A SEROTONIN-DEPENDENT DEOXYRIBOZYME THAT USES LIGHT TO REPAIR THYMINE DIMERS IN DNA

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ABSTRACT

Thymine dimers are the most common lesion formed in DNA as a result of exposure to ultraviolet (UV) light. Naturally occurring protein enzymes are known which repair these lesions in different ways. It was hypothesized that in early Earth it may have been RNA that played the functional role, which proteins play today. Also in early Earth it was suspected that the intensity of UV radiation was strong, which could produce dimer products within RNA. A previous *in vitro* selection has been performed to determine whether DNA was capable of catalyzing a photochemical reaction to reverse this damage. This selection yielded two different deoxyribozymes able to catalyze thymine dimer repair in DNA. The deoxyribozyme Sero1C, the topic of this thesis, uses light and serotonin as a cofactor to repair the thymine dimers. Characterization of Sero1C provided hypotheses for structure, the proposed reaction mechanism, and aspects of substrate specificity.

Keywords: Deoxyribozyme, Thymine Dimers.

To Danny, Mom, Dad, and Tyler
For their encouragement and support

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LIST OF ABBREVIATIONS

>	Greater than
^3H	Tritium
A	Adenosine
C	Cytidine
CPD	Cyclobutane pyrimidine dimer
dA	deoxy-Adenosine
dC	deoxy-Cytidine
dG	deoxy-Guanosine
DEPC	Diethyl pyrocarbonate
DMS	Dimethyl sulfate
DNA	Deoxyribonucleic Acid
DNAzymes	Deoxyribozymes
dT	deoxy-Thymidine
dU	deoxyuridine
ε	extinction coefficient
G	Guanosine
LDP	Pseudosubstrate (same sequence as TDP substrate but lacking actual thymine dimer)
nm	nanometers
nt	Nucleotides
PAGE	polyacrylamide gel electrophoresis
RDP	All RNA-containing substrate with a uracil dimer
RNA	Ribonucleic Acid
rT	ribothymidine

T	Thymidine
T=T	Thymine dimer
TDP	Thymine dimer-containing substrate
UDP	Uracil dimer-containing substrate with DNA flanking strands
UV	ultraviolet

CHAPTER 1:
INTRODUCTION TO NUCLEIC ACID CATALYSIS, *IN VITRO* SELECTION, AND THYMINE DIMER REPAIR

1.1 Introduction

The work described in this thesis will cover the characterization of a serotonin-dependent catalytic DNA that photorepairs thymine dimers in DNA.

This introductory chapter will provide background information to put the remainder of this thesis into context. I will first introduce thymine dimers in DNA. I will describe how they are formed, the resulting damage that can occur in a cell, and also describe several common mechanisms to repair this damage in DNA. I will then introduce the idea of catalytic DNA and how these catalytic DNAs were discovered through *in vitro* selections. Finally, I will describe the previously performed *in vitro* selection from which a serotonin-dependent catalytic DNA was discovered that was able to use light to repair thymine dimers in DNA.

1.2 Thymine dimers in DNA

Thymine (or pyrimidine) dimers are the most common lesion formed in DNA as a result of exposure to UV light (200 nm to 300 nm). These lesions in DNA are serious and can give rise to many unwanted responses in a cell. Such responses include: Inflammatory responses, suppression of immune function, and induction of mutations leading to skin cancer (Vink and Roza 2001). The transcription and replication

machineries in a cell cannot transcribe past thymine dimers; therefore thymine dimers can lead to mutations and ultimately cancer in a cell if they are not repaired prior to the transcription or replication event in a cell.

Two types of thymine dimers result from direct photoexcitation of adjacent thymines with UV light: The cyclobutane pyrimidine dimer (CPD), and also the 6-4 photoproduct (Figure 1-1). The cyclobutane thymine dimer is produced more commonly,

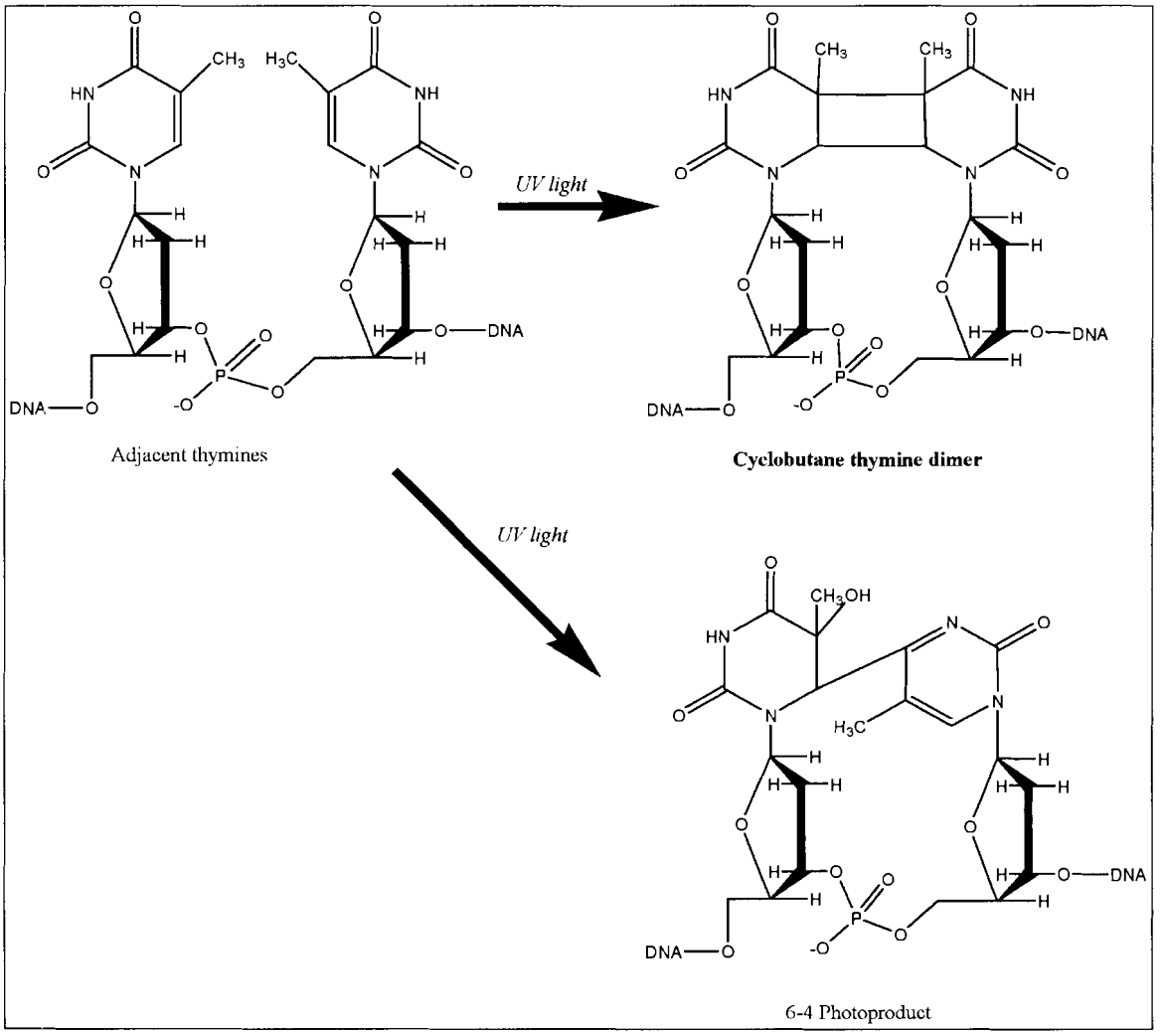


Figure 1-1 Schematic diagram of types of thymine dimers formed in DNA as a result of direct UV photoexcitation of adjacent thymines.

(approximately three times more often than the 6-4 photoproduct (Tornaletti and Pfeifer 1996)), and consists of covalent linkages between the C5 and C6 positions of the thymine bases. The thymine dimers discussed in this thesis are all cyclobutane thymine dimers: Therefore I will focus only on repair of these dimers, and not repair of the 6-4 photoproducts.

1.2.1 Repair of cyclobutane thymine dimers

There are natural photochemical and non-photochemical mechanisms by which cells can repair these UV-induced lesions, and other types of damage, in DNA. In particular, mammalian cells use either "nucleotide excision repair" (NER) (Costa *et al.* 2003) or "base-excision repair" (BER) (Fortini *et al.* 2003) to remove the damaged bases and replace them with their original nucleotides. These mechanisms are more general types of repair since they also repair lesions other than thymine dimers.

NER can repair both cyclobutane thymine dimers and 6-4 photoproducts, and other helix distorting damage in DNA. As indicated by the name, 'excision repair', the cell has to physically remove the damaged bases. In the case of nucleotide excision repair up to 14 bases surrounding the damaged bases are also removed. NER requires many different proteins, and several steps to achieve this overall goal of repair. Once the bases are removed DNA polymerase I and DNA ligase replace the excised nucleotides, and to ligate the ends of the new DNA to the original strand of DNA, respectively (reviewed in (Costa *et al.* 2003)).

BER also removes bases to repair damaged DNA, yet does not remove any surrounding bases. This mechanism also uses multiple protein enzymes in five different

steps, all to repair a single base or two. As well, within this repair pathway are protein enzymes which can remove other damaged bases, or mismatched thymines from a G-T wobble base pair.

In contrast, photochemical methods of repair, performed by photolyase enzymes via photoreactivation, do not require the physical removal of the damaged bases, and are performed by a single protein and its cofactors. These NER and BER mechanisms seem more energetically costly to a cell, compared to the mechanism of photolyases, and involve several proteins, and several steps, to achieve repair of the damaged DNA. Although they seem energetically costly to a cell, nature still evolved the NER and BER pathways. This is most likely due to the fact that the NER and BER pathways are not only specific for thymine dimer damage in DNA. The proteins involved in the NER and BER pathways are also involved in repair of many different types of damage that occur and are found in DNA. Alternatively, photolyase enzymes are very specific for thymine dimer damage recognition and repair in DNA, therefore this could be a reason for being less energetically costly to a cell.

1.2.1.1 Photoreversal of Cyclobutane Thymine Dimers

There are different types of protein photolyase enzymes, including cyclobutane pyrimidine dimer (CPD) photolyase enzymes which photoreverse thymine dimers using light (Matsunaga *et al.* 1991; Carell *et al.* 2001; Sancar 2003). These enzymes have been studied extensively over the past several years There are different photolyase enzymes used to repair CPD dimers and 6-4 photoproducts. Therefore these enzymes are specific for types of thymine dimer damage formed in DNA. Since the thymine dimer substrate

used in our experiments contained only cyclobutane thymine dimers, I will concentrate more specifically now on the background of CPD photolyases.

CPD photolyases are able to harness light at longer wavelengths, greater than 300 nm (lower energy), than that at which cyclobutane thymine dimers are naturally formed. Being able to harness longer wavelength light is beneficial to a cell since additional lesions are not created during repair of existing dimers (while repairing the already formed dimers). All CPD photolyases use the same catalytic cofactor, and other similar cofactors, to harness the longer wavelength light and therefore use similar mechanisms. Of specific interest, to us, was the *Escherichia coli* (*E. coli*) photolyase. This photolyase is a protein enzyme, part of the CPD family of photolyases, which photoreactivates cyclobutane pyrimidine dimers, including thymine dimers, with the aid of two cofactors. The two noncovalently bound cofactors of the *E. coli* photolyase are the flavin adenine dinucleotide (FAD) and methenyltetrahydrofolate (MTHF) chromophores (Figure 1-2).

These cofactors are both able to absorb light over a broad range of wavelengths, between 250 nm and 500 nm. The FAD cofactor is common to all CPD photolyases since it acts as the primary cofactor, and is necessary for specific binding to the damaged DNA and for catalysis of the photoreactivation process. The main role of the second chromophore, MTHF, is to absorb light energy at longer wavelengths and transfer it to FAD. MTHF is not crucial in the substrate recognition aspect of the photoreactivation process, but can prove useful under limited light reactions. Here MTHF can increase the rate of the reaction up to 100-fold due to its ability to absorb light energy at longer wavelengths when compared to the catalytic active form of FAD, $FADH^-$ (Sancar 2003).

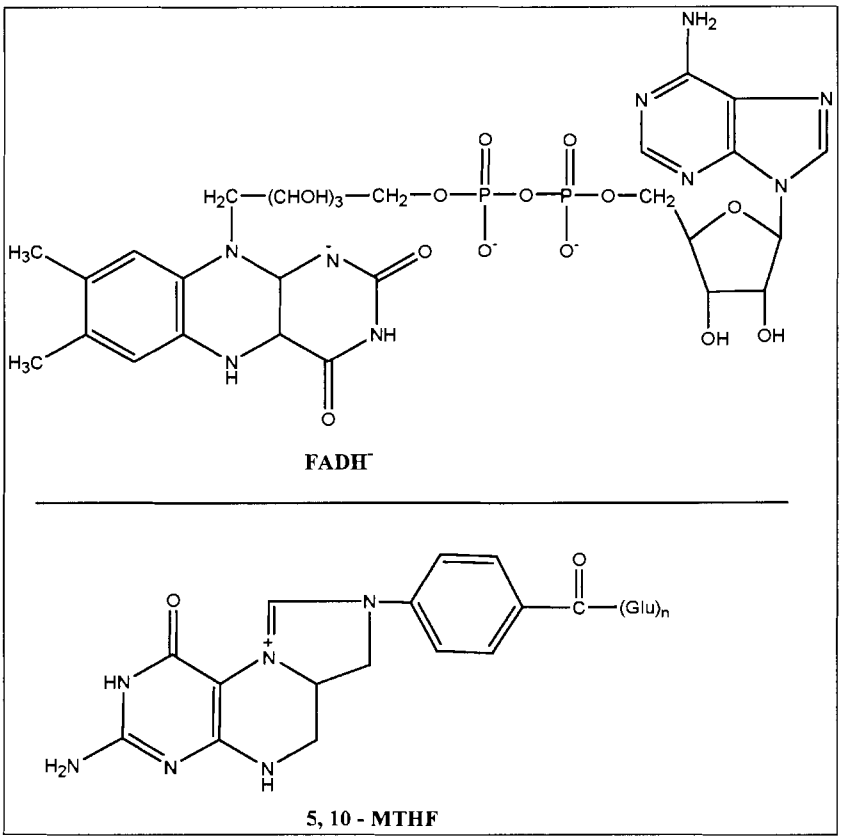


Figure 1-2 Structures of FAD and MTHF chromophores used as cofactors by the *E. coli* photolyase.

