protein that could be purified from 6 L growth media was 10 mg. The protein could be concentrated to a maximum of 150 μM, after which precipitation would occur. The SPL contains 1.8 mol Fe/mol protein and a stoichiometric amount of acid-labile sulfide.

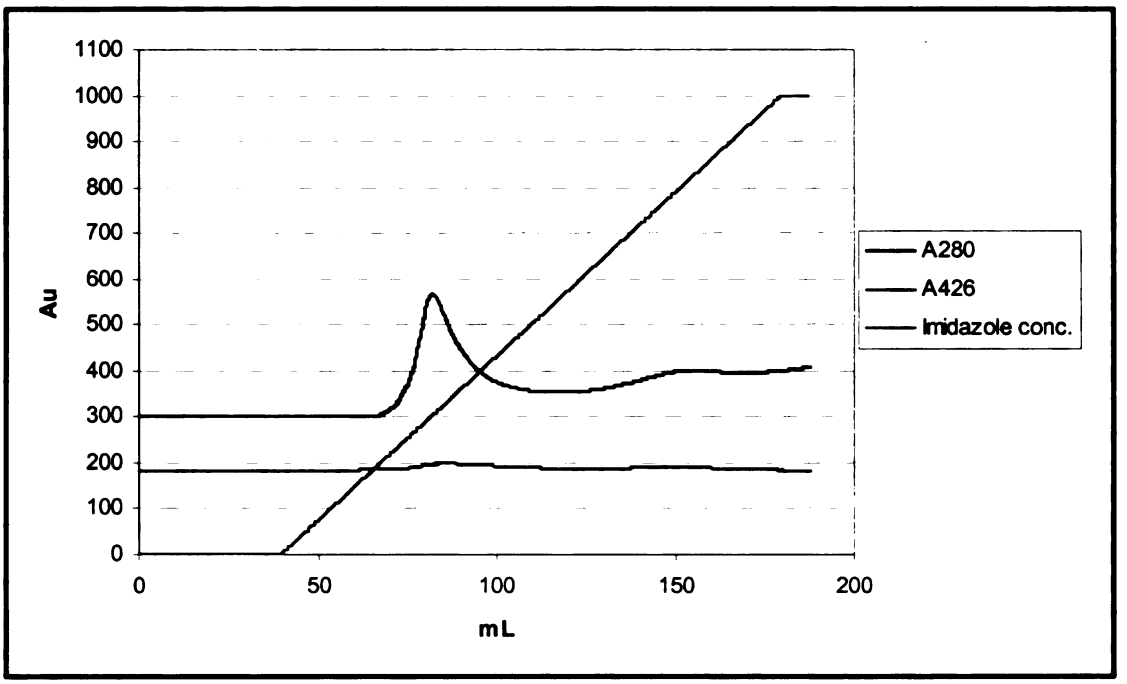


Figure II.5 Chromatogram of purification of SPL. Elution profile for the Ni-NTA Affinity column (Qiagen, 1.5 x 25 cm), gradient from Buffer A to Buffer B over 200 mL, flow rate 1 mL/min. SPL eluted through last half of the gradient. The peak at 70-80 mL is the other proteins and a broad band at ~140-170 is SPL.

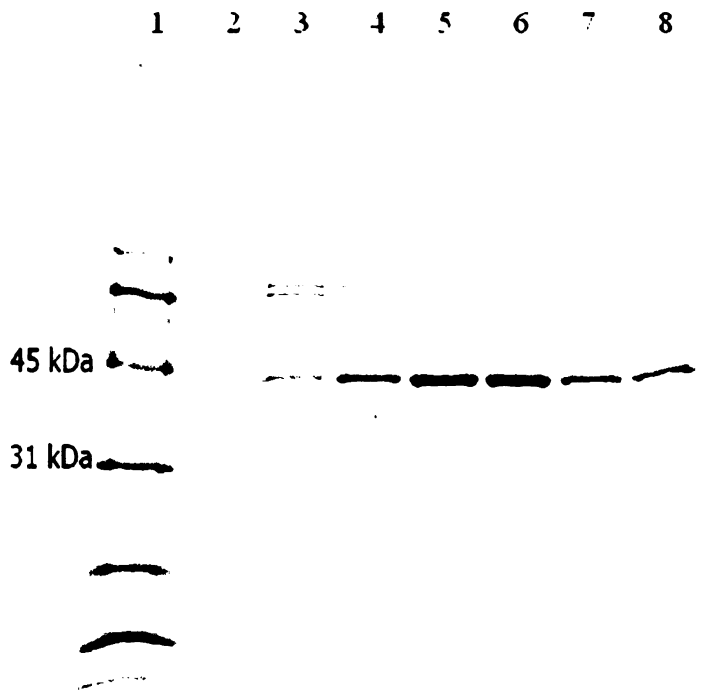


Figure II.6 SDS-PAGE analysis of SPL fractions off the Ni-NTA Affinity column. Lane 1, molecular marker (kDa); lanes 2-8, fractions 19-29 (odd fractions only). Fractions 21-27 were pooled.

CHAPTER III

GENERATION AND REPAIR OF ISOTOPICALLY LABELED SPORE PHOTOPRODUCT

III.1 Introduction

The different photochemistry between vegetative cells and spores is due to the change of the conformation of DNA. This conformational change might be triggered by environmental conditions and/or by the presence of small acid-soluble proteins. In the early papers it was shown that DNA conformation changes when relative humidity is lowered below 65% (10). At these conditions the DNA is disordered and a loss of base stacking leads to a reduction of cyclobutane dimer formation and increase in SP formation. But the enhancement in the yield of spore photoproduct as the relative humidity is reduced cannot be explained simply by formation of disordered or unstacked DNA, because no SP is observed upon irradiation of highly denatured DNA in solution (10). It was also proposed, that SP formation depends on DNA conformation (5). It was shown that in ethanolic solution native T7 DNA can undergo conformational transitions from the B conformation (0% ethanol) to the C-like (60% w/w ethanol) and the A (80% w/w ethanol) conformations (9). The formation of SP reaches a maximum when the concentration of ethanol is 80% in either native or denatured DNA, while cyclobutyl dipyrimidines are still the major class of photoproducts formed (9). It was assumed that the lowered water activity itself, at high ethanol concentration, rather than the conformational changes such an environment induces in DNA, is the critical factor in formation of SP.

It was later discovered, however, that DNA conformational changes in spores are due to the presence of small acid-soluble proteins (SASP)(15) and that SASP plays a major role in formation of spore photoproduct (40, 41). SASPs bind to and saturate spore DNA, causing structure perturbations in the DNA such that the structure is more A-like (42). It is believed that these structural perturbations induced by SASPs result in formation of SP, rather than cyclobutane pyrimidine dimers, upon UV irradiation (41). Although the presence of α/β-type SASP largely explains the production of SP by 254 nm UV radiation, it does not explain spore UV resistance (15). When UV irradiation was applied to [^{3}H] thymidine-labeled DNA, the major photoproduct was *cis,syn* cyclobutane dimer with smaller amounts of two other cyclobutane-type dimers, CT and *trans,syn* cyclobutane dimer. In contrast, irradiation of a 5:1 complex (wt/wt) of SspC with DNA in liquid produced very little pyrimidine dimer but a significant amount of SP (14). When the complexes with increasing SspC/DNA ratio were used, production of *cis,syn* cyclobutane dimer fell while production of SP increased. The SspC/DNA interaction in the dry DNA films also appeared to saturate at an SspC/DNA weight ratio of 4-5:1 and at this ratio *cis,syn* cyclobutane dimer formation was almost completely abolished (14). Photoreactivity of α/β-type SspC/DNA complexes in vitro is similar to that in the spore in the first minutes, when the spores go through the period of transiently increased UV resistance (14).

III.2 Experimental methods

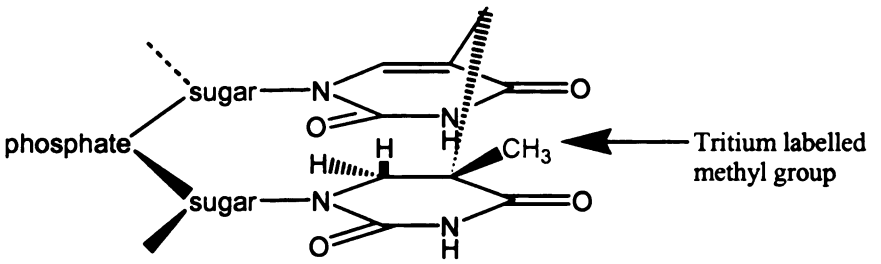
III.2.1 Materials

AdoMet was purchased from Sigma-Aldrich. [methyl-^{3}H] thymidine (0.74mBq/mL) was purchased from Amersham Pharmacia. pUC18 DNA was previously made in our laboratory. High flash point cocktail Safety-Solve for scintillation counting was purchased from Research Products International.

III.2.2 Generation of Spore Photoproduct

The plasmid DNA from pUC18 was transformed as described previously (Chapter II.2.1) into NovaBlue *E.coli* (Novagen). A single colony of these bacteria was placed in LB medium containing 50 μg/mL ampicillin, 0.45 mM deoxyadenosine, and 10 μM [methyl-^{3}H] thymidine and grown overnight for 16 hours at 37 °C in the shaker at 250 rpm. This process is intended to label the thymine bases in the DNA backbone at the position shown in Figure 12. The DNA from grown cells was extracted using a Promega Wizard Mini-Prep Kit. Specific activity of the purified DNA was about 1.5×10^7 cpm/μmol.

DNA (100, 0.186 μM) was placed on SaranWrap and left overnight to dry. The following day the SaranWrap was placed over saturated $ZnCl_2$ solution and left for 5-7 days to equilibrate. The tritium labeled DNA was then irradiated with ultraviolet light (UVGL-25 lamp, 2x4 W, UVP) at 254 nm for 5 hours, to generate the tritiated spore photoproduct (Scheme III.1).



Scheme III.1 Tritium labeled C-6 spore photoproduct

Another set of the experiments included irradiation of ^{3}H-labeled DNA in ethanol solution. DNA (100 μL) was added to 100 μL of water and 800 μL of ethanol (total – 80%). The resulting solution was placed on a watch glass and irradiated at 254 nm (UVGL-25, 2x4 W, UVP) for 1, 2, and 5 hours while stirring with the magnetic stir bar.

The third type of experiment involved making a DNA/ethanol dry film. DNA (50 mL, 0.186 μM) was added to 450 μL of ethanol and placed on a watch glass and dried overnight. The dry film was then irradiated at 254 nm (UVGL-25, 2x4 W, UVP) for 5 hours and resuspended in 100 μL of water.

III.2.3 Spore Photoproduct Repair Assays

SP lyase activity was determined in the following manner, using modified version of the assay developed by Nicholson and coworkers. All work was carried out under anaerobic conditions in an Mbraun glove box and the solutions were prepared inside the

glove box. The oxygen level was <1 ppm. Assay experiments were set up in parallel with one containing SP lyase (10 μg) and the other containing no enzyme. A reaction mix of 200 μL was created containing 25 mM Tris-acetate pH 7.0, 4 mM DTT, 3 mM dithionite, 30 mM KCl, 2 mM AdoMet and 10,000-50,000 cpm of the spore photoproduct DNA from pUC 18. This assay mix was placed in o-ring sealed eppendorfs and allowed to incubate at 37 °C for 1 hour. After the incubation, 0.5 mL trifluoroacetic acid was added to each assay mixture and transferred to quartz ampoules. These ampoules were sealed under vacuum and placed in the oven at 165 °C for 3 hours. Then the seals of ampoules were broken and trifluoroacetic acid was evaporated under the Schlenk line. Water (100 μL) was used to resuspend the solid. The resulting suspension was centrifuged at 12,000 rpm for 4 minutes and transferred to new eppendorf tubes. HPLC was used to separate hydrolyzed DNA and protein by loading 15 μL of the assay onto a Waters phenyl-Spherisorb column (4 mm x 25 mm) and running with water as mobile phase at 1.5 mL/min for 25 minutes. Fractions were collected every minute.

III.2.4 Re-irradiation of thymine photoproducts

For the re-irradiation experiments previously irradiated and hydrolyzed ^{3}H-labeled DNA was placed on SaranWrap and irradiated at 254 nm (UVGL-25, 2x4 W, UVP) for 2 or 4 hours. The following product was loaded onto a Waters phenyl-Spherisorb column (4 mm x 25 mm) running with water as mobile phase at 1.5 mL/min flow rate for 25 minutes. Elution was monitored by absorption at 254 nm. Fractions were collected every minute.

III.2.5 Scintillation Counting

Safety-Solve (15 mL) was added to each fraction from HPLC chromatographic runs. Then all the fractions were analyzed by scintillation counting (Wallac 1414-001). The first sample used as blank, contained Safety-Solve (15mL) and water (1 mL).

III.2.6 HPLC of DNA bases

The HPLC of DNA bases, adenine, guanine, thymine and cytosine, were carried out as follows. DNA bases were dissolved in water and filtered through 20 μm filter. The solution (15 μL) was injected onto Waters-phenyl-Spherisorb column (4 mm x 25 mm) and running water as mobile phase at 1.5 mL/min flow rate for 25 minutes. Elution was monitored by absorption at 254 nm.

III.3 Results and discussion

There are several ways to generate spore photoproduct in DNA. SP can be induced between two thymines while irradiating dry film DNA (10), DNA/ethanol solutions (9), frozen aqueous DNA solutions (5, 43), and irradiating DNA/SASP mixture (14). Experiments included various types of DNA, like calf thymus DNA (5), T7 DNA (9), pUC19 DNA or Bacillus spores (12, 14), which were labeled with tritium and irradiated with UV light. The following steps included the acid hydrolysis of DNA and quantification of thymine dimers. The best results were obtained while irradiating dry DNA films, where 5% of total thymine was converted into SP (44) and irradiating DNA/SASP complexes, where 5.8% of total thymine was converted to SP (14).

For our experiment, we first measured the elution time of the DNA bases from HPLC chromatography. The standard samples were injected onto the Waters-phenyl-Spherisorb column (4 mm x 25 mm) and elution was monitored by absorption at 254 nm. Adenine, cytosine and thymine eluted at 2.5 minutes and guanine eluted at 7.5 minutes (at a flow rate of 1.5 mL/min) (Table III.1). DNA that we chose for our experiments was pUC18 DNA due to its similarity to pUC19 DNA used to generate SP by Nicholson and co-workers (14). In order to make spore photoproduct, several experiments were carried out. The first one involved irradiating dry film DNA at 254 nm, the second involved irradiating 80% ethanol/DNA solution, and the third involved irradiating a DNA/ethanol dry film. The DNA was irradiated for 5 hours at 254 nm, with the total UV dosage of 130 kJ/m^2. When dry film DNA was irradiated, 3.8% of total thymine was converted to TT dimer (based on data from the scintillation counting). When DNA/ethanol dry film was exposed to UV light, 2.9% of total thymine was converted to TT dimer. However, when 80% ethanol solution of DNA was irradiated for 2 hours, only 0.3% of total thymine was converted to TT dimer (Figure III.1). The similar results were obtained when 80% ethanol solution of DNA was irradiated at 254 nm for 5 hours (data not shown). However, these results did not indicate which of the thymine dimers is formed – cyclobutane dimer, (6-4) photoproduct or SP.

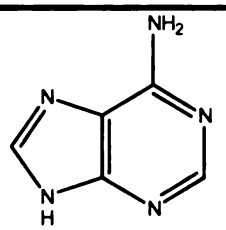
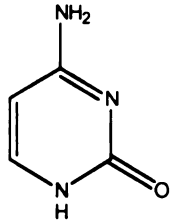
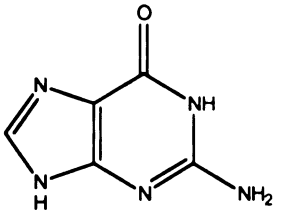
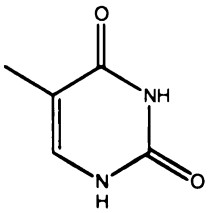
DNA base		Concentration, mg/mL	Elution time, min
	Adenine	0.036 mg/mL	2.5
	Cytosine	0.036 mg/mL	2.5
	Guanine	0.07 mg/mL	7.5
	Thymine	0.026 mg/mL	2.5

Table III.1 DNA bases and their elution times from HPLC (Waters phenyl-Spherisorb column). Eluent – water, flow rate – 1.5 mL/min, injection volume – 15 μL.

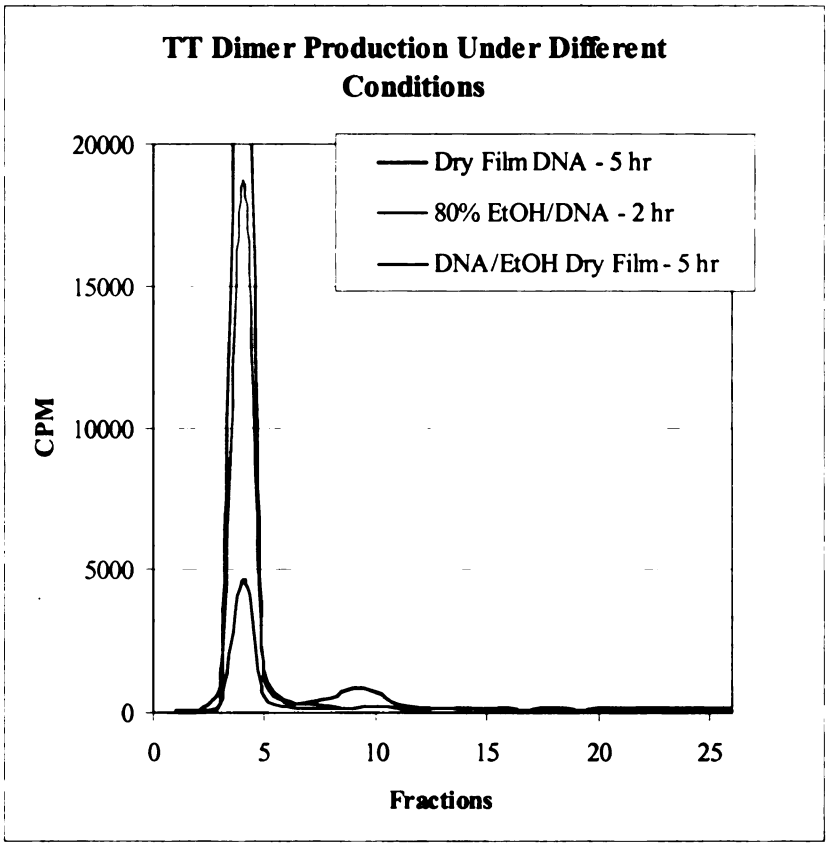


Figure III.1 Thymine dimer production depending on conditions. Waters phenyl-Spherisorb column (4 mm x 25 mm) was running with water as mobile phase at 1.5 mL/min flow rate for 25 minutes. Fractions were collected every minute. Thymine eluted after 3–4 minutes and thymine photoproducts eluted after 8-9 minutes.

In order to investigate how UV dosage affects formation of thymine dimers the following experiment was carried out. Based on research done by Nicholson and co-workers (44), our next experiments involved irradiation of dry ^{3}H-labeled DNA films at 254 nm for shorter time – 10, 20, and 30 minutes (UV dosage 4, 8 and 12 kJ/m^2, respectively). However these yielded no thymine-thymine photoproducts. The difference between the experiment carried by Nicholson and co-workers and ours was that they used DNA/SASP mixture and that could be a major reason that there was no SP formed in our experiment (Data not shown).

Since the irradiation of DNA dry film gave the highest yield of TT photoproduct, the next experiment was done by using photoproducts from dry film DNA. For the repair assay, 50 μL of DNA (9.3 pmol) were mixed with 2 μl (238 pmol) of SPL and other components as described in Materials and Methods, and incubated at 37 °C for different amounts of time. Then the samples were quenched with TFA and acid hydrolysis was performed. After hydrolysis the TFA was evaporated and the samples were resuspended in 100 μL of water. Each sample (15 μL) was injected onto a Waters phenyl-Spherisorb column and fractions were analyzed by scintillation counting. From the results (Figure III.2) we can see that TT photoproduct not treated with SPL elutes at 13-14 minutes; if incubated with SPL the peak elutes at 10-11 minutes. No significant repair was observed and therefore it might be possible, that TT dimer was not spore photoproduct. The difference in elution time between DNA and DNA/SPL mixtures could be explained the conformational change of thymine dimer or some unknown reasons. Other reason for the doubt was that under the same irradiation conditions thymine photoproduct was not reproducible, i.e. it eluted at different times from HPLC chromatography (the data not shown).

In order to investigate the hypothesis that the photoproduct eluting at 13-14 minutes was not SP, the following experiments were done. DNA, which was previously irradiated and hydrolyzed, was re-irradiated at 254 nm for 2 and 4 hours. Previous papers show (14, 45), that if cyclobutane dimers or (6-4) photoproducts are irradiated after acid hydrolysis, they are converted to thymines, while spore photoproduct remains unchanged.

The results from re-irradiation, either after 2 hours or 4 hours indicate that there were some changes between thymine-thymine dimers (Figures III.3 and III.4). The

unidentified thymine dimer eluted at 8-9 minutes before re-irradiation, but after 2 hours of re-irradiation it eluted at 11-12 minutes (Figure III.3). If the same DNA was re-irradiated for 4 hours, thymine dimers eluted after 9-10 minutes. This indicates that thymine dimer underwent some conformational changes or the thymine dimer was converted into new thymine dimer and therefore, it might be spore photoproduct. However, these results need further investigations, like mass spectrometry of thymine dimers.