The overall scheme of the photoreactivation process performed by the *E. coli* photolyase follows the mechanistic pathway shown in Figure 1-3. First, the MTHF chromophore is excited by light between 300 nm and 500 nm. This excitation energy is subsequently transferred to the fully reduced $FADH^-$ (the catalytically active form) by fluorescence resonance energy transfer (FRET). Now $FADH^-$ in its excited state donates an electron directly to the bound pyrimidine dimer (reviewed in (Sancar 2003)). The pyrimidine dimer undergoes a [2+2] cycloreversion to generate pyrimidine monomers, and the electron is presumable returned to $FADH^*$. This will return the FAD to its fully reduced, active form, $FADH^-$, completing the catalytic cycle. Throughout this

photoreversal mechanism there is no loss or gain of electrons. The quantum yield of thymine dimer repair by photolyase is approximately 1, therefore essentially every photon absorbed is used to repair a dimer. Overall, this is a highly efficient process.

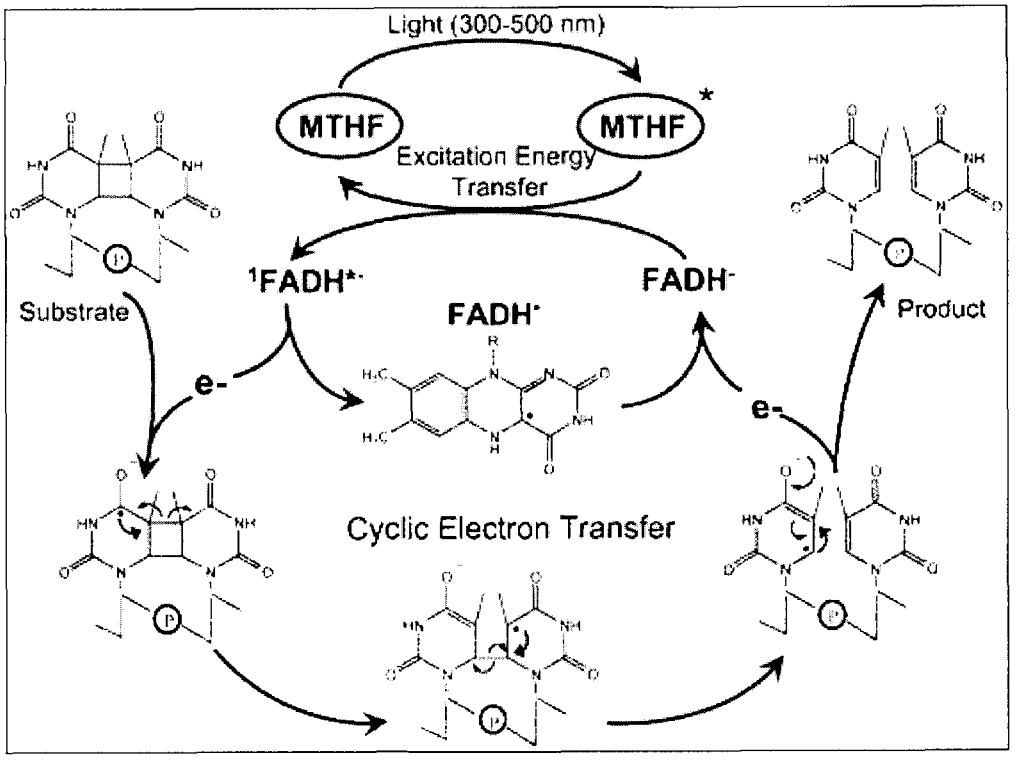


Figure 1-3 Scheme of energy transfer from MTHF* to FADH⁻ and subsequent electron transfer from FADH*⁻ to the thymine dimer, leading to repair.

1.2.1.1.1 Tryptophan's role in thymine dimer repair

It was a tryptophan residue, tryptophan-306, within the *E. coli* photolyase, that was the first amino acid within the photolyase discovered to play a role in photolyase. (Li *et al.* 1991). This involves the *in vitro* donation of an electron to $FADH^*$ via an intra-protein photoinduced electron transfer. This photoreduction of $FADH^*$ to the catalytically active $FADH^-$ occurs *in vitro*, but is not thought to play a role in thymine dimer repair.

There were, in fact, model studies which suggested that indoles are able to act as photosensitizers, to reversibly transfer an electron to a thymine dimer, resulting in cleavage of the intermediate thymine dimer radical anion (Helene and Charlier 1977). This hypothesis led some researchers to investigate further the roles of some tryptophan residues within the *E. coli* photolyase since tryptophan residues are electron rich indole compounds. Further probing into the possible roles of tryptophan residues that lie in close proximity to the thymine dimers did in fact lead to the discovery of a catalytic tryptophan residue, tryptophan-277, within the *E. coli* photolyase. Tryptophan-277 was also shown to be involved in recognition of the thymine dimer substrate and DNA binding (Li and Sancar 1990). These roles are very similar to those of the FAD cofactor. Later, Sang-Tae Kim (Kim *et al.* 1992) also investigated the roles of the many tryptophan residues within the *E. coli* photolyase. Through mutagenesis experiments of the tryptophan residues, they discovered that the tryptophan-277 residue was able to repair thymine dimers, at a shorter wavelength of 280 nm, independent of the two regular cofactors, FAD and MTHF. This research group termed the tryptophan-277 residue the "third chromophore" of the *E. coli* photolyase.

These two tryptophan residues, tryptophan-277 and tryptophan-306, were shown to play roles in the catalysis of thymine dimer repair, and FAD photoreduction, respectively, and provide evidence for amino acid "cofactors", within *E. coli* photolyase.

1.2.1.2 Repair of Thymine Dimers by Catalytic Antibodies

Around the same time, another group of researchers was able to generate antibodies to catalyze photocleavage of thymine dimers (Cochran *et al.* 1988). Mechanistic studies of these antibodies resulted in the conclusion that a single excited

tryptophan residue within the antibody, and in close proximity to the thymine dimer, was responsible for photosensitizing the thymine dimer cleavage (Jacobsen *et al.* 1995). This finding also correlates well with the hypothesis of Helene and Charlier that indoles are capable of electron donation to repair thymine dimers (Helene and Charlier 1977). However, no mechanism describing how the tryptophan residue, within the antibody, catalyzes repair of the thymine dimer, has yet been proposed.

1.3 Nucleic Acids

In the early 1950s Francis Crick suggested a unidirectional flow of genetic information between DNA, RNA and proteins respectively. DNA is transcribed to RNA, which is in turn translated into proteins. This flow of information was labelled the central dogma of molecular biology (Crick 1970). In this scheme for the flow of genetic information, DNA has been thought solely to be the means for storage of genetic information, RNA mediates the transfer of information between DNA and protein, and proteins are the functional molecules being coded for in the original DNA (Figure 1-4).

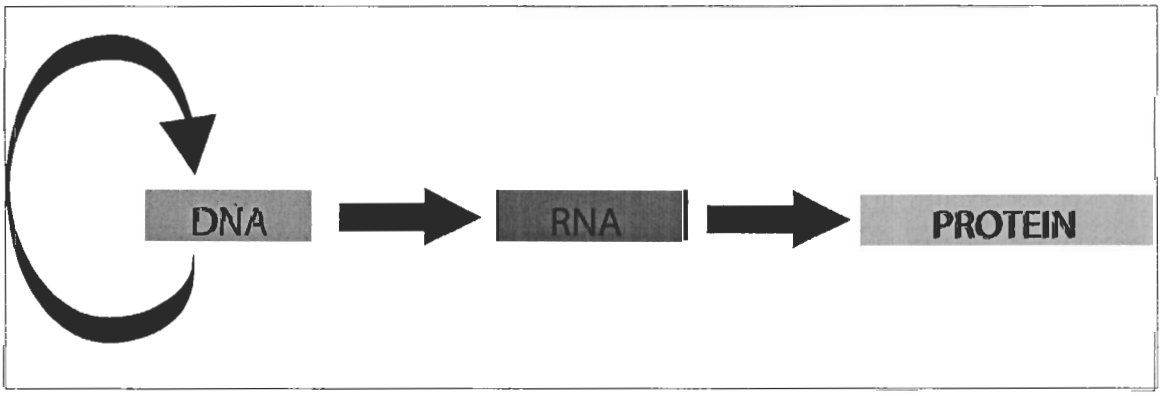


Figure 1-4 Scheme of central dogma of molecular biology.

This basic scheme is generally true, although, there are a few additions to this scheme that have been identified over the years to broaden our knowledge of the critical roles, functions, and possibilities of both DNA and RNA.

1.4 Discovery of Ribozymes – Catalytic RNA

The overall concept of the central dogma of molecular biology makes sense. The feature of proteins possessing all the catalytic and functional abilities is logical due to their sequence and structural complexity and diversity compared to DNA or RNA. Amino acids, and the proteins they create, would seem to be more suited for the catalytic tasks, as opposed to nucleic acids for many reasons (discussed further in section 1.5.2). Until the early 1980s nucleic acids seemed to have many disadvantages compared to proteins in terms of catalytic activity. Therefore, the discovery that RNA can indeed have catalytic properties was surprising. It was Sydney Altman and Thomas Cech (Kruger *et al.* 1982; Guerrier-Takada *et al.* 1983) who first showed that RNA not only plays a role of mediator in the transfer of information from DNA to proteins, but that RNA was actually capable of catalytic function. Not only have nucleic acids now been shown to be capable of catalysis, but most of the earlier discovered ribozymes, such as the hammerhead (Tuschl *et al.* 1995) and the HDV (Shih and Been 2002) ribozymes, are in fact naturally occurring. In addition to the discovery of these catalytic RNAs, many researchers have effectively demonstrate the different catalytic abilities of nucleic acids using a process known as *in vitro* selection, which will be discussed later in this chapter (Breaker 1997; Wilson and Szostak 1999; Joyce 2004).

The above mentioned ribozymes indeed have catalytic abilities, but none have catalytic functions critical in the central dogma of molecular biology. Recently, a

naturally occurring ribozyme was discovered with a critical role in this central dogma. The ribosome is a ribonucleoprotein complex, composed of RNA and protein components, with the function of synthesizing proteins in a cell. The RNA subunit was hypothesized to play strictly a structural role until crystallographic studies determined that the large RNA subunit of the ribosome was indeed a ribozyme (Ban *et al.* 2000; Nissen *et al.* 2000). This ribozyme was discovered through crystallography, by mapping the catalytic region of the ribosome to the RNA subunit of the enzyme, where no protein moieties were found, thereby concluding that the RNA was responsible for the catalysis.

Other ribozymes (discovered through *in vitro* selections) provide further examples of ribozymes with biologically significant catalytic functions. The recently discovered RNA polymerase ribozyme (Johnston *et al.* 2001) demonstrates that RNA is capable of diverse functions critical in the central dogma of molecular biology, since polymerase enzymes are necessary to assemble new DNA and RNA strands from templates.

1.4.1 RNA World Hypothesis

The discovery of RNA having significant and varying catalytic abilities led some researchers to rethink the original central dogma of molecular biology, especially the notion that proteins are the only players of catalytic function. Not only was the description of RNA, simply as a mediator between DNA and proteins, being transformed, but it was also hypothesized that, in early life, RNA could have been the primary molecule responsible for expressing both genotype and phenotype (Gilbert 1986; Benner *et al.* 1989; Benner and Ellington 1991). The theory is now called the 'RNA world hypothesis'. This 'RNA world hypothesis' states that all of the tasks, including catalytic functions, that are now carried out by proteins, could have been performed by RNA in

early evolution, before proteins were able to be translated from RNA. Now, RNA has been shown to both code for genetic information, and to catalyze important biological reactions (as discussed in section 1.4). Since the structural properties of RNA nucleotides and DNA nucleotides are not all that different, one might assume that if RNA is capable of catalytic function, then so should DNA. At the nucleotide level, the only structural difference between RNA and DNA is that DNA nucleotides lack a 2' hydroxyl group on the sugar (Figure 1-5). In other words, RNA contains ribose sugar moieties and DNA contains 2' deoxyribose sugars. This single atom difference has important structural consequences on nucleic acid polymers. For instance, RNA had been shown to be able to fold into complex structures, especially single-stranded motifs (reviewed in (Moore 1999)), whereas DNA was primarily found to be in a double-stranded helical structure with limited flexibility.