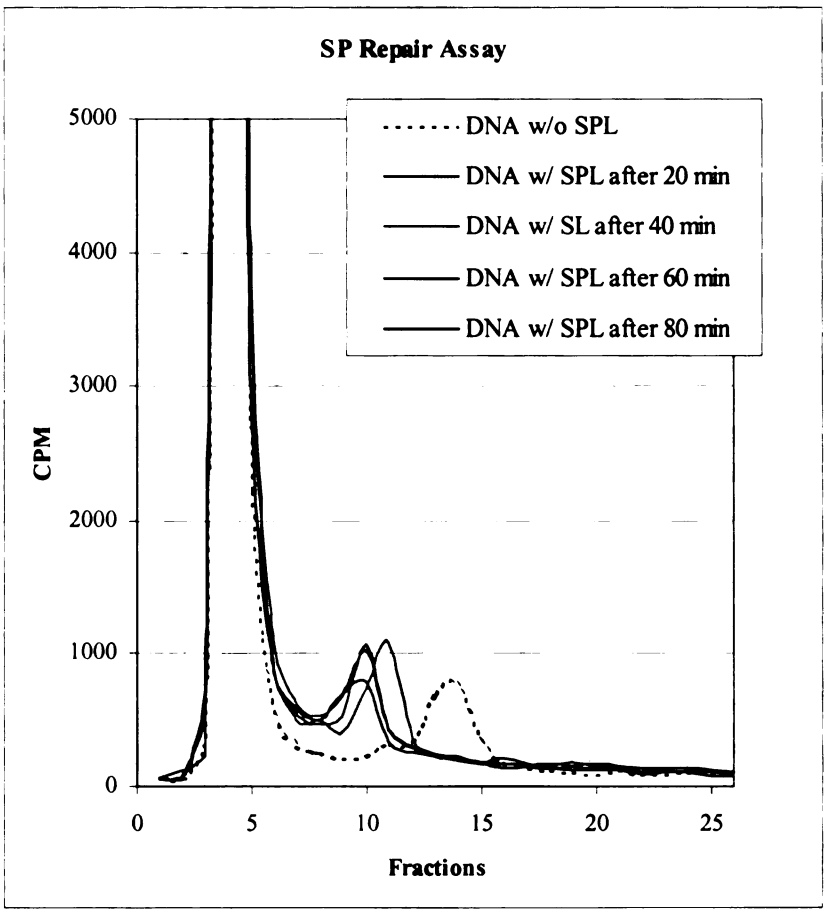


Figure III.2 SP repair assay. ^{3}H-labeled spore photoproduct was incubated with SPL for 20, 40, 60, and 80 minutes at 37 °C. Waters phenyl-Spherisorb column (4 mm x 25 mm) was running with water as mobile phase at 1.5 mL/min flow rate for 25 minutes.

Fractions were collected every minute. Thymine eluted after 3–4 minutes and thymine photoproducts eluted after 8-9 and 12 minutes.

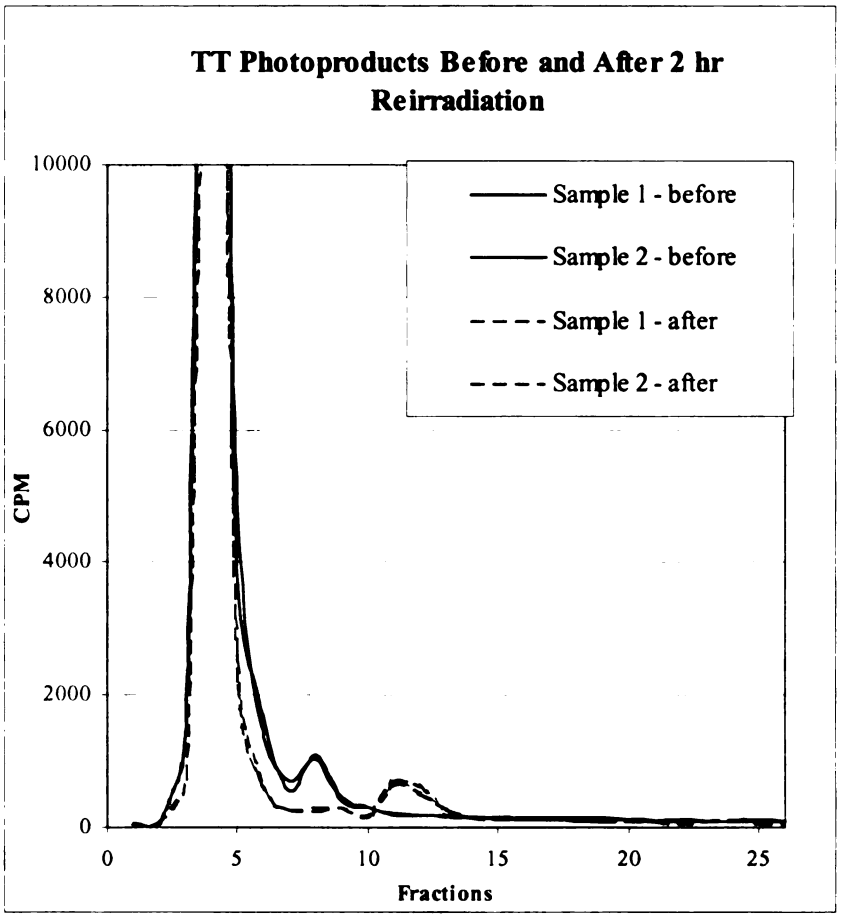


Figure III.3 Thymine dimers before and after 2 hours of re-irradiation at 254 nm wavelength. Waters phenyl-Spherisorb column (4 mm x 25 mm) was running with water as mobile phase at 1.5 mL/min flow rate for 25 minutes. Fractions were collected every minute. Thymine eluted at 3-4 minutes, thymine dimers – at 8-9 and 11-12.

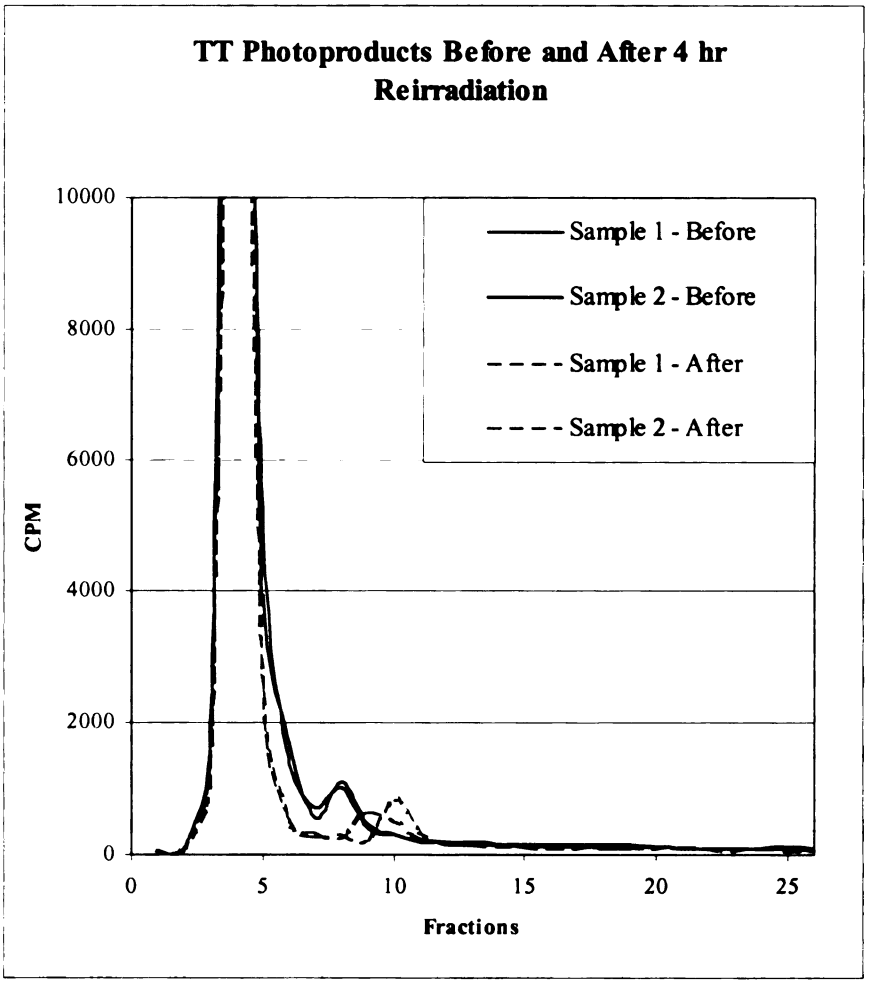


Figure III.4 Thymine dimers before and after 4 hours of re-irradiation at 254 nm wavelength. Waters phenyl-Spherisorb column (4 mm x 25 mm) was running with water as mobile phase at 1.5 mL/min flow rate for 25 minutes. Fractions were collected every minute.

CHAPTER IV

SPORE PHOTOPRODUCT LYASE BINDING TO DNA AND OLIGONUCLEOTIDES

IV.1. Introduction

The enzyme SP lyase is the first identified non-photoactivatable pyrimidine dimer lyase, and, together with the SASP's, confers on *Bacillus* spores their unusual resistance to UV radiation (18). SP lyase shares some sequence homology with the members of the DNA photolyase/(6-4) photolyase/blue light photoreceptor protein family (18). The conserved residues are in the general region believed to be involved in substrate binding by DNA photolyase (31), although no structures of DNA photolyase bound to pyrimidine dimers have been published. Computer-assisted comparisons performed between the deduced amino acid sequence of SP lyase and other DNA repair proteins revealed that a subset of the carboxyl half of SP lyase (amino acids from 170 to 250) shares sequence homology with the DNA photolyases from number of prokaryotes and lower eukaryots, suggesting that these two classes of enzymes may have descended from common ancestral protein (46).

The involvement of DNA repair systems in spore resistance has been studied most thoroughly for 254 nm radiation, where spores lacking NER or SP-specific repair are 2-3 times more UV-sensitive than wild-type spores (11). The NER system in *B. subtilis* appears generally similar to that in *E.coli* and the gene products involved are also similar (33). The NER system involves the products of a number of different genetic loci, while SP–specific repair is due only to products of the *spl* locus. Cloning and mapping of DNA

that complements an SP lyase-defective mutant revealed that the SP lyase (*spl*) locus is located at 118° on the *B.subtilis* map, immediately downstream from *ptsHI* operon, which

SPL	SPLDKRIEA**A**VKVAKA**GYP**LGFIVAPIYIHE**GW**EEGYRHLF	250
N.c.	WSYNVDHFH**A**WTQGRT**GFP**IIDAAMRQVLST**G**YMHNRLRMI	477
S.c.	WENNPVAFEKWCTGNT**GIP**IVDAIMRKLLYT**G**YINNRSRMI	454
E.c.	WQSNPAHLQ**A**WQEGKT**GYP**IVDAAMRQLNST**GW**MHNRLRMI	346
H.h.	WRDDPAALQ**A**WKDGET**GYP**IVDAGMRQLRAEAYMHNRVRMI	353
A.n.	WENREALFT**A**WTQAQT**GYP**IVDAAMRQLTET**GW**MHNRCRMI	353
S.g.	WRSDADEMH**A**WKSGLT**GYP**LVDAAMRQLAHE**GW**MHNRARML	334

Figure IV.1. One of the regions of sequence homology between SPL and DNA photolyases from *Neurospora crassa (N.c.), Saccharomyces cerevisiae (S.c.), Escherichia coli (E.c.), Halobacterium halobium (H.h.), Anacystis nidulans (A.n.), Streptomyces griseus (S.g.).* The conserved amino acids are shown in bold. The potential SPL binding site to DNA (helix-turn-helix) is underlined (47).

is the part of phosphoenolpyruvate:sugar phosphotransferase (PTS) system (48). Nucleotide sequence of the *B.subtilis spl* locus revealed that it is a two-gene operon, where the first gene (*splA*) encodes a protein of nearly 9 kDa and which in some way regulates expression of *spl* operon, and the second gene, *splB*, encodes an SP lyase, a protein of nearly 40 kDa (18, 49). The *spl* operon is expressed only in sporulation in the developing forespore, and is expressed in parallel with α/β-type SASP and transcribed by RNA polymerase with the same forespore–specific sigma factor (σ^G) that transcribes *ssp* genes (18). The *spl* operon is not expressed during spore germination and is not induced

by DNA damage in growing cells (48). The analysis of *splAB* operon revealed that (a) SP lyase may function in the cell as (at least) a homodimer, and (b) iron appears to be necessary for optimal SP lyase activity during spore germination (48, 50). Also, spores of mutants lacking the entire *splAB* operon exhibited the same UV sensitivity as spores lacking only *splB* and only deletion of *splB* resulted in loss of SP lyase repair capability (48).

It was shown that SplB containing a C-terminal 10His tag purified from an *E.coli* overexpression system bound to 35-bp double stranded DNA oligonucleotide containing a single SP. However, this (10His)SplB was unable to complete catalysis unless its N-terminal 10-His tag was first proteolytically removed at the engineered Xa site (44). Thus, the SP-specific binding and cleavage functions of SP lyase appear to be separable. SPL also specifically cleaved SP, but not *cis,syn* cyclobutane dimer, probably due to the fact that (10His)SplB does not bind to pyrimidine dimer-containing DNA with high affinity(44).

Although the three dimensional structure of DNA containing SP has not yet been elucidated, it is known that *cis,syn* cyclobutane dimer distorts DNA, producing a helical kink of 27^{o} and unwinding of 19.7^{o} (2). Because SPL binds SP but not Pyr<>Pyr with high affinity, it presumably recognizes an SP-specific helical distortion in DNA which differs in its geometry from distortion caused by *cis,syn* cyclobutane dimer (44). Binding of SPL to SP apparently introduces additional distortion in the helix (44). Enhancement in distortion of Pyr<>Pyr containing DNA by binding of DNA repair proteins has also been seen in the Uvr(A)BC exonuclease, DNA photolyase, and phage T4 endonuclease V. Once SP-specific binding occurs, the [4Fe-4S] cluster of SP lyase interacts with

AdoMet, presumably resulting in the creation of a 5'-adenosyl radical, which participates in reversal of SP to two thymines, likely by radical fragmentation (44).

Despite what we know about DNA photolyase and how it recognizes and binds to DNA, there is very little knowledge about how SPL binds to DNA. Therefore a lot of research has to be done to investigate SPL-DNA interactions, how SPL recognizes DNA damage and if SPL binding depends on DNA sequence.

IV.2. Experimental methods

IV.2.1. Materials

Restriction enzyme EcoR I was purchased from New England BioLabs. The oligonucleotides JC1, JC2, TK4-72a, and TK4-72b were purchased from Integrated DNA Technologies. [γ-^{32}P]-ATP (10 μCi/μL, 3000 Ci/mmol) was purchased from MP Biomedicals.

IV.2.2 Agarose gel-based gel-shift assay

IV.2.2.1 Digestion of pUC18 DNA

NE buffer (9 μL) (50 mM NaCl, 100 mM Tris-HCl, 10 mM EDTA, 1 mM dithiothreitol, 0.15% Triton X-100, 200 μg/ml BSA and 50% glycerol), 9 μL BSA, 6 μL EcoR I (111 units), 66 μL pUC18 DNA (40 μg) (total volume 90 μL) were mixed and incubated at 37 °C for 1 hour. Then the reaction mixture was inactivated at 65 °C for 20 minutes. The linear DNA was stored at –20 °C.

IV.2.2.2 The extraction of linear pUC 18 DNA from agarose gel

Linearized pUC18 DNA was purified from a 1% agarose gel (1g of agarose, 100 mL TAE Buffer and 30 μL ethidium bromide (10 mg/mL)) as follows. Loading dye (2 μL) was added to 25 μL of DNA mixture and loaded onto agarose gel. The standard 1 kb DNA Ladder (500 μg/mL, New England BioLabs) was also loaded. A 100 V current was applied to the gel. DNA was extracted using the Compass DNA Purification Kit (American BioAnalytical) and stored at –20 °C.

IV.2.2.3 SPL binding to pUC 18 DNA

An agarose gel (0.5 %) was made of 0.5 g of agarose, 100mL of TAE Buffer and 30 μL ethidium bromide (10 mg/mL). The samples, containing either circular or linear pUC18 DNA, were mixed in the Coy anaerobic chamber at 4 °C. The samples also contained 4 μL of 50% glycerol and 2 μL of binding buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT). Then samples were incubated for 2 hours at 4 °C. Then 2 μL of loading dye was added to each of the sample and mixed. The samples were electropheresed through 0.5% agarose gel at 100 V and 4 °C.

IV.2.3 Gel-shift assay using polyacrylamide gel electrophoresis

IV.2.3.1 The 5'-end labeling of 35-bp oligonucleotide

Two complementary oligonucleotides were synthesized (Integrated DNA Technologies), one 35-mer containing a single pair of adjacent thymines (JC1 oligonucleotide, MW = 10,749 Da, 5'- CCC GGG GAT CCT CTA GAG **TT**G ACC TGC AGG CAT GC –3'), and its complementary oligonucleotide (JC2 oligonucleotide,

MW = 10,758 Da, 5'- GCA TGC CTG CAG GTC AAC TCT AGA GGA TCC CCG GG –3')).

JC1 oligomer (1 μL, 5.8 pmoles or 0.17 μg) was added to 5X Exchange Reaction Buffer (Invitrogen), 5 μL [γ-^{32}P] ATP (10 μCi/μL, 3000 Ci/mmol), 0.5 μL T4 polynucleotide kinase (Invitrogen), 13.5 μL autoclaved MQ water. Total volume was 25 μL. The mixture was incubated for 10 minutes at 37 °C followed by heat inactivation at 65 °C for 10 min. Ethanol precipitation was carried out as follows. To the Eppendorf tube containing labeled oligomer was added 250 μL 4.67 M NH_4OAc and 750 μL 100 % ethanol (ice cold). Then the sample was kept on ice for 30 minutes and centrifuged at 12,000 rpm for 20 minutes. The supernatant was removed and 500 μL of 80 % ethanol (ice cold) was added. The sample was centrifuged at 12,000 rpm for 20 minutes and supernatant was removed. The oligomer was then dried under a N_2 stream and resuspended in 50 μL TAE Buffer. The labeled strand was hybridized to the unlabeled complementary strand by heating at 90 °C for 2-3 minutes and then allowing to cool down to room temperature. The hybridized oligo was purified using a mini Quick Spin Oligo Column (Roche Applied Science). The oligo was stored at –80 °C in the freezer.

IV.2.3.2 Gel-shift assay of 35-bp oligonucleotide

The gel-shift assay reactions were prepared in a total volume f 20 μL containing 50% glycerol, 10 mM Tris-HCl pH 7.5; 0 or 50 or 200 mM NaCl, 0 or 0.5 or 2 mM DTT, 0.1% NP-40, poly(dI-dC) (to prevent non-specific interactions – omitted for some experiments), 0.22 pmol of the labeled double-stranded oligonucleotide, and spore photoproduct lyase. The assay reactions were prepared anaerobically in an Mbraun glove

box and incubated for 1 hour at room temperature. The assays were electrophoresed through a 12% or 8% non-denaturing polyacrylamide gel at 20 V/cm. Then the gel was taken out and placed in a dish with 7% acetic acid for 10 minutes. After that the gel was placed between two gel drying films, put in the frame and dried overnight. The electrophoresis products were visualized by autoradiography (X-Omat AR-5 film (Kodak)).

IV.2.3.3 5'-End labeling of 94-bp oligonucleotide

Two other complementary oligonucleotides were synthesized (Integrated DNA Technologies), based on *Bacillus subtilis* sequence (from 322456 to 322550). Oligonucleotides had 94 nucleobases:

TK4-72a (MW = 28,902 Da) 5'- CGG GAT CAA CCA GAG CAT CAT GCT TGC GTT ATC AAT GGT TGT TAT CGC CGC AAT GGT CGG TGC ACC GGG ACT TGG TTC TGA AGT ATA CAG TGC C –3' and

TK4-72b (MW = 29,057 Da) 5'- GGC ACT GTA TAC TTC AGA ACC AAG TCC CGG TGC ACC GAC CAT TGC GGC GAT AAC AAC CAT TGA TAA CGC AAG CAT GAT GCT CTG GTT GAT CCC G -3'

TK4-72a oligomer (1 μL, 5.8 pmoles or 0.17 μg) was added to 5X Exchange Reaction Buffer (Invitrogen), 5 μL [γ-^{32}P] ATP (10 μCi/μL, 3000 Ci/mmol), 0.5 μL T4 polynucleotide kinase (Invitrogen), 13.5 μL autoclaved MQ water. Total volume was 25 μL. The mixture was incubated for 10 minutes at 37 °C followed by heat inactivation at 65 °C for 10 min. Then oligomer was purified by ethanol precipitation as described above.

IV.2.3.4. Gel-Shift assay of 94-bp oligomer

The 8% polyacrylamide gel was prepared using 10 mL 40% acrylamide stock solution, 1 mL 50X TAE Buffer, 0.35 mL ammonium persulfate (0.1 g/mL) and 17.5 μL TEMED. The mixture was poured between two glass plates using pipette. The gel polymerized within 1 hour. The gel then was pre-electrophoresed (100 V, 10 V/cm).

The samples were mixed in the Coy anaerobic chamber at 4 °C and were incubated for 2 hours at 4 °C. Then 2 μL of loading dye were added to each sample and the samples were loaded onto the gel. Gel was run for 3 hours (200 V, 20 V/cm). Then the gel was taken out and placed in a dish with 7% acetic acid for 10 minutes. After that the gel was placed between two gel drying films, put in the frame and dried overnight. The electrophoresis products were visualized by autoradiography and shown in figures IV.5 and IV.6.

IV.3 Results and discussion

The gel-shift assay provides a method for detecting DNA-binding proteins. The assay is based on the observation that complexes of protein and DNA migrate through a gel more slowly than free DNA fragments or double-stranded oligonucleotides. The gel-shift assay is performed by incubating a purified protein with a non-labeled DNA or ^{32}P end-labeled DNA fragment containing the putative protein-binding site. The reaction products are then analyzed on an agarose or on a non-denaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using DNA fragments or oligonucleotides containing a binding

site for the protein of interest or other unrelated DNA sequences. SPL binding properties were tested using pUC18 DNA and short oligonucleotides. Two types of gels were used for this experiment – agarose and polyacrylamide. Agarose gel was chosen because of the experiment's simplicity and because DNA doesn't require radioactive labeling. Disadvantage of this method is that it is not as accurate as polyacrylamide gel, but it is sufficient for the preliminary studies. The resolving power of polyacrylamide gel is much greater and it can separate molecules of DNA whose length differ by as little as 0.2% (ref). For polyacrylamide gel-shift assay two types of short oligonucleotides were used. The 35-bp length oligonucleotide had only two adjacent thymines; the 94-bp length oligonucleotide had more than two adjacent thymines. The experiments were carried to investigate if SPL binding to DNA, if this binding is specific and if it depends on the number of adjacent thymines.