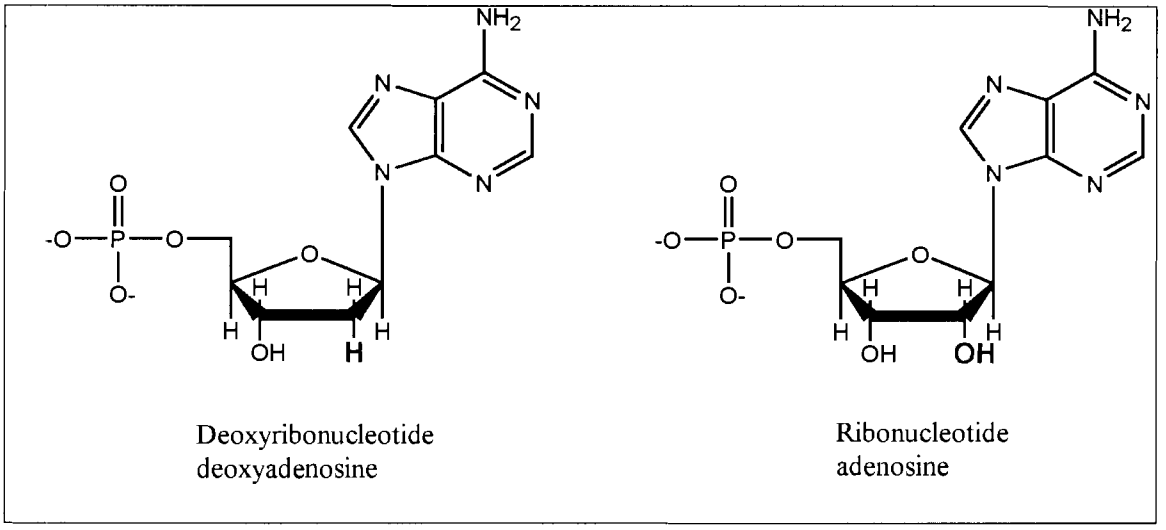


Figure 1-5 Structure of a DNA nucleotide versus an RNA nucleotide using adenosine as an example.

1.5 Discovery of Deoxyribozymes – Catalytic DNA

DNA was thought to be primarily the repository of genetic information in a doubled-stranded helical structure, which is not very suitable for catalytic purposes. Yet, some researchers started to test the idea of whether or not DNA could also have catalytic abilities (Cech and Bass 1986). It was Breaker and Joyce who discovered the first known deoxyribozyme (Breaker and Joyce 1994).

This single-stranded catalytic DNA (deoxyribozyme) was able to catalyze the cleavage of an RNA substrate in the presence of Pb^{2+}. This deoxyribozyme was the first of many artificial deoxyribozymes, capable of a wide variety of catalytic functions, that were discovered using a process known as *in vitro* selection or SELEX (**S**ystematic **E**volution of **L**igands with **Ex**ponential Enrichment). This process will be discussed in section 1.5.2. The catalytic function of DNAzymes include catalyzing reactions such as RNA cleavage (Santoro and Joyce 1997), DNA phosphorylation (Wang *et al.* 2002), DNA capping (Li *et al.* 2000), porphyrin metallation (Li and Sen 1996), and more.

Another question regarding catalytic DNA was whether these deoxyribozymes were catalytically comparable to the known and naturally occurring ribozymes. Santoro and Joyce (Santoro and Joyce 1997) set out to try to engineer a versatile deoxyribozyme that can cleave any RNA sequence, which could be later used for gene silencing. They were successful in their attempts, through *in vitro* selection, and discovered the 10-23 deoxyribozyme, which to date exemplifies the versatility and catalytic potential possible with deoxyribozymes. This deoxyribozyme functions such that the 'binding arms' of the enzyme can be designed to bind to any RNA substrate sequence, thereby allowing cleavage of a desired RNA substrate. Also, the 10-23 deoxyribozyme has now been

shown to be applicable *in vivo* as a therapeutic agent (reviewed in (Cairns *et al.* 2002; Khachigian 2004)). Finally, upon detailed investigations with the 10-23 deoxyribozyme, it was found to have a high catalytic efficiency comparable to that of the known RNA-cleaving ribozymes.

1.5.1 Proteins versus Nucleic Acids as catalysts

To date, all of the mechanisms shown to repair thymine dimers, within a cell, involve protein enzymes. Naturally, this agrees with the original hypothesis of the central dogma of biology. In conjunction with the central dogma of molecular biology, it makes sense that proteins would be more suitable for catalytic tasks for many reasons, which I will describe here. Now that we are uncovering the catalytic potential of nucleic acids, I would argue that it is especially interesting given the supposed limitations of nucleic acids in comparison to proteins and amino acids. First of all, proteins are composed of combinations of 20 amino acids, whereas strands of DNA or RNA are composed of combinations of only four nucleic acid bases. This is the first sign that proteins may be more suitable for catalytic tasks, due to their ability to create a larger number of different possible combinations of amino acids to form a protein for a desired task. For example, assuming a length of 10 amino acids, or 10 bases, the number of different possible proteins that could be formed is 20^{10}, whereas nucleic acids would only be able to produce 4^{10} different combinations in attempt to produce an equivalent catalytic oligonucleotide. Second, the structural and chemical properties of amino acids compared to those of nucleic acid bases are more complex and arguably more favorable for catalytic purposes. Proteins are all composed of amino acids with side chains with different functionalities, degrees of hydrophobicity, and charge (either positive, negative, or

neutral). Proteins do indeed have a diverse range of functional groups, which also undergo many post-translational modifications, therefore the complexity of the primary structure is higher. The degree of complexity of secondary and tertiary structures in proteins may also be correspondently higher than that of nucleic acids. Proteins may often have several distinct and complex secondary structural motifs, such as β sheets, α helices, helix-turn-helix motifs, and more. In contrast, DNA secondary structures primarily involve hydrogen bonding between complementary base pairs, and stacking, to form double helical structures (double-stranded DNA duplexes). Another difference between catalytic proteins and nucleic acids are their overall size. Proteins are usually very large, where most ribozymes and deoxyribozymes discovered are much smaller. One common feature of proteins and nucleic acid enzymes is their ability to form different domains, such as a catalytic core, and binding domains.

Finally, it has been shown that specific protein enzymes, including proteins involved in the NER pathway, may also be involved in other pathways, with different functions (Cline and Hanawalt 2003). Conversely, deoxyribozymes (especially since they are derived from *in vitro* selections) usually have one specific function, yet may be able to act on different substrates. Overall, the significance and importance of catalytic nucleic acids is expanding, and still changing the original theme of the central dogma of molecular biology.

1.5.2 SELEX – In vitro selections

How is it possible to engineer and produce new artificial deoxyribozymes? This is achieved by using a well known technique called *in vitro* selection, or SELEX (Figure 1-6). This technique is a very powerful tool that was developed by three independent

research groups (Joyce 1989; Ellington and Szostak 1990; Tuerk and Gold 1990) to help identify new catalytic nucleic acids with differing functions. RNA and DNA molecules can fold into many different structures depending on their size and sequence. Therefore, a large number of random sequence DNA or RNA molecules (on the order of 10^{15} different sequences) are taken and subjected to particular salt, pH, and temperature conditions in order to induce folding into their particular individual structures. Once folded, these RNA or DNA molecules are then subjected to a screening process, which involves the RNA or

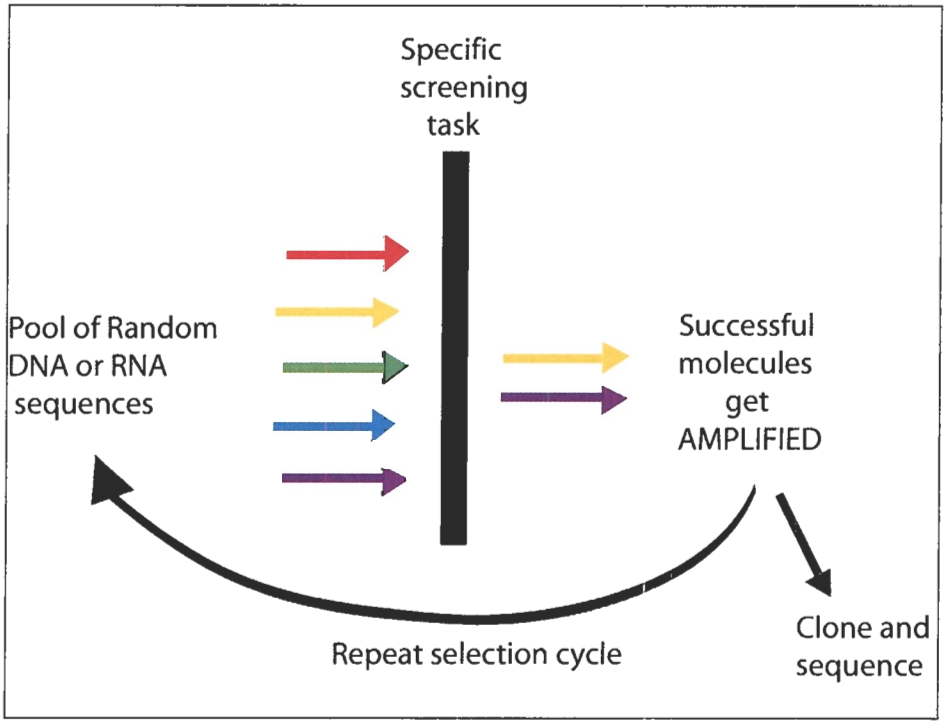


Figure 1-6 General scheme of *in vitro* selections

DNA molecules being able to catalyze a specific reaction of interest, or bind a specific molecule. The RNA or DNA molecules that are successful in completing the given task can then be separated from those molecules that are not successful in the screening

process. Next, these successful nucleic acid molecules are amplified by PCR to create double-stranded DNA, which is later made into single-stranded DNA or transcribed to RNA, in order to regenerate a starting pool, which will now contain a higher concentration of the successful molecules. The successful molecules that have been amplified are then subjected to the same screening process, and amplification, for several more rounds until a positive signal of catalysis is sufficiently higher than that of the background. Finally, after a desired level of positive signal is achieved, the remaining successful molecules are cloned and sequenced.

Throughout the selection process, it is also possible to increase the specificity or efficiency of the desired nucleic acid catalyst. For example, one could decrease the amount of time the nucleic acid molecule has to be able to perform the screening process task. In this way, the slower catalytic nucleic acids get eliminated, due to their inability to successfully complete the task in less time, and only the faster deoxyribozymes get amplified. In making such changes to the reaction conditions, one can increase the stringency of the selection, thereby finally selecting the 'strongest' nucleic acid catalyst.

In addition to being able to change the stringency of an *in vitro* selection, one can also design the selection to discover a nucleic acid that would perform a specific desired function, or possibly more than one function. By changing the task that the nucleic acid molecule would have to complete successfully, in order to get amplified, one can search for different types of nucleic acid molecules. For example, *in vitro* selections have most commonly been designed to find catalytic nucleic acids, although the screening process within the selection scheme has also be designed to find a nucleic acid molecule that binds a particular molecule of interest, in other words to discover a nucleic acid aptamer.

Overall, the theory of *in vitro* selections can be manipulated to select for nucleic acids of many different functions.

The specific *in vitro* selection discussed next (in section 1.6) describes how Chinnapen and Sen (Chinnapen and Sen 2004) selected for a deoxyribozyme with the ability to repair thymine dimers, using serotonin as a cofactor.

1.6 *In vitro* selection to yield serotonin-dependent deoxyribozyme

To add to the list of deoxyribozymes and their different catalytic functions, an *in vitro* selection was performed that relates to the findings mentioned above regarding cofactor mediated thymine dimer repair by photolyases. I will now describe and explain the basis for the design of the selection that Dan Chinnapen performed (Chinnapen and Sen 2004), in an attempt to find a deoxyribozyme capable of repairing thymine dimers using serotonin as a cofactor. This selection yielded two distinct deoxyribozymes both with the ability to catalyze photoreversal of thymine dimers.

In the previously mentioned experiments probing thymine dimer repair by photolyase, a tryptophan residue was shown to act as a "cofactor" for catalysis, and was able to play other roles in repair of thymine dimer repair, including substrate recognition. One concern with tryptophan as a possible "cofactor" is its absorption spectrum. The maximum excitation wavelength of tryptophan is 279 nm. Therefore, exciting a tryptophan residue at this wavelength to repair thymine dimers, may also create more thymine dimers, since the DNA also absorbs light at that wavelength. This was one problem that the *E. coli* photolyase has overcome by using cofactors that absorb light at longer wavelengths. Again, researchers have found that electron rich, indole containing

compounds, such as tryptophan, may be good photosensitizers for electron transfer reactions (Helene and Charlier 1977). With this in mind, serotonin was chosen as a cofactor for the selection since it has a structure very similar to tryptophan. Yet, it has a broader, more red-shifted absorption spectrum (Figure 1-7). Serotonin is an indole containing compound, lacking a carboxylic acid group and containing an extra hydroxyl group in comparison to tryptophan (Figure 1-8). The maximum absorption spectrum of serotonin is approximately 285 nm, yet it has a second peak around 300 nm, where tryptophan absorbs very little light at 300 nm (approximately 4% of that which was absorbed by serotonin at 305nm) (Figure 1-7). Also, DNA is not able to signigicantly absorb light at 300 nm either. Therefore this wavelength would be less harmful when attempting to repair thymine dimers.