IV.3.1 Agarose gel-based gel-shift assay

First SPL binding properties were tested on circular pUC18 DNA. However, the results were somewhat not reliable due to DNA tendency to be in two forms – supercoiled and nicked which caused appearance of two bands on agarose gel (data not shown). Therefore for the next experiment we included only supercoiled DNA purified separately from the nicked DNA. The other type of DNA used for this experiment was linear pUC18 DNA. The samples, containing pUC18 DNA and different amounts of binding buffer (Table IV.1), were mixed with SPL and incubated for 1 or 2 hours and loaded onto agarose gel. Lanes 1-7 had supercoiled pUC18 DNA, lane 8 had a 1 kb DNA Ladder standard, and lanes 9-12 contained linear pUC18 DNA. For the supercoiled DNA

the effect of salt concentration (binding buffer) was also tested. Therefore lanes 1-4 had 2 μL of binding buffer and lanes 5-7 contained no binding buffer (Figure IV.2). Lanes 1, 5 and 9 had no SPL; lanes 2-4, 6-7 and 10-12 had increasing number of protein. Band shifting in lanes 2-4, 6-7, and 10-12 clearly indicates SPL binding; DNA bands were more retarded as the concentration of enzyme was increased. For supercoiled DNA, however, we can see two bands (lanes 1 and 5), which suggest that some of the DNA became nicked even though supercoiled DNA was purified from nicked DNA prior running the gel. Therefore it is hard to say if SPL is binding supercoiled DNA, nicked DNA or both (from the results we can see, that both bands were shifted after adding SPL - lanes 2-4 and 6-7). The other problem that occurred, was that some of the DNA, both supercoiled and linear, were stuck on the wells of the gel. The reason of that could be the aggregation of SPL and to avoid this problem for later experiments the DNA and protein mixtures were incubated at 4 °C temperature and the gel was run at 4 °C as well. Due to instability of supercoiled DNA, later experiments included only linear pUC18 DNA, which was cut with the restriction enzyme EcoRI and purified from agarose gel.

For the second gel (Table IV.2, Figure IV.3) lane 1 had no SPL and lanes 2-5 had increasing amount of enzyme; lane 6 had a 1 kb DNA Ladder standard. The second gel also had broader concentration range than the previous one. From this gel we can see that much less of DNA-protein adduct is stuck on the wells of the gel. Also, one SPL molecule can bind up to 12 DNA base pairs, which means that SPL might be binding not only to thymines. From these results we can make several conclusions, (i) SPL does bind to DNA, (ii) SPL binding might be non-specific, (iii) SPL can dimerize or bind to itself.

Table IV.1 DNA and SPL binding conditions for agarose gel in Figure IV.2

	Lanes											
	Supercoiled DNA							Std.	Linear DNA			
	1	2	3	4	5	6	7	8	9	10	11	12
DNA, μL	3	3	3	3	3	3	3		3	3	3	3
Binding Buffer, μL	2	2	2	2	0	0	0		2	2	2	2
50% Glycerol, μL	4	4	4	4	0	0	0		4	4	4	4
MQ Water, μL	11	9	7	5	17	15	13		11	9	7	5
SPL, μL	0	2	4	6	0	2	4		0	2	4	6
Conc. of SPL, nmol	0	0.04	0.09	0.14	0	0.04	0.09		0	0.04	0.09	0.14
Molecules per base pair	0	0.027	0.055	0.083	0	0.027	0.055		0	0.02	0.04	0.077

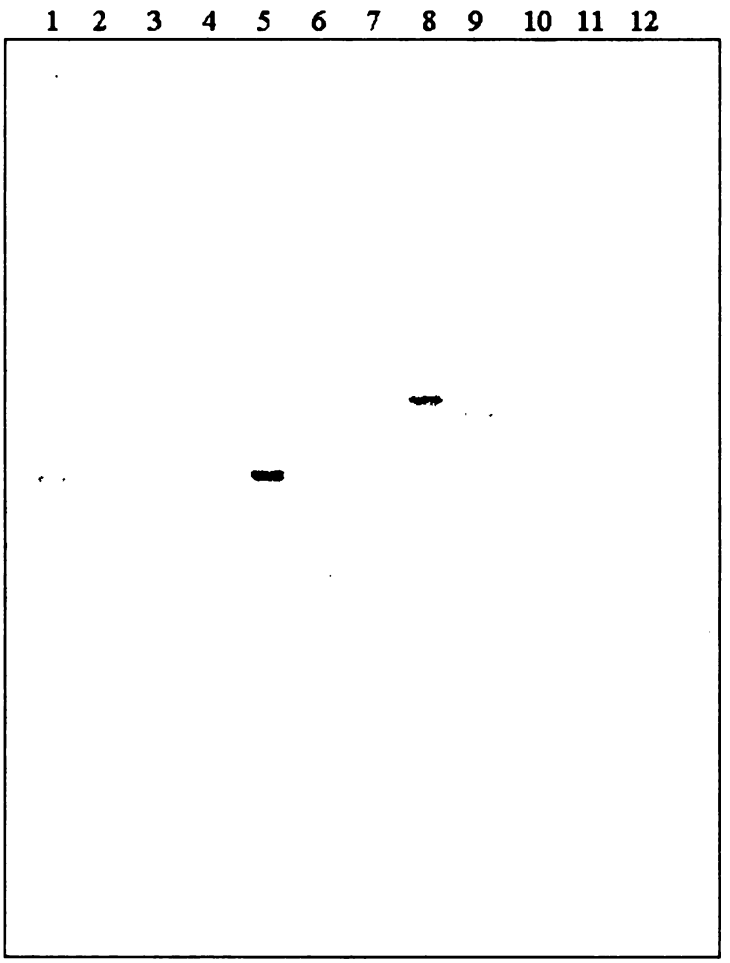


Figure IV.2 Agarose gel of circular pUC18 DNA (lanes 1-4), supercoiled pUC18 DNA (lanes 5-7) and linear pUC18 DNA (lanes 9-12), standard – lane 8.

Table IV.2 Linear DNA and SPL binding conditions for agarose gel in Figure IV.3

	Lanes					
	1	**2**	**3**	**4**	**5**	**6**
Binding Buffer, μL	2	2	2	2	2	
50% Glycerol, μL	4	4	4	4	4	
DNA, μL	4	4	4	4	4	
MQ Water, μL	10	8	6	4	2	
SPL, μL	0	2	4	6	8	
Conc. of SPL, nmol	0	0.04	0.09	0.14	0.18	
Molecules per base pair	0	0.02	0.04	0.077	0.083	

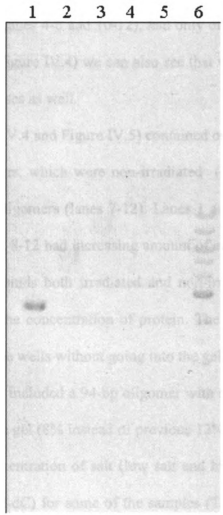


Figure IV.3 Agarose gel of linear pUC18 DNA (lanes 1-5); standard – lane 6. The samples were incubated and the gel was run at 4 °C.

IV.4.2 Gel-shift assay using polyacrylamide gel electrophoresis

The gel-shift assays were done in order to confirm, that SPL binds to DNA and to find the binding constant. Binding assays were carried out for two different oligonucleotides, 35-bp with only two adjacent thymines and 94-bp with several adjacent thymine dimers. Also, the SPL binding was tested for non-irradiated oligomers and irradiated at 254 nm oligomers.

When 35-bp oligomer was not purified, lanes 1-3 and 7-9 (Table IV.3, Figure IV.4), it had too much contamination, while the oligomer, which was purified with mini Quick Spin Oligo Column (lanes 4-6 and 10-12), had only oligomer and it's adduct with the protein. From this gel (Figure IV.4) we can also see that while increasing the amount of protein the binding increases as well.

The next gel (Table IV.4 and Figure IV.5) contained only purified oligomers. Half of the samples had oligomers, which were non-irradiated (lanes 1-6) and another half with irradiated at 254 nm oligomers (lanes 7-12). Lanes 1 and 7 had only oligomer and no SPL, while lanes 2-6 and 8-12 had increasing amount of enzyme. From the results we can see, that SPL equally binds both irradiated and non-irradiated oligomers; binding increases while increasing the concentration of protein. The only problem was, that all bound oligomer stayed on the wells without going into the gel.

The next experiment included a 94-bp oligomer with more adjacent thymines and lower percentage acrylamide gel (8% instead of previous 12%). The other conditions that were changed, was the concentration of salt (low salt and high salt or even no salt), as well as skipping the poly(dI-dC) for some of the samples (Table IV.5 and Figure IV.6). The incubation was carried out at 4 ^{o}C for 1 hour and gel was running at 4 ^{o}C to help to

prevent the precipitation or aggregation. The best binding was achieved, when reaction mix had low concentration of salt and no poly(dI-dC), which probably binds to SPL as well.

The gel-shift assay was repeated with oligomer at low concentration of salt and decreased concentration of poly(dI-dC) (10 fold, lanes 1 to 7). Lanes 8-14 had no poly(dI-dC). Lanes 1 and 8 had no enzyme; lanes 2-7 and 9-14 had increasing amounts of the SPL (Table IV.6 and Figure IV.7). It was observed, that the binding is better without the poly(dI-dC) and the full binding was observed when the concentration of SPL was 0.8 molecules of enzyme per base pair of oligomer.

The conclusions from this set of the experiments are, (i) the change of acrylamide concentration from 12% to 8% did not change the results and DNA-SPL adducts were stuck on the wells of the gel, (ii) the temperature change for the incubation (from 22 to 4 ^{o}C) also did not make big difference, (iii) the best binding was observed at low salt concentration.

Table IV.3 The binding conditions for gel-shift assay

	Lanes											
	Non-irradiated Oligo						Irradiated Oligo					
	Non-purified			Purified			Non-purified			Purified		
	1	2	3	4	5	6	7	8	9	10	11	12
Binding Buffer, μL	2	2	2	2	2	2	2	2	2	2	2	2
50% Glycerol, μL	4	4	4	4	4	4	4	4	4	4	4	4
poly (dI-dC), μL	1	1	1	1	1	1	1	1	1	1	1	1
DNA, μL	2	2	2	2	2	2	2	2	2	2	2	2
MQ Water, μL	11	10.8	10.6	10.4	10.2	10	11	10.8	10.6	10.4	10.2	10
SPL, μL	0	0.2	0.4	0.6	0.8	1	0	0.2	0.4	0.6	0.8	1
Conc. of SPL, nmol	0	2.4	4.8	7.1	9.5	11.9	0	2.4	4.8	7.1	9.5	11.9
Molecules per oligo	0	109	218	318	437	583	0	109	218	318	437	583

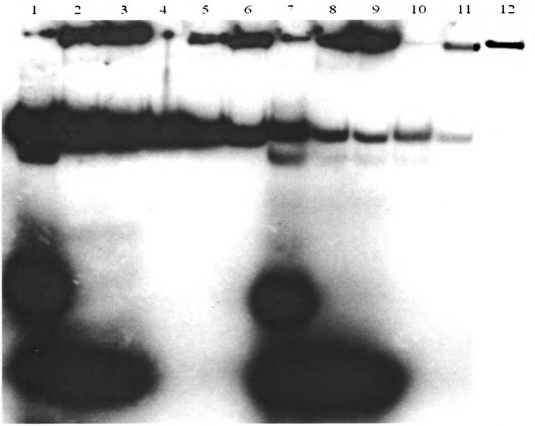


Figure IV.4 Gel-shift assay of the ^{32}P-labeled 35-bp oligonucleotide. Lanes 1 to 6 had non-irradiated oligomer and lanes 7 to 12 had irradiated oligomers. Samples 1-3 and 7-8 were not purified and the samples 4-6 and 10-12 were purified by using mini Quick Spin Oligo Column (Roche Applied Science).