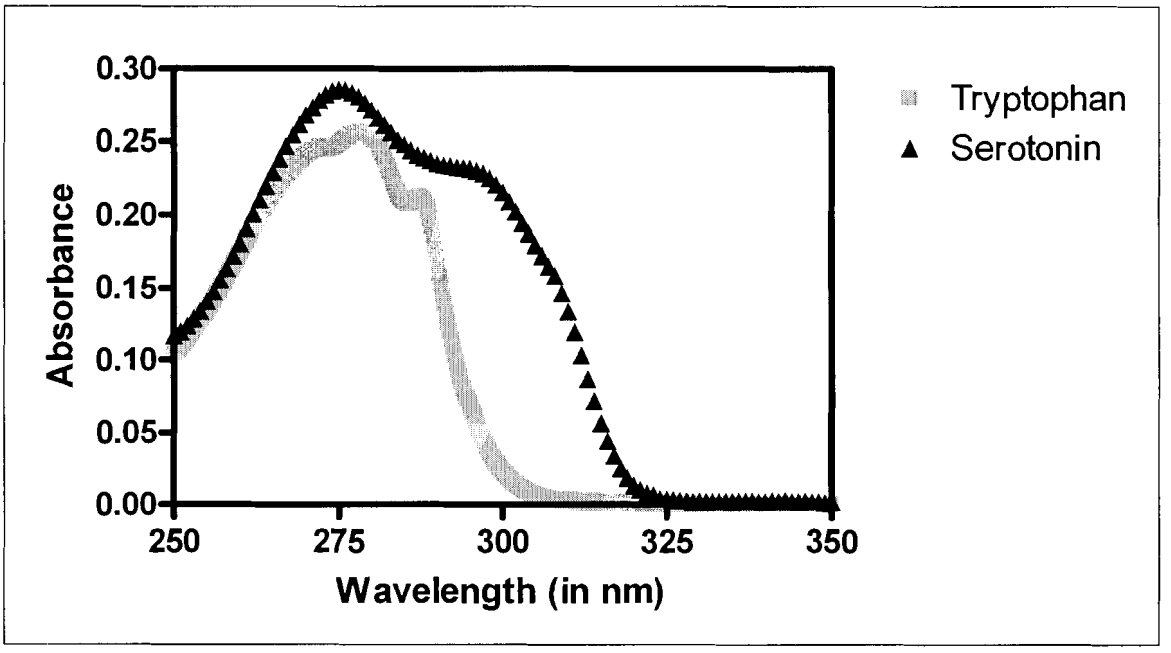


Figure 1-7 Absorption spectra of 50 μM serotonin and 50 μM tryptophan. Extinction coefficient of serotonin at 300 nm is approximately 2500 M^{-1} cm^{-1}

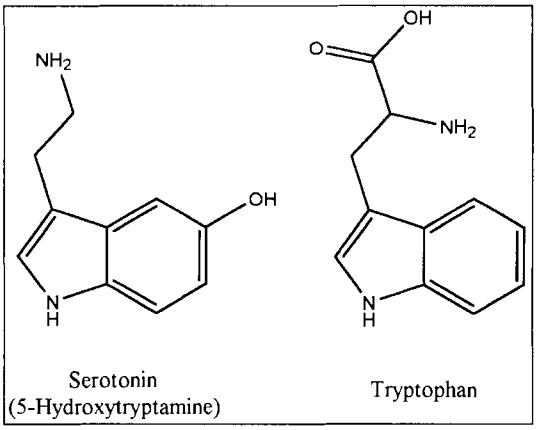
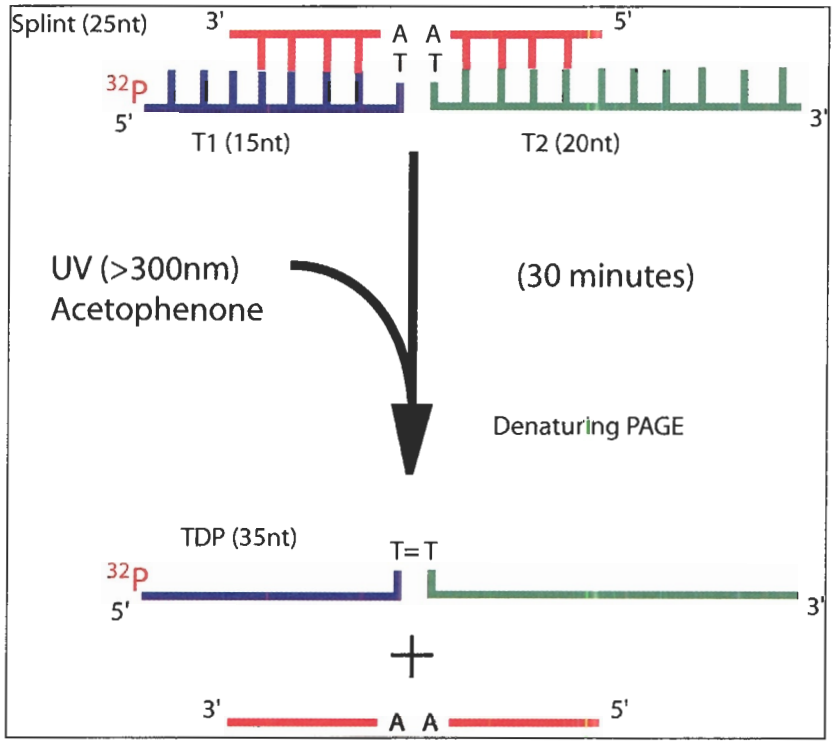


Figure 1-8 Structures of serotonin and tryptophan

After choosing a cofactor, a cyclobutane thymine dimer-containing substrate was needed. A thymine dimer substrate was designed such that repair of the dimer was easily detected by radioactive methods. This thymine dimer substrate was created such that there was no phosphodiester bond between the adjacent thymines involved in the dimer. To form this thymine dimer substrate, a splint oligonucleotide base pairs to two separate oligonucleotides, such that it brings two thymines adjacent to one another (Figure 1-9). Upon irradiation of these oligonucleotides at a wavelength >300 nm, in the presence of acetophenone, a thymine dimer was created and separated from all the other oligonucleotides by denaturing gel electrophoresis, and purified. Therefore, upon repair of the thymine dimer substrate, which was radiolabeled with ^{32}P on the 5' end, strand separation was achieved and visible with denaturing polyacrylamide gel electrophoresis analysis. These substrates were also tested to verify that the thymine dimers were a cyclobutane product, and not a 6-4 photoproduct (Chinnapen 2005).



Modified with permission from (Chinnapen and Sen 2004) Copyright (2004) National Academy of Sciences, U.S.A.

Figure 1-9 Schematic of how cyclobutane thymine dimer-containing substrate was formed.

The complete selection scheme is summarized in Figure 1-10. Upon forming the thymine dimer substrate, it was first incorporated by PCR into oligonucleotides each containing a random region comprised of 40 bases. The number of random DNA molecules contained within the starting pool was approximately 10^{14}. Therefore, the original selection was selecting for a *cis*-acting deoxyribozyme; meaning that the thymine dimer substrate and potential deoxyribozyme (from the 40 base random region) were all part of the same strand of DNA. Subsequently, a negative selection step was performed to remove any DNA sequences that may repair the thymine dimer where there was no serotonin present upon irradiation of light >300 nm. Interestingly, Dan Chinnapen (Chinnapen and Sen 2004) noticed an increase in signal of repair of the thymine dimer in

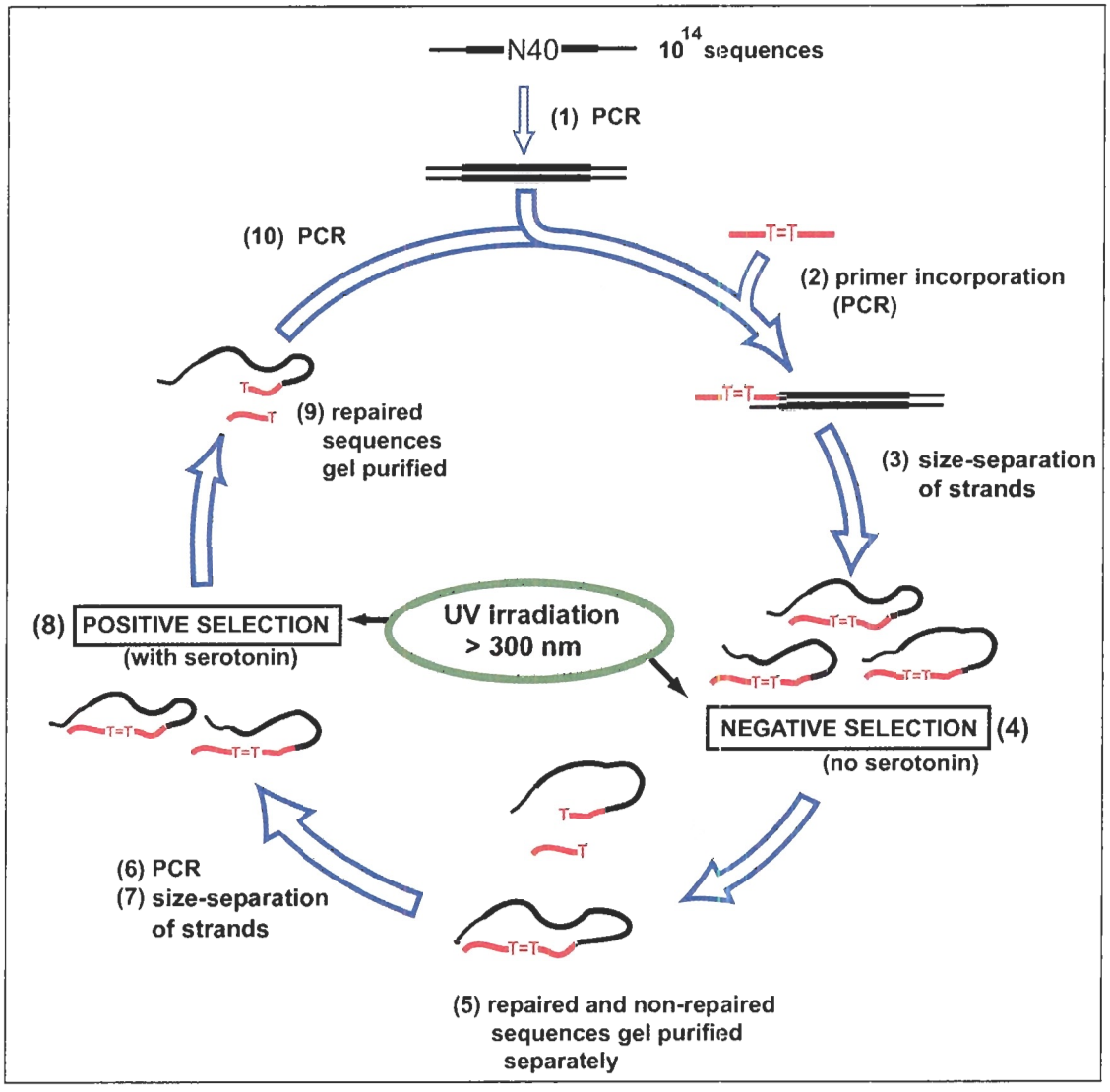


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Figure 1-10 ***In vitro*** **selection cycle scheme.**

this negative selection step. The pool of serotonin-independent repair molecules was then separated from the serotonin-dependent pool and subjected to further rounds of only the negative selection step. From this, a *trans*-acting deoxyribozyme, later named UV1C, was discovered and characterized. This deoxyribozyme did not need a cofactor to repair thymine dimers. Through structural and mechanistic studies, they discovered that UV1C formed guanine quadruplexes as part of its secondary structure. They further

hypothesized that it was the guanine quadruplex structures which absorbed the light energy and transferred it to the thymine dimer to split it back to monomers (Chinnapen 2005).

Returning to the original selection, the next step in the selection cycle involved the positive selection step, in which serotonin was added to these DNA molecules, and they were then tested for their ability to repair thymine dimers upon irradiation of light >300 nm. The DNA molecules that were able to successfully repair the thymine dimers in the presence of serotonin were purified, amplified, and subjected to further rounds of negative and positive selection.

Upon cloning and sequencing of the pool of successful DNA molecules after 25 rounds of selection, two prominent sequences were obtained for the 40 base random region of the *cis*-acting serotonin-dependent deoxyribozyme (Figure 1-11). Sero1 was chosen for further characterization.

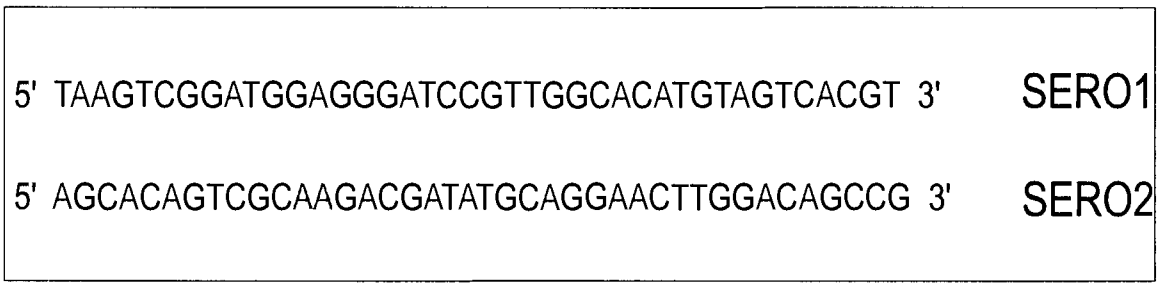


Figure 1-11 Sequences of the two most common random regions selected from round 25.

As mentioned previously, the deoxyribozymes were originally selected as *cis*-acting deoxyribozymes. To be more applicable *in vivo*, one of the first experiments performed by Chinnapen and Sen (Chinnapen 2005) involved testing the ability of Sero1,

(the full length 90 nucleotide substrate and enzyme containing strand of DNA), to be able to act *in trans*. This involved separating the substrate and enzyme DNA sequences into two different strands of DNA (Figure 1-12). The substrate, named TDP, was created by detaching the 5'-35 nucleotide constant region, containing the thymine dimer, from the random region. The deoxyribozyme, named Sero1C, was created from the 40 nucleotide random region from the original construct. In fact, Sero1C was able to act *in trans* to repair the thymine dimer containing substrate (Chinnapen 2005). All remaining characterizations and experiments were performed utilizing the ability of Sero1C being able to act *in trans*.