Table IV.4 The binding conditions for gel-shift assay

	Lanes									
	Non-irradiated Oligo					Irradiated Oligo/SP				
	1	**3**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Binding Buffer, μL	2	2	2	2	2	2	2	2	2	2
50% Glycerol, μL	4	4	4	4	4	4	4	4	4	4
poly(dI-dC), μL	1	1	1	1	1	1	1	1	1	1
1% NP-40, μL	1	1	1	1	1	1	1	1	1	1
Oligo, μL	1	1	1	1	1	1	1	1	1	1
MQ Water, μL	11	10.6	10.4	10.2	10	11	10.6	10.4	10.2	10
SPL, μL	0	0.4	0.6	0.8	1	0	0.4	0.6	0.8	1
Conc. of SPL, mmol	0	4.8	7.1	9.5	11.9	0	4.8	7.1	9.5	11.9
Molecules per oligo	0	437	700	875	1166	0	437	700	875	1166

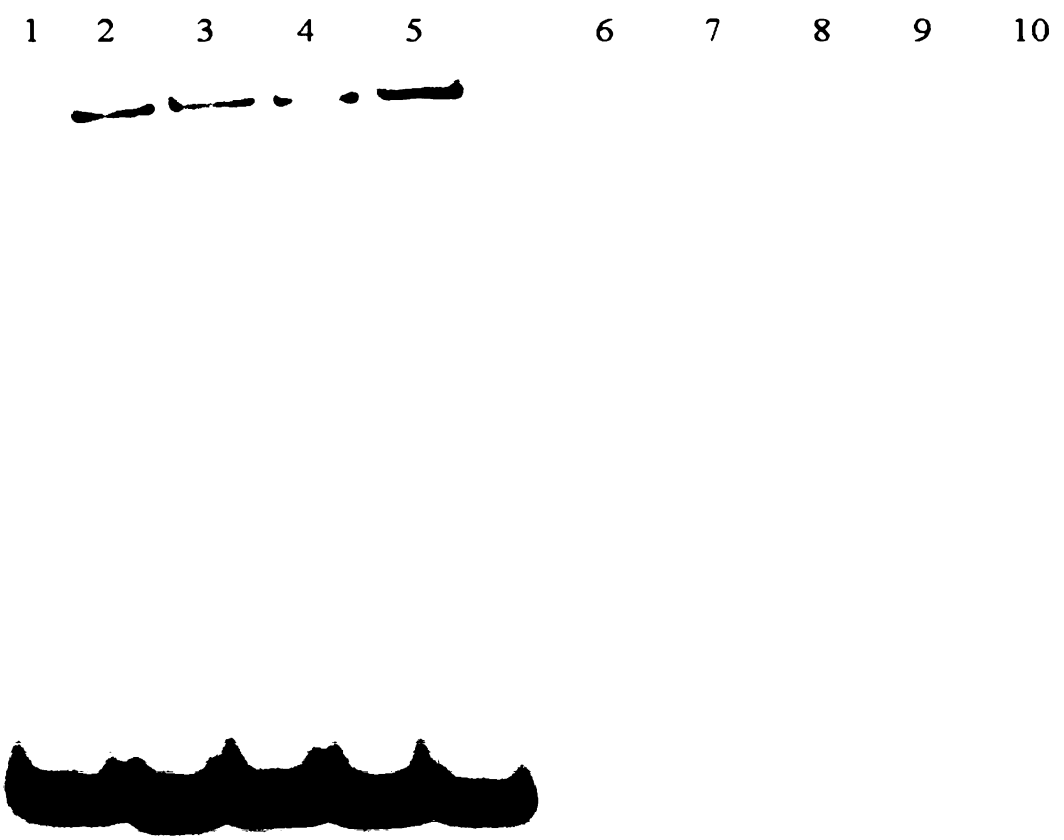


Figure IV.5 Gel-shift assay of the ^{32}P-labeled 35-bp oligonucleotide. Lanes 1-5 had non-irradiated oligomer and lanes 6-10 had irradiated oligomers. Lanes 1 and 6 had no SPL; lanes 2-5 and 7-10 had increasing amounts of SPL.

Table IV.5 The binding conditions for gel-shift assay

	Lanes												
	With poly (dI-dC)					Without poly (dI-dC)							
	Low Salt					No Salt				High Salt			
	1	2	3	4	5	6	7	8	9	10	11	12	13
Binding buffer, μL	2	2	2	2	2	0	0	0	0	8	8	8	8
50% Glycerol, μL	4	4	4	4	4	4	4	4	4	4	4	4	4
Poly (dI-dC), μL	1	1	1	1	1	0	0	0	0	0	0	0	0
1% NP-40, μL	1	1	1	1	1	1	1	1	1	1	1	1	1
Oligomer, μL	1	1	1	1	1	1	1	1	1	1	1	1	1
SPL, μL	0	1	3	5	7	0	1	3	5	0	1	3	5
MQ Water, μL	11	10	8	6	4	14	13	11	9	6	5	3	1
Conc. of SPL, pmol	0	2.26	6.78	11.3	15.8	0	2.26	6.78	11.3	0	2.26	6.78	11.3
Molecules per oligo	0	19	58	95	134	0	19	58	95	0	19	58	95

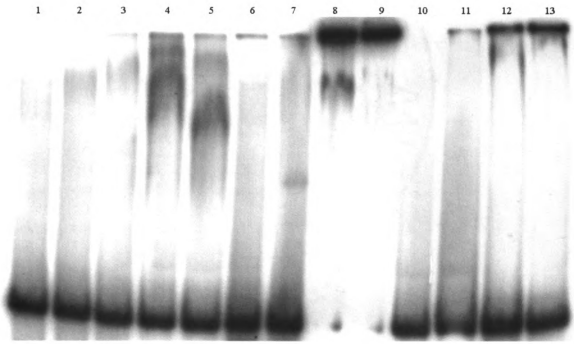


Figure IV.6 Gel-shift assay of the ^{32}P-labeled 94-bp oligonucleotide. Lanes 1-5 are the samples incubated with poly (dI-dC) at low ionic strength (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT); lanes 6-9 are the samples that had no poly(dI-dC) and no salt; lanes 10-13 are the samples incubated without poly(dI-dC) at high ionic strength (40 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM DTT). Lanes 1,6,10 had no SPL.

	Lanes													
	With poly(dI-dC)							Without poly(dI-dC)						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Binding buffer, μL	2	2	2	2	2	2	2	2	2	2	2	2	2	2
50% Glycerol, μL	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Poly (dI-dC), μL	1	1	1	1	1	1	1	0	0	0	0	0	0	0
1% NP-40, μL	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Oligomer, μL	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MQ Water, μL	11	10	9	8	7	6	5	12	11	10	9	8	7	6
SPL, μL	0	1	2	3	4	5	6	0	1	2	3	4	5	6
Conc. of SPL, pmol	0	2.26	4.52	6.78	9.04	11.3	13.6	0	2.26	4.52	6.78	9.04	11.3	13.6
Molecules per oligo	0	19	37	58	78	94	117	0	19	37	58	78	94	117

Table IV.6 The binding conditions for gel-shift assay

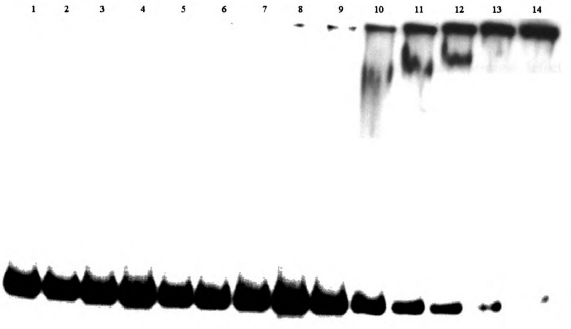


Figure IV.7 Gel-shift assay of the ^{32}P-labeled 94-bp oligonucleotide. Lanes 1-7 are the samples that were incubated with poly (dI-dC) at low ionic strength (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT); lanes 8-14 are the samples that were incubated without poly(dI-dC) at low ionic strength (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT); the lanes 1 and 8 had no SPL.

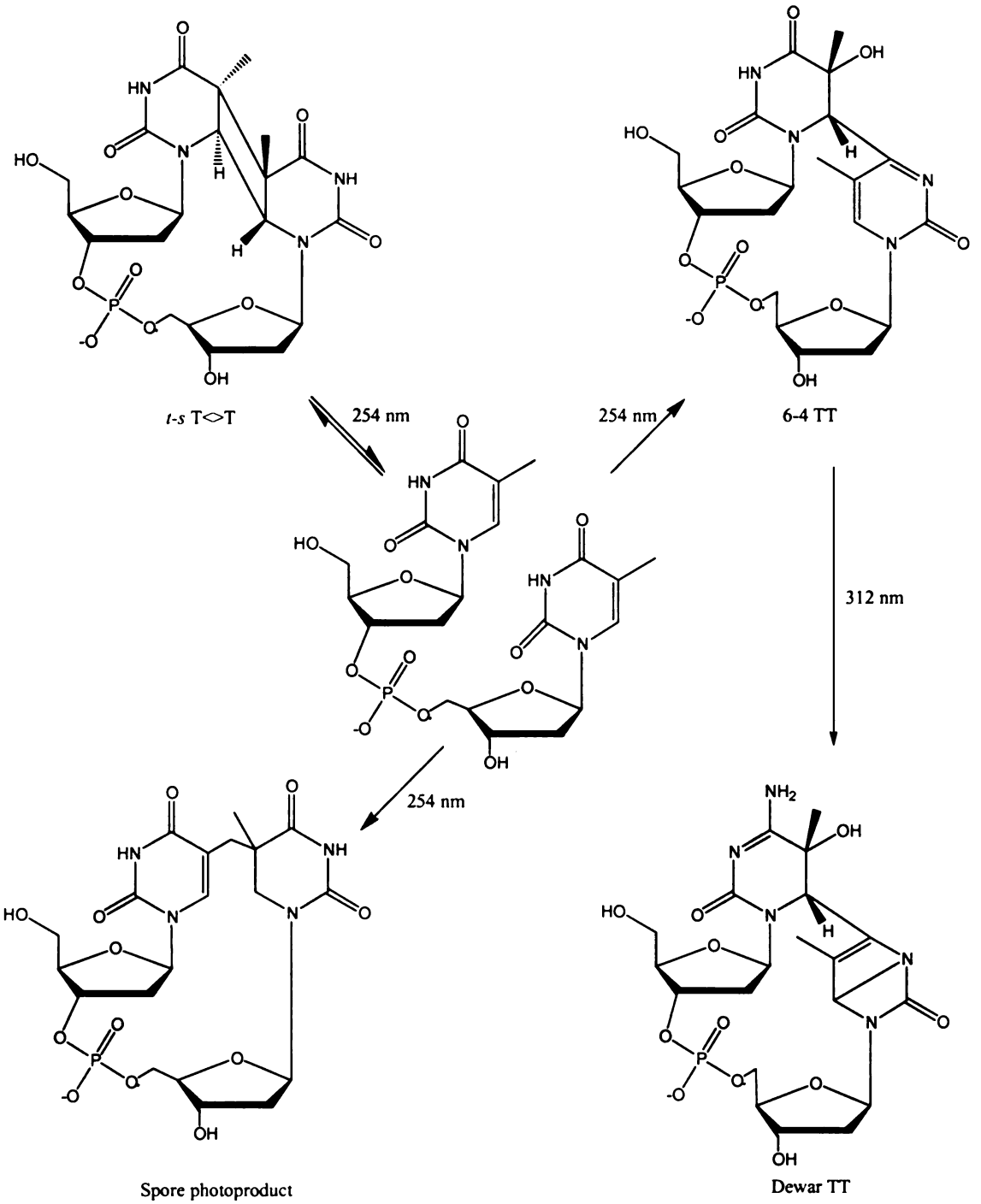
CHAPTER V

PHOTOSYNTHESIS OF SPORE PHOTOPRODUCT FROM THYMIDINE

V.1 Introduction

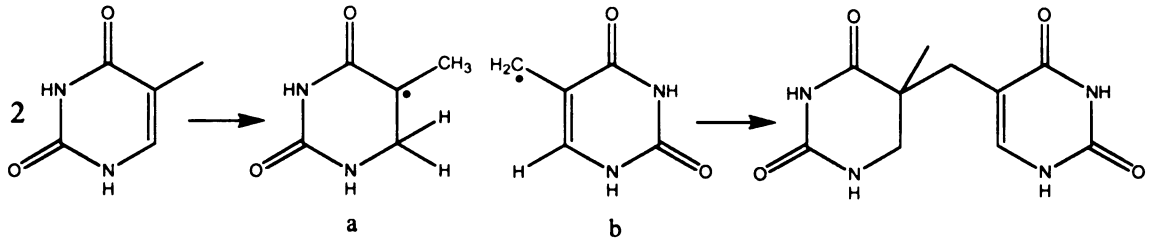
DNA bases are the specific chromophores of nucleic acids in the ultraviolet region of the spectrum. Unlike other chromophores involved in photobiology, such as chlorophyll in photosynthesis or retinaldehyde in vision phenomena, some of the photochemical reactions that are induced in nucleic acids by UV light give rise to unwanted biological effects at the cellular level (3, 51). Those effects could be cell death, photomutagenesis, and photocarcinogenesis. A bacterium lacking the normal repair mechanism has a 0.5 death probability when only 10 molecular photochemical defects are created per DNA molecule ($\sim 10^7$ nucleotides)(6).

DNA structure contains four different bases – adenine (A), guanine (G), thymine (T), and cytosine (C). Within nucleic acids, the hydrogen atoms linked to the nitrogen N(1) of the purine bases (A and G) and to the nitrogen N(1) of the pyrimidine bases (T and C) are substituted by a five-carbon sugar (2-deoxy-D-ribose in DNA). The complete nucleic acid building blocks involve the presence of phosphoric acid. DNA damage is induced by the direct absorption of incident UVB and UVC photons by nucleobases, whose excited states can be populated by direct UV irradiation. As a result, dimeric photoproducts, such as various isomeric cyclobutane type dimers, pyrimidine (6-4) pyrimidone photoadducts, and 5,6-dihydro-5-(α-thyminyl)-thymine (spore photoproduct) are produced between adjacent pyrimidine bases (Scheme V.1)(3).



Scheme V.1 The formation of various thymidine photoproducts under UV irradiation

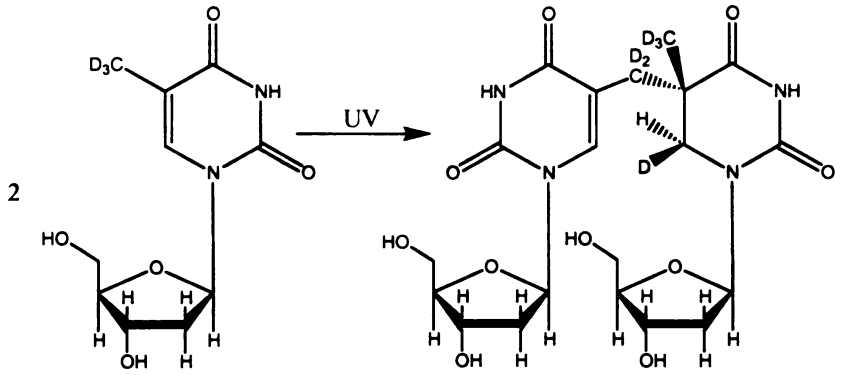
Cyclobutane pyrimidine dimers are generally the most common type of UV-induced DNA damage in cells. Early experiments have shown, that the cyclobutane pyrimidine dimers are no longer the main DNA lesions induced by monochromatic UV light in highly dehydrated microorganisms such as bacterial spores(11), or when DNA is irradiated either at low temperature or as a solid film (43, 52). Under these conditions the major photoproduct is SP, which has two diastereomers (5R* and 5S*) and which can be separated on reversed phase or silica gel HPLC columns and characterized by FAB mass spectrometry or ^{1}H and ^{13}C NMR analysis (53). Interestingly, only one of the two possible SP diastereomers was obtained when thymidine was irradiated as a dry film and while both diastereomers were obtained in frozen solution (54). Different stereochemistry of the photoreaction is due to different stacking of nucleosides in different forms of DNA (5). Two mechanisms have been proposed for the formation of SP. The first one involves the recombination of the photogenerated 5-(2'-deoxyuridyl)methyl and the 5,6-dihydrothymid-5-yl radicals (53). From electron spin resonance studies many investigators have reported the formation of thymyl radical (Scheme V.2, a (5)) in DNA and thymidine as a result of ultraviolet irradiation. The thymyl radical is formed by the addition of a hydrogen atom at the C(6) position of a thymine moiety. In situations such as may exist in dry or frozen DNA or thymidine, the methyl group of an adjacent



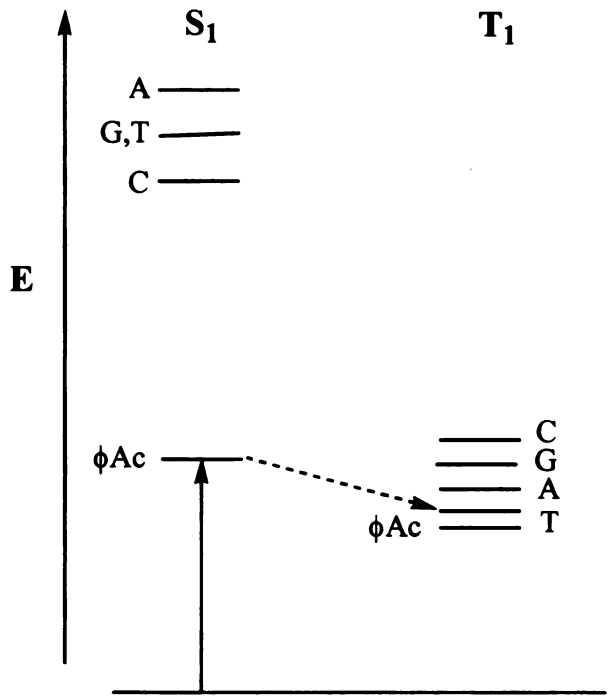
Scheme V.2 The UV-light induced formation of thymyl and thyminyl radicals (55)

thymine residue may serve as the hydrogen donor. The resulting thyminyl radical (Figure V.2, b) adds on to the thymyl radical, forming stable spore photoproduct (56).

The second proposed mechanism is a concerted mechanism (Scheme V.3) (53), which has been shown to take place in the frozen state while using methyl deuterated [CD_3] thymidine as a substrate (53, 54). Interestingly, the deuteration of the C(6) carbon was found to take place in highly stereospecific way, since only one singlet corresponding to one of to H(6) protons is observed in the ^1H NMR spectrum of the hexadeuterated diastereomers which has nonmagnetically equivalent methylene protons (53). The photosensitization experiment of thymidine by benzophenone in the dry state also suggests that a concerted mechanism is involved in the formation of SP in dry films of thymidine (52, 54). Photosensitization is reaction when DNA damage can be resulted from wavelengths in the electromagnetic spectrum, which, though not absorbed directly by bases, are absorbed by other molecules (sensitizer molecules) that then transfer energy to bases of in DNA. Sensitizing molecules can behave as a photocatalyst or it can be consumed. The photosensitization reactions that damage DNA may include the formation of thymine dimers by triplet excitation transfer. Various ketones promote pyrimidine dimer formation, a notable example being acetophenone. The lowest triplet energy state of acetophenone is slightly higher than that of thymine but lower that the triplet states of other DNA bases (Scheme V.4). Upon irradiation of DNA in the presence of acetophenone at wavelength of ~300 nm, the triplet energy state of the photosensitizer is transferred to thymine, facilitating the formation of thymine dimers (1). However, some investigators used benzophenone for the generation of the SP instead (54).



Scheme V.3 Concerted mechanism for the formation of the hexadeuterated 5,6-dihydro 5-(α-thymidyl) thymidine upon exposure to far-UV light in frozen aqueous solutions (53).



Scheme V.4 The energy levels of the lowest excited singlet states (S_1) and lowest triplet states (T_1) of adenine (A), guanine (G), cytosine (C), thymine (T), and acetophenone (ϕAc) (Adapted from Ref. (1))

V.2. Experimental methods

V.2.1 Materials

Thymidine and benzophenone were purchased from Sigma-Aldrich.

V.2.2 Recrystallization of thymidine and benzophenone

Benzophenone was recrystallized from ethanol. Benzophenone (100 mg) was dissolved in minimum amount of ethanol while heating, filtered through filter paper (Whatman) into an Erlenmayer flask, and the filtrate was placed on ice to crystallize. The crystals were filtered using vacuum filtration, washed with a small amount of ice-cold ethanol, and dried.

Thymidine (2 g) was added to 20 mL of ethanol and heated to boiling. Since the solution was not clear, 10 mL of water and some activated carbon was added. This hot mixture was filtered through paper filter into an ice-cold Erlenmayer flask kept on ice. White precipitate formed. The precipitate was filtered and dried.

V.2.3 Photosynthesis of SP

The synthesis of spore photoproduct from thymidine in the solid state was carried out as follows. Recrystallized thymidine (100 mg) and 50 mg of recrystallized benzophenone were dissolved in 50 mL of ethanol in a 6" test tube (Pyrex). The ethanol was then evaporated overnight in the fume hood. The samples were irradiated either in a Rayonet photoreactor, which was equipped with 8 UV lamps (>290 nm) or in a custom made photoreactor, which was equipped with 2x40 W 350 nm lamps (see Table V.1).

Then some of the sample was dissolved in D_2O and transferred to an NMR tube for ^1H NMR analysis.

The synthesis of spore photoproduct from thymidine in the liquid state was done as follows. Thymidine (50 mg) and 25 mg of benzophenone were dissolved in 25 mL of either ethanol or water and the argon was purged for 1 hour. Then the samples were placed in photoreactor, which was equipped with 2x40 W 350 nm ultraviolet lamps and irradiated for 21 hour. After the reaction was done, ethanol or water was evaporated under the Schlenk line and some of the sample was dissolved in D_2O for ^1H NMR analysis.

The third type of reaction involved irradiation of thymidine in frozen aqueous solution. 100 mg of thymidine was dissolved in 16.6 mL of MQ water in a Petri dish. The Petri dish with the solution was placed over dry ice and frozen. The solution was irradiated with UVGL-25 UV lamp (4 W, 254 nm, UV Products) for 6 hours while keeping the Petri dish over dry ice. After the irradiation was done, the sample was thawed, the water was evaporated and some of the sample was dissolved in D_2O for ^1H NMR analysis.

V.2.4 NMR spectroscopy

^1H NMR spectra were recorded at room temperature on Varian Inova-300 spectrometer (300 MHz) or on Varian VRX-500 spectrometer (500 MHz).