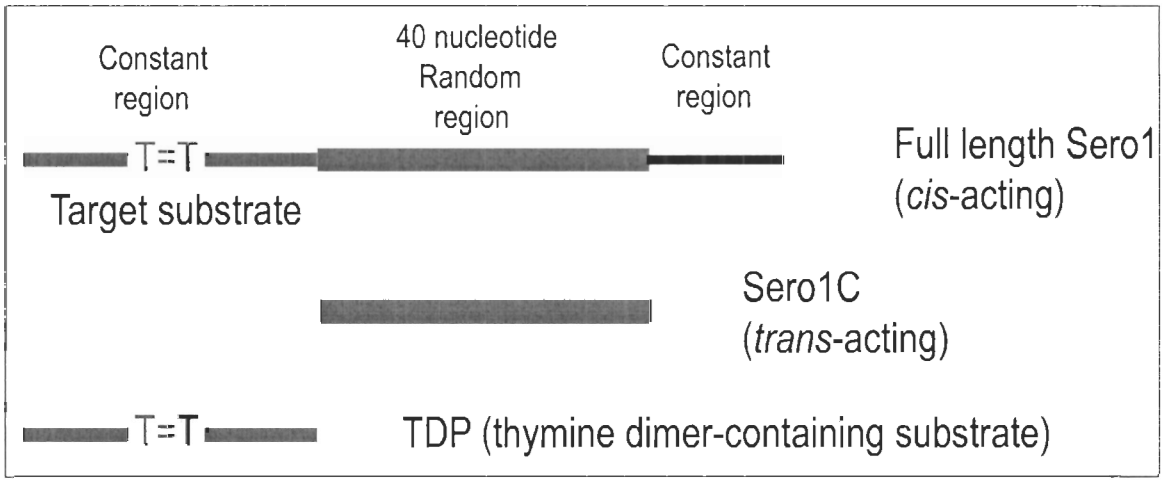


Figure 1-12 Devised constructs used to create *trans*-acting deoxyribozyme.

Therefore, Sero1C was the *trans*-acting serotonin-dependent deoxyribozyme that was able to harness light energy in order to catalyze repair of thymine dimers in DNA, and will be the topic of this thesis.

The main component of such photochemical reactions is light. There are many different components of light that must be considered while employing photochemical techniques. I will briefly introduce photochemical components such as absorbance, light flux, and fluence rate, to put my experiments (discussed in section 2.3) into context. First, I employed the use of absorbance measurements in several cases, such as in Figure 1-7. An absorbance measurement of a specific compound is a measurement of the difference between the light intensity going into a cuvette and the light intensity going through the cuvette. Therefore, it is the loss of intensity of light being measured to determine how much light energy has been absorbed by the serotonin molecules. Specifically, absorbance measurements are the logarithm of the intensity of light going into a cuvette divided by the light intensity passing through the cuvette. As well as recording absorbance measurements, I also use other wavelengths of light to excited serotonin in the reactions. In both of these cases the light flux is an important aspect to consider. The light flux is the number of photons entering a cuvette per unit area per unit second (per cm^2 per second). Further, the fluence rate is defined as the light flux multiplied by the area of the cuvette. The fluence rate is measured in einsteins min^{-1}, where an einstein is defined as the energy per mole of photons carried by the beam of light. To ensure that a shadow effect is not occurring in my reactions, a low concentration of serotonin was used in order to ensure a lower absorbance value (under a value of one). A lower absorbance value is indicative that light is still passing all the way through the sample, therefore there are more photons going through the sample than there are molecules to react with the photons. The extinction coefficient (ε) of serotonin is approximately 2500 $M^{-1} cm^{-1}$ at 300 nm, and using the following equation where absorbance = (ε)(concentration of

molecule)(path length), I obtained approximately expected values for absorbance when using serotonin at a concentration of 50 μM.

1.7 Thesis Overview

Early Earth was thought to be a very reducing environment with high UV intensity (Cockell 1998). It is known that UV light can cause damage to RNA and DNA, such as uracil and thymine dimers respectively. Chinnapen and Sen (Chinnapen and Sen 2004) had asked the question of whether DNA itself was capable of catalyzing photochemical reactions, such as photoreversal, to repair this damage and survive early Earth conditions when proteins may not have existed. My thesis will cover the characterization of a serotonin-dependent deoxyribozyme that uses light to repair thymine dimers. It will also demonstrate the potential of catalytic DNA and its significance to the RNA world hypothesis

Chapter 2 will outline and describe general characteristics of the 40-nucleotide serotonin-dependent deoxyribozyme, named Sero1C, that repairs thymine dimers in DNA upon irradiation of wavelengths >300 nm. Characteristics such as its wavelength dependence, salt dependence, serotonin dependence, and catalytic abilities will be described. As well, more detailed studies involving the substrate specificity of Sero1C will be investigated, leading into attempts to determine a secondary structure. Finally, from several experiments, I will outline a theory on the photochemical mechanism of Sero1C.

Chapter 3 will summarize the major findings on Sero1C and discuss the significance of these results. Also, I will outline several future experiments to be

performed to further help the understanding of the mechanism and structure of this deoxyribozyme.

CHAPTER 2:
CHARACTERIZATION OF A SEROTONIN-DEPENDENT DEOXYRIBOZYME THAT HARNESSES LIGHT TO REPAIR THYMINE DIMERS IN DNA

2.1 Introduction

The RNA world hypothesis (Gilbert 1986) states that RNA, and RNA-like polymers, which are capable of genetic and catalytic function, may have constituted primitive "life" in early evolution due to their wide range of abilities. Early in evolution, it was assumed that there was a higher level of powerful UV rays, that were not shielded by an ozone layer (Cockell 1998), which forced organisms to evolve mechanisms to repair the damage caused by this UV light.

Thymine dimers are the most common lesion formed in DNA as a result of exposure to UV light. Several natural mechanisms have evolved to repair these lesions, as discussed in the previous chapter. These mechanisms either involve several protein enzymes, with their cofactors, or light to photoreactivate the thymine dimers.

The overall goal of the *in vitro* selection of Chinnapen and Sen, was to determine whether or not and how deoxyribozymes were capable of carrying out such photochemical reactions (Chinnapen and Sen 2004).

As mentioned previously (in section 1.6), the *in vitro* selection of Chinnapen and Sen yielded two different deoxyribozymes capable of repairing thymine dimers using light: UV1C, a cofactor-independent deoxyribozyme, and Sero1C, a serotonin-dependent deoxyribozyme. The remainder of this thesis involves experiments and characterization

of the cofactor-dependent deoxyribozyme Sero1C. As mentioned in chapter 1, the deoxyribozymes were originally selected for as *cis*-acting deoxyribozymes, which means that the thymine dimer substrate was part of the same strand of DNA as the catalytic region. To be more applicable *in vivo*, one of the first experiments performed by Dan Chinnapen involved testing the ability of Sero1, the full length 90-nucleotide substrate and enzyme containing strand of DNA, to be able to act *in trans*. This involved separating the substrate and enzyme into two different strands of DNA. The substrate was then a 35-nucleotide strand containing the thymine dimer, and the deoxyribozyme, Sero1C, was then solely the 40 nucleotide random region from the original construct. In fact, Sero1C was able to act *in trans* to repair the thymine dimer containing substrate (Chinnapen and Sen 2004). All remaining characterizations and experiments outlined in this thesis were performed utilizing Sero1C acting *in trans*. Figure 2-1 depicts a hypothetical schematic of Sero1C, acting *in trans*, with serotonin as a cofactor to repair the thymine dimer-containing TDP.

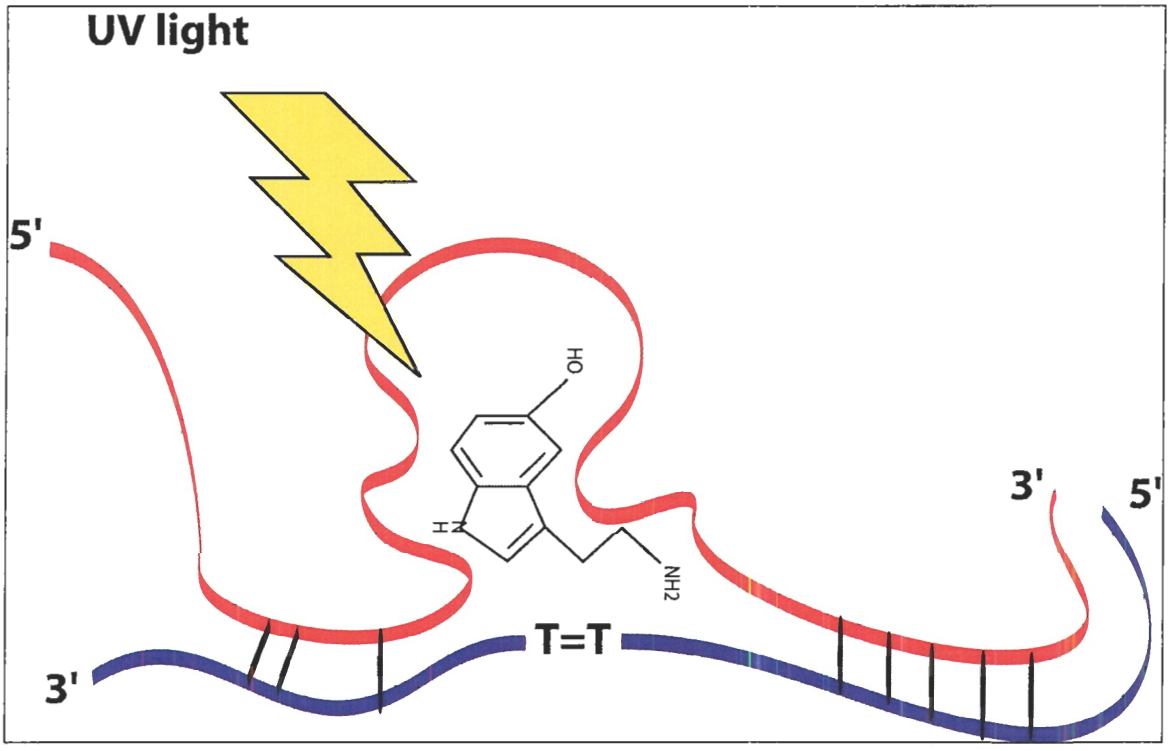


Figure 2-1 **Rough schematic of Sero1C: A *trans*-acting serotonin-dependent deoxyribozyme that repairs thymine dimers.**
Red indicates Sero1C; blue indicates TDP; black indicates hypothetical possible base pairs.

2.2 Materials and Methods

2.2.1 DNA oligonucleotides and Reagents

DNA oligomers were obtained from University of British Columbia NAPS Unit, and size-purified on denaturing polyacrylamide gels. The Sero1C deoxyribozyme had the sequence 5'-TAAGT CGGAT GGAGG GATCC GTTGG CACAT GTAGT CACGT-3'. The TDP substrate had the sequence 5'-AGGAT CTACA TGTAT=TGTGT GCGTA CGAGT ATATG-3'. In the instances where the "pseudosubstrate" was used, this means that the sequence was identical to that of the TDP substrate, but the actual thymines that normally participate in the thymine dimer were not cross-linked, i.e. it was a continuous strand of DNA. 5-iodouracil-containing DNA was synthesized by University Core DNA Synthesis Lab (UCDNA) using 5'dimethoxytrityl-5-iodo-2'deoxyuridine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. Single base mutations were made on the substrate. Ribothymidine constructs were purchased from UCDNA. Deoxyuridine constructs were synthesized using 5'-dimethoxy-2'deoxyuridine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. DNA was end-labelled using T4 polynucleotide kinase from Invitrogen. Serotonin powder was purchased from Sigma-Aldrich.

2.2.2 Synthesis of Thymine Dimer-containing Oligonucleotide

The TDP substrate was formed by combining unlabelled T2 and 5'-^{32}P-labelled T1 oligonucleotides, and annealing them at 400μM concentrations to a complementary splint oligonucleotide in buffered 50mM $MgCl_2$. These solutions were then degassed by freezing and thawing under vacuum three times. 20mM of the triplet sensitizer acetophenone (which has been shown previously to specifically favour the formation of the cyclobutane thymine dimer (Wang 1976)) and 50% acetone were added to the

solution. The DNA solution was then irradiated at wavelengths greater than 300 nm with a Fotodyne transilluminator, at a fluence rate of 3.4×10^{-9} einsteins min^{-1} illumination, for 30 minutes. The TDP product was ethanol precipitated and size-purified on 12% denaturing polyacrylamide gels and further tested for the presence of the cyclobutane thymine dimer (as opposed to the 6-4 photoproduct). This was done by direct photoreversal experiments at 250 nm.

2.2.3 Kinetic Analysis

The kinetic properties of Sero1C were measured in 20 mM sodium phosphate, pH 7.0, 240 mM NaCl, at room temperature. The DNA was heated to 90 °C for 3 minutes and then allowed to cool slowly back down to room temperature in the above mentioned buffer. To create the action spectrum (wavelength dependence of Sero1C) shown in Figure 2-8, 100 μL solutions containing 5' ^{32}P-labelled DNA were irradiated in a Hellma glass quartz cuvette, with a 1 cm path-length, and cell holder using an Ushio Xenon short arc lamp, with the different wavelengths being isolated with a SLM MC200 model monochromator, and bandwidth set at 8 nm. Time points were taken, and the DNA, following separation on 12% denaturing polyacrylamide gels, was quantified using Molecular Dynamics ImageQuant software. Single turnover kinetics were all carried out with 2 μM Sero1C and 20 nM TDP substrate, and either 50 μM or 500 μM serotonin as specified, in 20 mM sodium phosphate, pH 7.0, with 240 mM NaCl. The photon flux of the lamp source at the indicated wavelengths was determined using standard ferrioxalate actinometry (Calvert and Pitts 1966), which was also used to determine light intensity corrections (further described in section 2.2.4). Rate enhancement data was calculated by

dividing the background rates (TDP repair monitored in the presence of an unrelated DNA oligomer) by the initial rates of the reactions.

All remaining kinetic reactions were 100 μL reactions, which were placed into a flat-bottom 96-well polystyrene ELISA plate (with the surface having been pre-blocked with glycogen) and irradiated on the Fotodyne transilluminator, at a fluence rate of 3.4×10^{-9} einsteins min^{-1} illumination. 5 μL aliquots were removed at the specific time points and subsequently separated on 12% denaturing polyacrylamide gels. Fragments were quantitated using Molecular Dynamics ImageQuant software, and rates were calculated using GraphPad Prism4 software using a first order rate equation to yield the initial rates of the reactions.