V.3 Results and discussion

The UV-induced DNA damage usually is from the direct absorption of photons by bases in DNA. However, photosensitizers, like acetophenone, benzophenone or other ketones, can induce thymine dimers as well. For our experiments as a photosensitizer was chosen benzophenone, based on work done by Douki and co-workers (54).

First, the ^{1}H NMR of pure thymidine in D_2O was carried out and it had following peaks in ^{1}H NMR spectra: 1.88 (3H, methyl), 2.32-2.38 (2H, H2'), 3.70-3.88 (2H, H5'), 3.98-4.40 (1H, H4'), 4.42-4.48 (1H, H3'), 4.84 (D_2O peak), 6.24-6.30 (1H, H1'), 7.64 (1H, H6). Because D_2O was used a solvent, there are no peaks in spectrum representing hydrogens from NH and OH (Figure V.1).

The first set of experiments involved irradiation of thymidine/benzophenone in solid state. Some of the reactions were carried out under argon to prevent the scavenging effect of oxygen radicals. However, the irradiation of solid thymidine at >290 nm resulted no photodimers (Table V.1). Following experiment involved irradiation of frozen aqueous thymidine solution at 254 nm, which also was unsuccessful. That could be due to short irradiation time, weak UV source or poor interaction between thymidine and benzophenone. The results are summarized in Table V.1

Since the interaction between molecules is more intense, the next set of the experiments included irradiation of thymidine/benzophenone ethanolic solutions. The irradiation of thymidine/benzophenone in ethanol solution at 350 nm gave photoproduct, which is likely to be (6-4) photoproduct. But the yield of the obtained photoproduct was very low and from ^{1}H NMR spectrum it was hard to confirm the identity of photoproduct (see Figure V.1). Besides the peaks from the starting material thymidine, there were

additional peaks due to benzophenone 7.20-7.50 and (6-4) photoproduct - 2.32-2.38 (1H, H2'), 2.65 (1H, H2"), 2.78 (3H, methyl), 2.94 (3H, methyl), 3.00 (1H, H2"), 3.10 (1H, H2'), 3.80 (2H, H5'), 4.10 (1H, H4'), 4.42 (1H, H3'), 4.84 (D_2O peak), 4.90 (1H, H6), 5.55 (1H, H1'), 5.82, 5.88 (1H, H1').

But when thymidine/benzophenone ethanolic solution was irradiated for prolonged time, 37 hours, some of the peaks, like at 2.78 ppm and 2.94 ppm, were gone, which indicates of a loss of thymine dimer (Figure V.1). When the amount of benzophenone (thymine:benzophenone 1:1 ratio and 1:2 ratio) was increased to see if the yield of unknown photoproduct can be improved, it gave no significant changes (spectra not shown). The similar result to what was obtained from irradiating thymidine at 350 nm for 18 hours was obtained by irradiating thymidine in ethanol solution (no benzophenone added) at 254 nm in a quartz test tube for 23 hours (Figure V.1). ^1H NMR peaks of this photoreaction are: 2.60 (1H, H2'), 2.65 (1H, H2"), 2.71 (3H, methyl), 2.89 (3H, methyl), 3.00 (1H, H2"), 3.10 (1H, H2'), 3.80 (2H, H5'), 4.10 (1H, H4'), 4.42 (1H, H3'), 4.84 (D_2O peak), 4.90 (1H, H6), 5.50 (1H, H1'), 5.85 (1H, H1'). However, when the same type of reaction was carried out in water, there was no photoproduct formed neither at 350 nm nor at 254 nm (Table V.1).

Table V.1 Various conditions for making spore photoproduct from thymidine

#	Phase	Argon	Solvent	Benzophenone	λ	Time, h	T<>T Dimer
1	Solid	-	EtOH	+	>290 nm	22	-
2	Solid	+	EtOH	+	>290 nm	24	-
3	Solid	+	EtOH	+	>290 nm	24	-
4	Solid	-	EtOH	+	>336 nm	24	-
5	Frozen	-	Water	-	254 nm	6	-
6	Liquid	-	EtOH	+	>290 nm	21	-
7	Solid	-	EtOH	+	350 nm	24	-
8	Solid	-	EtOH	+	350 nm	48	-
9	Liquid	-	Water	-	350 nm	24	-
10	Liquid	+	EtOH	+	350 nm	24	+
11	Liquid	+	Water	+	350 nm	24	-
12	Liquid	+	Water	-	254 nm	21	-
13	Liquid	+	EtOH	-	254 nm	23	+
14	Liquid	+	EtOH	1:1 ratio	350 nm	18	+
15	Liquid	+	EtOH	1:1 ratio	350 nm	18	+
16	Liquid	+	EtOH	1:2 ratio	350 nm	37	+
17	Liquid	+	EtOH	1:2 ratio	350 nm	37	+

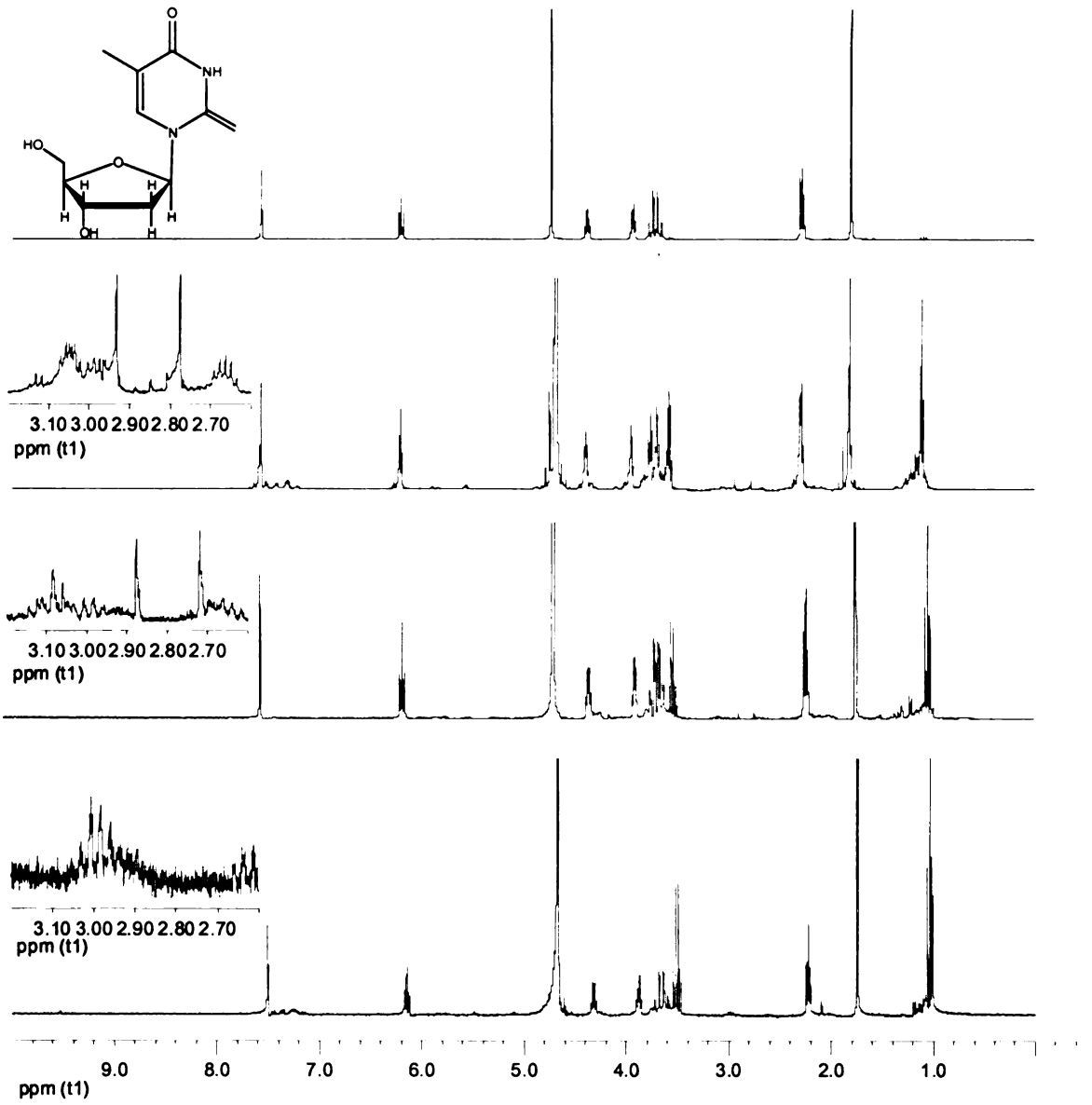


Figure V.1 ^{1}H NMR spectrum of thymidine and its dimers (in D_2O). Top spectrum – thymidine in D_2O (Varian Inova-300, 300 MHz), insert – the schematic representation of thymidine; the second from the top – thymidine and benzophenone ethanol solution after 18 hours of irradiation at 350 nm (Varian VRX-500, 500 MHz); the third from the top – thymidine and benzophenone ethanol solution after 23 hours of irradiation at 254 nm (Varian Inova-300, 300 MHz); the bottom spectrum – thymidine and benzophenone solution in ethanol after 37 hours of irradiation at 350 nm (Varian Inova-300, 300 MHz).

CHAPTER VI

CONCLUSIONS

Spore photoproduct lyase was overexpressed and purified under anaerobic conditions as described; approximately 10 mg of purified protein was obtained from 6 L of bacteria culture. This as-isolated SPL contains 1.86 mol Fe/mol protein.

For SPL binding activity gel-shift assay experiments were established. The preliminary binding studies were carried out between pUC18 DNA and SPL on an agarose gel. It showed that SPL binds to DNA with the ratio 1 molecule of SPL to ~12 base pairs of DNA. The more insightul studies were done by using polyacrylamide gel-shift assays. Two types of double-stranded oligonucleotides were end labeled with ^{32}P and reactions were carried out. The first double-stranded oligonucleotide, which had 35 base pairs and had one adjacent TT pair, was found to bind to SPL. The second oligomer, which had 94 base pairs, also was found to bind to SPL. Various assay conditions and reactions were tried; the best conditions for binding were at low salt concentration and without specific non-competitor poly(dI-dC). However, more work has to be done, including quantification of bound oligonucleotides, finding SPL binding constant, and determining how binding depends on the sequence of the DNA bases.

Attempts to generate spore photoproduct from tritium labeled DNA were also made. It was found that best yield of thymine dimer was obtained under irradiation of dry film DNA at 254 nm wavelength for 5 hours. However, further investigations failed to prove that this photoproduct is spore photoproduct and more research has to be done, including optimization of irradiation conditions to maximize photoproduct production. In

addition, analytical tools like NMR and mass spectrometry need to be used characterize the photoproducts and correlate to specific peaks on the HPLC chromatogram.

The effect of irradiation on thymidine was also carried out. The reactivity of thymidine was tested under various conditions, including the experiments in solid state, liquid phase and in frozen solution. The irradiation of thymidine/benzophenone ethanol solution at 350 nm led to formation of a photodimer, which is likely to be (6-4) photoproduct, but the yield of the reaction was very low and therefore it is hard to make a conclusion. More research has to be done, including finding the conditions for creating spore photoproduct. The ultimate goal of this work is to develop a more efficient method of SP generation. The resulting SP could then be used for SP repair assays and other experiments, like mass spectrometry etc.

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