Multiple turnover kinetics were performed in the presence of 20 nM Sero1C and varying concentrations of TDP substrate (between 0.1 μM and 10 μM) in 20 mM sodium phosphate, pH 7.0, 240 mM NaCl. Experiments were performed at least in duplicate and error values were calculated from the fit of the curves using GraphPad Prism4 software.

The pH dependence reactions were carried out using two separate buffers: Tris(hydroxymethyl)aminomethane was used for pH values of 7.4, 7.8, 8.2, 8.6, 9.0, and 9.2, to a final concentration of 50 mM. A Sodium Carbonate-Bicarbonate buffer was used for pH values of 9.0, 9.2, 9.6, 10.0, and 10.2, to a final concentration of 25 mM. All solutions were calibrated to the appropriate pH using a Beckman pH meter, after the proper standardizations. All reactions were carried out with 200 mM NaCl, 2 μM Sero1C, 50 μM serotonin, and negligible amounts of radiolabeled TDP.

The reactions in which I tested the ability of different cofactors to be able to repair TDP, were prepared as first described in this section, except 50 μM of each possible cofactor (Tryptophan, Tryptophol, Tryptamine, Tryptophan-Glycine, Tryptophan-Tryptophan, Lysine-Tryptophan-Lysine, or 5-Hydroxyindole) was added to separate reactions as opposed to serotonin. The positive control in which 50 μM serotonin was present was also performed.

2.2.4 Potassium Ferrioxalate Actinometry

Standard ferrioxalate actinometry was performed to normalize the light intensity at different wavelengths (on the spectrofluorimeter) as previously described (Calvert and Pitts 1966). 8.7 mM solutions of $K_3Fe(C_2O_4)3{\cdot}3H2O$ in 50 mM H_2SO_4 (made in the dark) were irradiated at different wavelengths for 3 minutes. To these solutions, 30 mM NaOAc, 9 mM H_2SO_4, and 0.04% 1-10 phenanthroline were added, and the solutions were allowed to equilibrate for 45 minutes. The absorbances of these solutions were then measured at 510 nm on a Cary 300Bio UV-Visible spectrophotometer to detect the production of Fe^{2+}. The highest absorbance reading was then used to standardize the rates of the reactions at other wavelengths.

2.2.5 Spectrophotometric Analysis

The difference spectra were taken on a Cary dual beam UV spectrophotometer, with Sero1C and LDP (the "pseudosubstrate") both at 2 μM concentrations, folded in 50 mM Tris, pH 8.0, with the addition of either 200 mM NaCl or LiCl. The absorbance spectrum of the DNA in the lithium buffer was subtracted from the absorbance spectrum

of the folded DNA in the sodium buffer, and averaged over 5 scans, to obtain the final difference spectrum.

2.2.6 Chemical Probing

For the methylation protection reactions 2 μL of a freshly prepared 4.4% aqeous dimethyl sulfate (DMS) stock was added to 1.5 μL of 5' ^{32}P-labeled Sero1C, in 20 mM potassium cacodylate buffer, pH 7.5, and 240 mM NaCl, with either 100 μM, 500 μM, or 1 mM serotonin. These reactions were allowed to proceed for 30 minutes on ice, and terminated by adding 80 μL of ddH_2O followed by ethanol precipitation. The recovered DNA was resuspended in 10% piperidine and incubated at 90 °C for 30 minutes, followed by 3 lyophilizations. Samples were resuspended in denaturing gel loading buffer (containing 90% formamide and 10 mM EDTA).

DEPC (diethyl pyrocarbonate) reactions were performed on ice for 3 hours with 2.5% DEPC per reaction. The reaction conditions were such that 2 μM Sero1C with negligible amounts of ^{32}P-radiolabeled Sero1C (as a marker), was folded in 5 mM Tris, 50 μM EDTA, 260 mM NaCl (except in ladder), with 10 μM LDP (when present), and 500 μM serotonin (when present). After 3 hours, 50 μL of ddH_2O was added to the reactions and the DNA was ethanol precipitated. The recovered DNA was resuspended in 10% piperidine and incubated at 90 °C for 30 minutes, followed by 3 lyophilizations. Samples were resuspended in denaturing gel loading buffer, and equals counts were loaded in each lane of the 12% denaturing polyacrylamide gel.

For the $KMnO_4$ protection assays, varying amounts of $KMnO_4$ between 1 mM and 10 mM were assayed, at room temperature and on ice, with the reaction times

varying from 30 seconds to 2 minutes. The reactions were quenched with 5% allyl-alcohol. The reactions conditions otherwise were the same as stated above for the DEPC reactions.

2.2.7 Iodouracil Cross-linking

Cross-linking reactions were performed with the "pseudosubstrate", LDP, containing the iodouracil modifications, named T15IdU and T16IdU. 5' ^{32}P-labelled Sero1C, diluted with 2 μM non-labelled Sero1C, was folded in 50 mM Tris-base, and 260 mM NaCl, with 2 μM of either T15IdU or T16IdU. These solutions were irradiated for 30 minutes in an ELISA plate on a Fotodyne transilluminator (as described previously) with a maximum output at 300 nm and also filtered to block out any wavelengths shorter than 300 nm. These samples were then ethanol precipitated and resuspended in denaturing gel loading buffer (as described previously). The samples were then size purified on 8% denaturing gels after visualization with X-ray film and phosphorimager screens. The cross-linked species were eluted from the gel into a buffer containing 10 mM Tris-base, pH 8.0, and 0.1 mM EDTA. The cross-linked DNA species were ethanol precipitated then resuspended in 10% piperidine and incubated at 90 °C for 30 minutes. The samples were lyophilized 3 times, resuspended in denaturing gel loading buffer, and separated on 12% denaturing polyacrylamide gels.

2.2.8 Mapping of position of serotonin

The mapping reactions involved using a ^{3}H-radiolabelled serotonin (5-hydroxy[^{3}H]tryptamine trifluoroacetate) purchased from Amersham Biosciences. 2 μM Sero1C was folded in 20 mM sodium phosphate, pH 7.0, 240 mM NaCl, at room

temperature, in the presence of ^{32}P-radiolabeled Sero1C, or ^{32}P-radiolabeled T15IdU. The DNA was heated to 90 °C for 3 minutes and then allowed to cool slowly back down to room temperature in the above mentioned buffer. 50 μM serotonin (when present in the reactions) and 0.17 μM 3H-radiolabeled serotonin (as a marker) was then added and allowed to fold for another 30 minutes. These reactions were irradiated for 30 minutes on a Fotodyne transilluminator, followed by ethanol precipitation. These samples were loaded onto a 10% denaturing gel. Next, the ^{32}P-radiolabeled bands in the gel were either cut out and put through a scintillation counting process to monitor ^{3}H levels if present, or the gel was dried and exposed to a tritium screen (courtesy of Brad Cooney) and the screen was scanned on the Typhoon phosphorimager.

2.2.9 Spectrofluorometric Analysis

Using the spectrofluorimeter (as mentioned previously) with the band width set at 8 nm and the gain set at 100, I monitored the emission of 5 μM serotonin, after being excited at 302 nm, in the presence of varying concentrations of Sero1C and LDP. In order to ensure accurate readings in duplicate, Hamilton syringes were used to pipette volumes less than 10 μL. The emissions readings were monitored between 315 nm and 450 nm. Sero1C and LDP concentrations were varied between 1 μM and 100 μM. When an oligonucleotide was present at a constant concentration, it was present at 5 μM.

2.3 Results and Discussion

2.3.1 Post-Selection Characterization of the Sero1C Deoxyribozyme

Originally, the serotonin-dependent deoxyribozyme, Sero1, obtained from the previously mentioned *in vitro* selection, was a 95 nucleotide self-repairing deoxyribozyme, that used light and serotonin as a cofactor, to repair the thymine dimers solely under single turnover conditions. To be considered a true enzyme, one of the criteria that needed to be satisfied was the ability of the deoxyribozyme to perform multiple turnover catalysis, such that the deoxyribozyme was not consumed or depleted during catalysis. Dan Chinnapen (Chinnapen 2005) had tested whether this deoxyribozyme and substrate could be separated into distinct oligonucleotides. Therefore, the original 95-nucleotide sequence was separated into a 35-nucleotide substrate sequence (which was the 5' constant region in the original sequence, named TDP), and a separate enzyme sequence (the 40 nucleotide random region, named Sero1C - see Figure 1-12). Using the deoxyribozyme and substrate oligonucleotides, he was able to detect significant repair of the thymine dimer in the substrate, relative to negative controls. This was where I started my characterizations of Sero1C. When performing any reactions with Sero1C there were two negative controls that were performed in parallel to every reaction. One negative control had no serotonin, and the second had no deoxyribozyme present (in its place, a 42 nucleotide unrelated sequence was present). In order to ensure that it was the deoxyribozyme, together with serotonin, which was responsible for the catalysis of thymine dimer repair, the two negative controls answered these two questions: 1) Can another unrelated DNA sequence substitute for Sero1C and work with serotonin to repair the thymine dimers? 2) Will Sero1C work in the absence of serotonin

to catalyze the photoreactivation of the thymine dimers? This was where my initial characterizations of Sero1C began. Figure 2-2 shows a denaturing gel that was loaded with individual time points from a reaction. The 35-nucleotide substrate product, TDP, indicates unrepaired thymine dimer, and the 15-nucleotide product is the 5' ^{32}P-labelled end of TDP that starts to appear as the thymine dimer is being repaired (refer back to Figure 1-9). These results are indicative that another DNA sequence was not able to replace Sero1C and catalyze repair of the thymine dimers. Second, Sero1C was not able to catalyze repair of the thymine dimers in the absence of serotonin. Figure 2-3 shows the analysis of the results, from Figure 2-2, from which k_{init} was calculated.

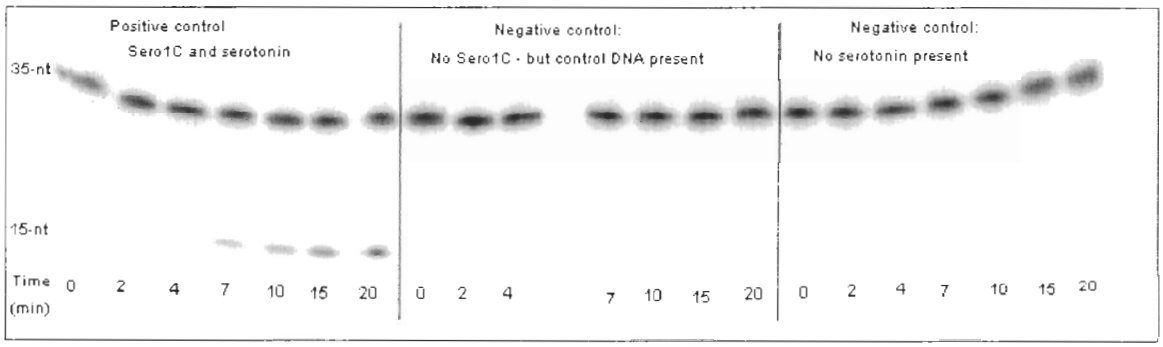


Figure 2-2 Example of time points from a kinetic reaction loaded onto a 12% denaturing polyacrylamide gel electrophoresis system and scanned onto the Typhoon phosphorimager.

We noticed however, that the rate of repair from Sero1C, versus the original 95 nucleotide self-repairing deoxyribozyme, was decreased. Sero1 in the presence of 50 μM serotonin could repair its internal thymine dimer at a k_{init} of approximately 0.198 min^{-1}, yet Sero1C with 50 μM serotonin only had a k_{init} of 0.0163 min^{-1}, almost ten times slower than Sero1. These reactions (shown in Figure 2-2) were performed under single turnover

conditions, assuming that all of the substrate, TDP, was bound to Sero1C and serotonin. Even though the overall rate of repair of the thymine dimer was decreased when the deoxyribozyme was acting *in trans*, all further experiments were carried out with the

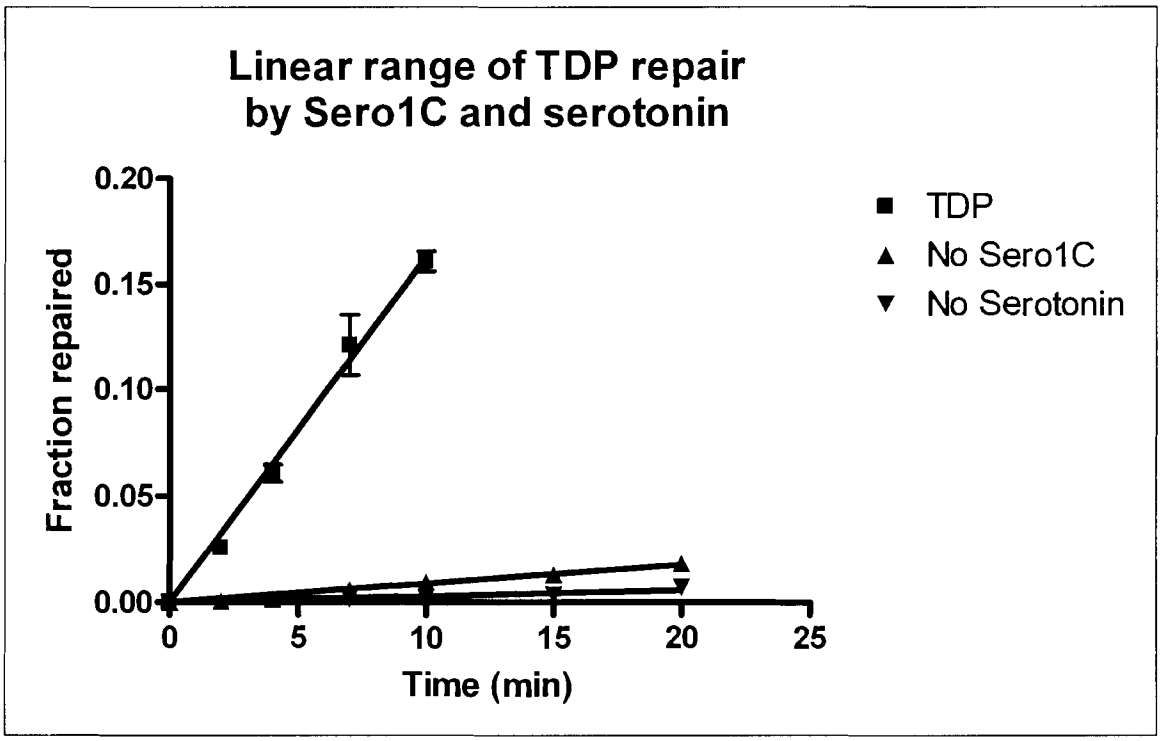


Figure 2-3 **Typical analysis of linear range of repair of the thymine dimer substrate, TDP. 50 μM serotonin, 2 μM Sero1C, and negligible amounts of TDP are present to determine k_{init}. No Sero1C is the negative control where no deoxyribozyme is present, but a control DNA is present with serotonin. No Serotonin is the negative control where Sero1C is still present, but no serotonin is present.**

40-nucleotide Sero1C deoxyribozyme. However, I did want to optimize further the conditions of the *trans* reactions to see if we could increase the rate of repair. One area of possible improvement was the concentration of serotonin present in the reactions. One explanation, for the decrease in rate of the *trans*-acting Sero1C (versus the *cis*-acting Sero1), could be that more serotonin was needed to bind the deoxyribozyme in an

intermolecular *trans*-system as compared to the *cis* construct where both enzyme and substrate were part of a single strand of DNA. In other words, it may have been easier for Sero1 to 'trap' serotonin somewhere between the catalytic region and the substrate region when they were part of one strand of DNA.

2.3.1.1 Serotonin Dependence of Sero1C revealed approximate K_d

With separate strands of DNA, the serotonin may have to position itself simultaneously while Sero1C and TDP were forming a secondary structure together. Therefore, my next experiment involved testing the serotonin concentration necessary for efficient repair of the thymine dimer substrate while Sero1C acted *in trans*. The original *in vitro* selection had been performed with a 10 μM serotonin concentration. I tested serotonin concentrations ranging from 10 μM to 4 mM, as shown in Figure 2-4. From these results we were able to calculate an approximate "functional" K_D of serotonin to be 73 μM. The fastest initial rate obtained was at a serotonin concentration of 1 mM, with an initial rate of approximately 0.07 min^{-1}. This rate was closer to that of the *cis* acting deoxyribozyme. Figure 2-5 also shows that the optimal rate enhancement over background was produced when serotonin was present at 50 μM. This rate enhancement was defined as the rate of Sero1C performing the catalytic reaction divided by the rate of the negative control reaction (where the 'control' DNA was present and not Sero1C).

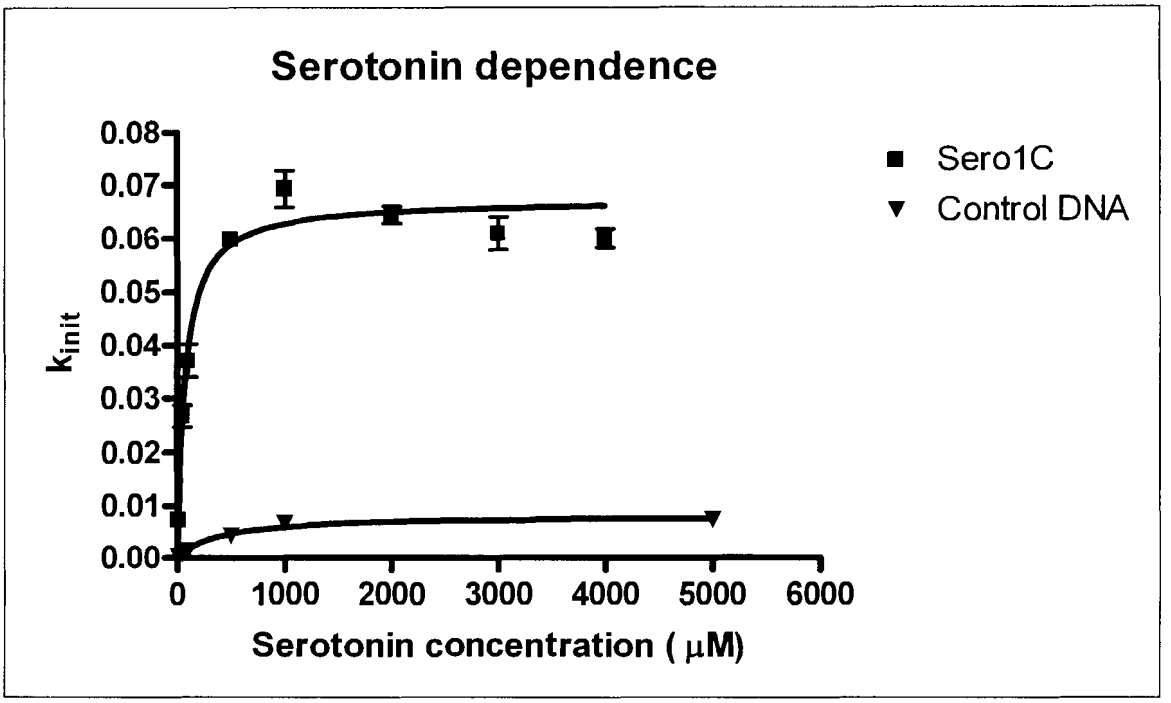


Figure 2-4 Analysis of serotonin dependence of initial rate of repair by Sero1C, revealing approximate K_d of serotonin.

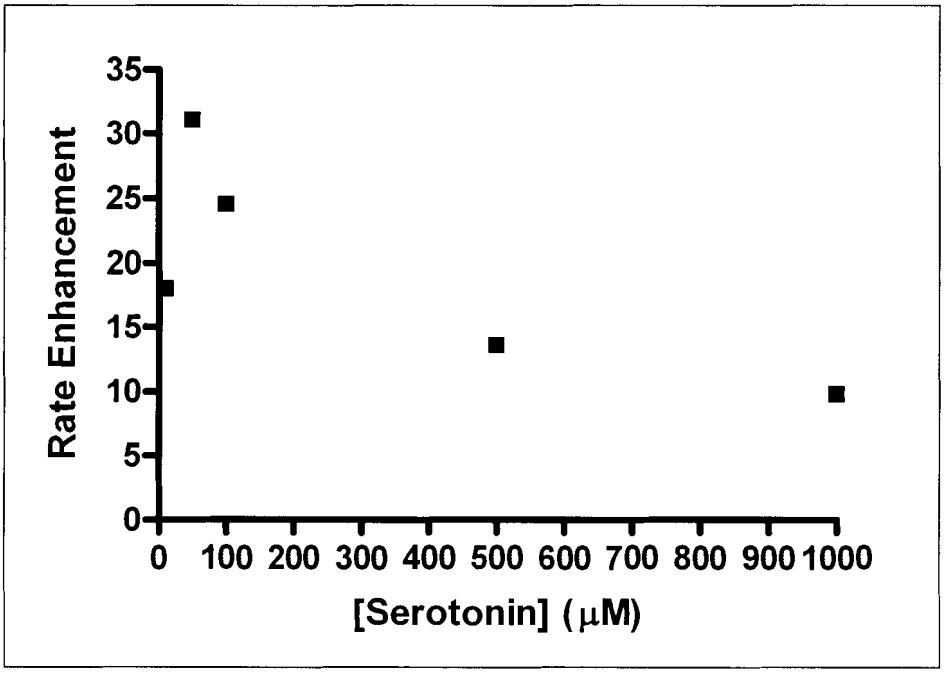


Figure 2-5 Rate enhancement analysis of Sero1C catalysis over the background reaction.

2.3.1.2 A Multiple turnover deoxyribozyme

Having optimized the serotonin concentration at which the deoxyribozyme was able to best catalyze repair of the thymine dimer *in trans*, I tested the ability of Sero1C to perform multiple turnover catalysis, in order to determine k_{cat} and K_M. From the approximate K_d value of serotonin, I am able to calculate the amount of Sero1C in complex with serotonin, assuming a 1:1 ratio, at any certain serotonin concentration, according to Equation 1:

$$K_d = \frac{[\text{Sero1C}]\,[\text{serotonin}]}{[\text{Sero1C}\cdot\text{serotonin complex}]} \qquad \text{Equation 1}$$

For most of my reactions, I used 2 μM Sero1C and 500 μM serotonin (for saturation). The amount of Sero1C complexed with serotonin can be calculated by substituting the following values into the above equation: [Sero1C] = (2 μM – X), X being the amount of Sero1C in complex with serotonin. [Serotonin] = (500 μM – X), and [Sero1C·serotonin in complex] = X. This then gives the following:

$$K_d = \frac{(2-[X])\,(500-[X])}{[X]} \qquad \text{Equation 2}$$

Solving the above equation for X, via a quadratic equation, yields a value of 1.7 μM. Therefore under these conditions, 87% of Sero1C was in complex with serotonin. Using this value, I was able to adjust the serotonin concentration so that the amount of Sero1C in complex with serotonin remained the same and comparable to other data and experiments. Therefore, when I decreased the amount of Sero1C present in the

multiple turnover conditions, in order to obtain a higher degree of substrate in excess, we adjusted the serotonin concentration accordingly to obtain the fastest rates. Verified by the same equations as above, when the Sero1C in the reaction was reduced to 20 nM, the presence of 500 μM serotonin still assured that the same percent of Sero1C would be in complex with serotonin.

The multiple turnover catalysis reactions were therefore carried out with 20 nM Sero1C, 500 μM serotonin, and the TDP substrate varying from 0.1 μM up to 10 μM, corresponding to a five-fold to 500-fold excess of substrate, respectively, compared to the deoxyribozyme. These multiple turnover conditions (Figure 2-6A) did not yield either a k_{cat} or K_M value since we were unable to reach a saturation point. Figure 2-6B and 2-6C are examples depicting that I used the slope of the linear region of repair, for each concentration of TDP, as the V_{init} data point to create Figure 2-6A.The fact that saturation could not be reached may be due simply to the fact that more data points, at higher TDP concentrations, were needed. Overall, this indicates that compared to UV1C, Sero1C would have a higher K_M. It was difficult to increase the substrate concentration, as the yield of the TDP formation reaction was low. Regardless, this experiment still showed that Sero1C is capable of multiple turnover catalysis.

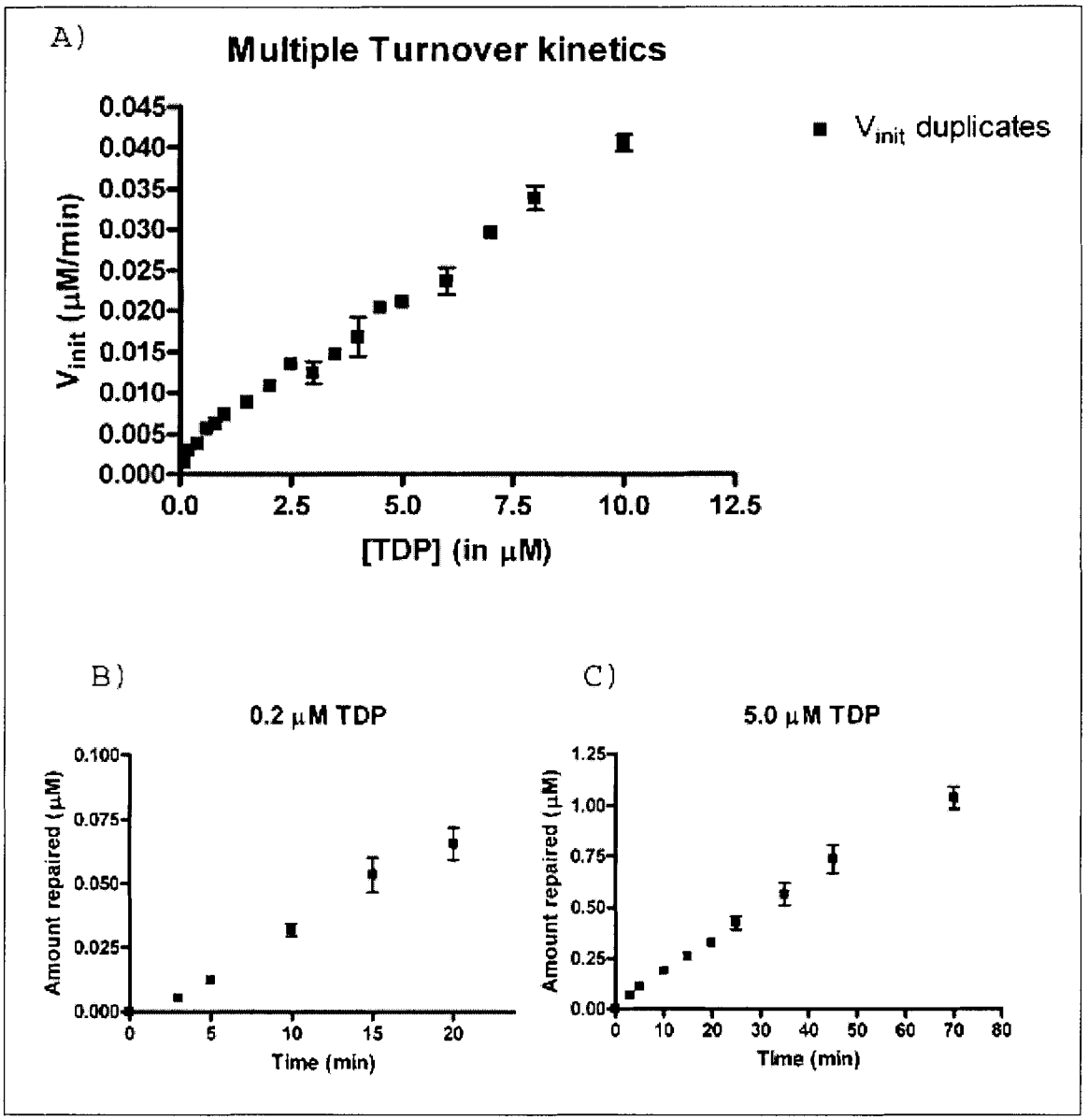


Figure 2-6 **Final analysis of multiple turnover reactions.**
A) Final graph of multiple turnover kinetics of Sero1C: 20 nM Sero1C and 500 μM serotonin.
B) Linear region of repair by 20 nM Sero1C of 0.2 μM TDP with 500 μM serotonin.
C) Linear region of repair by 20 nM Sero1C of 5 μM TDP with 500 μM serotonin.

2.3.1.3 Wavelength Dependence of Sero1C

The absorption spectrum of serotonin dissolved in water shows two main peaks (see Figure 1-7). The maximum absorbance of serotonin is at 285 nm, but there is also

another broad peak of absorbance ranging from 290 nm to 310 nm. This later peak was of more interest since we can excite serotonin selectively in the presence of the deoxyribozyme at greater wavelengths than 300 nm. But, is exciting serotonin at wavelengths greater than 300 nm better for catalysis? I wanted to determine an optimal wavelength of catalysis for Sero1C, therefore I investigated the wavelength dependence of Sero1C catalysis to produce an action spectrum.

I used a spectrofluorimeter to excite the serotonin, within the reactions, at different wavelengths. Before creating a final action spectrum (wavelength dependence of Sero1C), I performed experiments to correct for the different values of light intensity at each wavelength. One of the best chemical actinometers, to do so, is the potassium ferrioxalate ($K_3Fe(C_2O_4)_3$) system developed in the early 1950s (Hatchard and Parker 1956). Upon irradiation of ($K_3Fe(C_2O_4)_3$) solutions the Fe^{III} reduces to Fe^{II}. The more light intensity being directed at the sample, the more Fe^{III} will reduce to Fe^{II}. A 1,10-phenanthroline solution is then added, and the formation of $[Fe(phen)]^{II}$ is then detected via absorption at 510 nm. Therefore, the more $[Fe(phen)]^{II}$ detected, the greater the light intensity at that particular wavelength. Figure 2-7 shows that the light intensity is generally increasing as I increased the wavelength of irradiation. These values are expected with the use of the Xenon Short Arc Lamp used in our spectrofluorimeter. My values obtained for this experiment on our spectrofluorimeter, shown in Figure 2-7, were used to determine correction factors to standardize the initial rate values obtained in the analysis of the reactions at each wavelength, such that we then assumed constant light intensity for each wavelength.

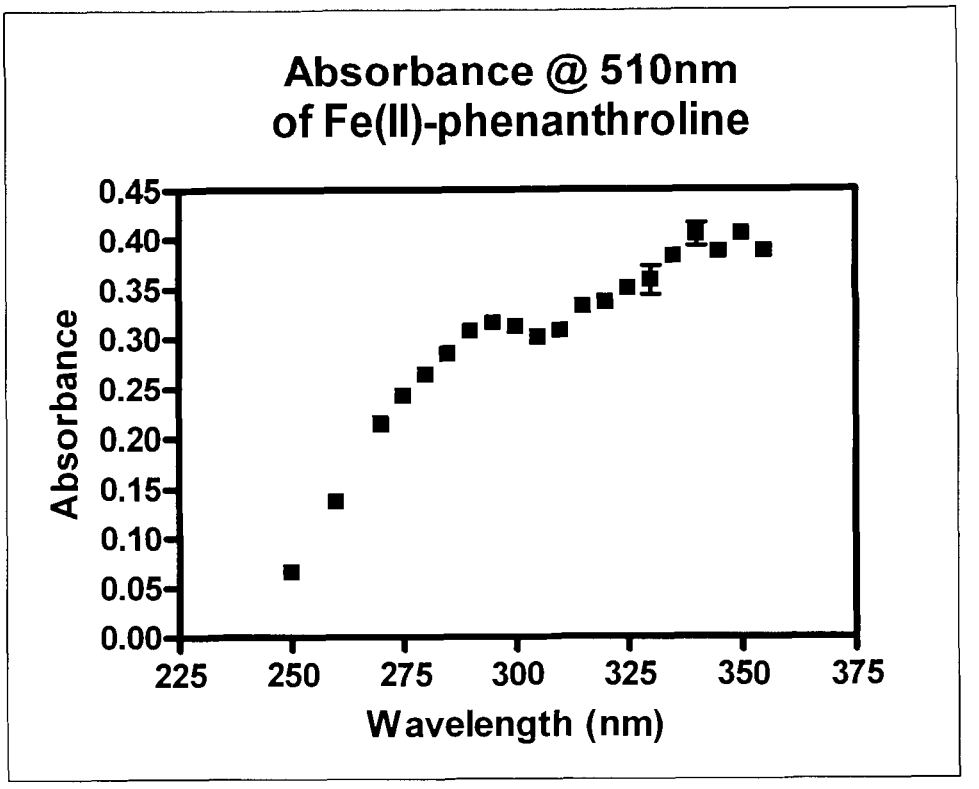


Figure 2-7 Potassium ferrioxalate actinometry analysis; monitoring production of Fe(II) at 510nm.

The final action spectrum of Sero1C with serotonin is shown in Figure 2-8, and indicates that the optimal wavelength for repair of the thymine dimer substrate by the deoxyribozyme, and not by direct photoreversal, was at 300 nm. Again, both negative controls were performed to ensure that direct photoreversal, or direct repair by serotonin were not playing a significant role in the catalysis. These confirm that catalysis requires both by Sero1C and serotonin (Figure 2-8). However, the rate enhancements plotted on top of the action spectrum (Figure 2-9) shows that the optimal wavelength for rate enhancement, over the background rates, was at approximately 315 nm. Therefore, even where serotonin naturally absorbs the most amount of light energy, at 285 nm, there was little rate enhancement over the background reactions at this wavelength.

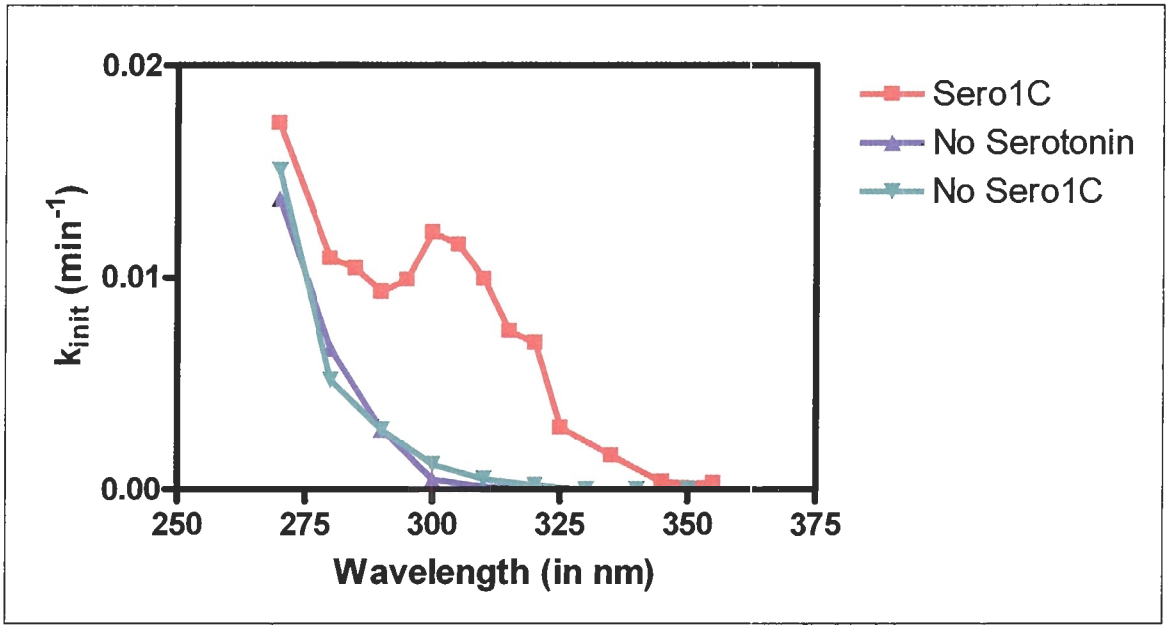


Figure 2-8 **Wavelength dependence of Sero1C: Complete action spectrum corrected for light intensity.**

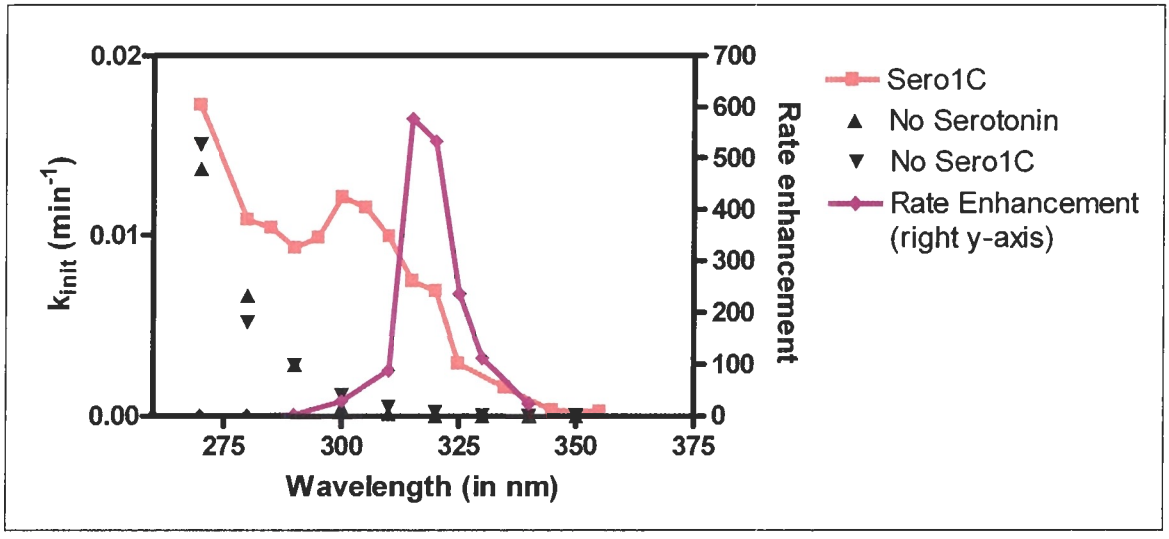


Figure 2-9 **Action spectrum overlapped with rate enhancements over background rates.**

Another point of interest, shown in Figure 2-10, is revealed when the absorption spectrum of serotonin is overlaid onto the action spectrum. It is notable that the second absorption peak of serotonin, between 290 nm and 310 nm, corresponded well with the most efficient repair of the thymine dimers by Sero1C and serotonin. This indicates that the serotonin is directly involved in the repair of the thymine dimers. From previous absorbance spectra, such as for tryptophan and serotonin (refer back to Figure1-8), it appears that the second peak in the absorption spectrum of serotonin is associated with the influence of the hydroxyl substitutions on the indole ring.

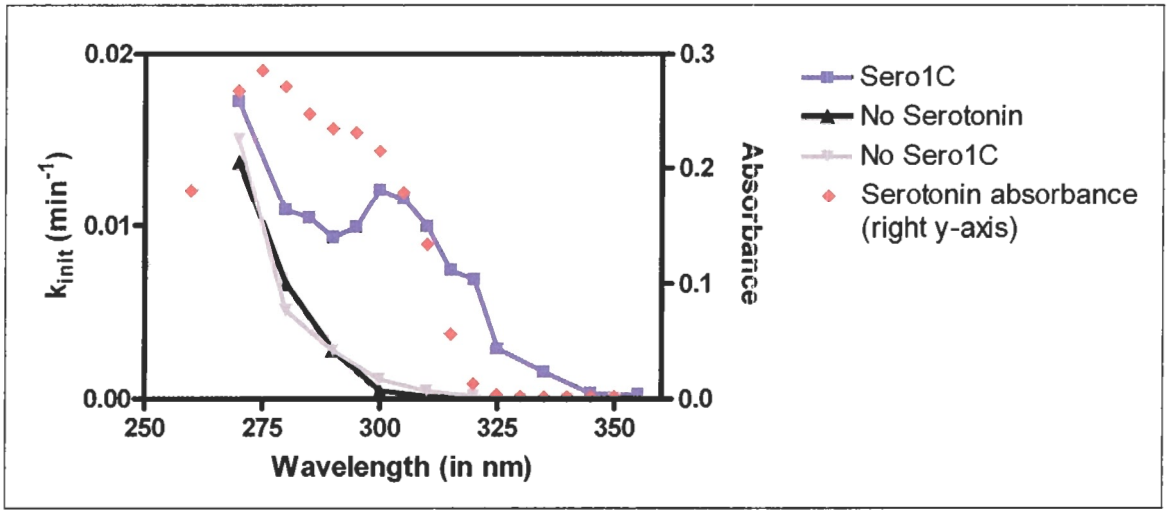


Figure 2-10 Action spectrum overlapped with absorbance spectra of serotonin.

2.3.1.4 Salt Dependence of Sero1C

Since serotonin was obviously playing a significant role as a cofactor in catalysis, I wished to investigate if there were any other catalytic requirements that Sero1C may have, such as specific salt requirements. It was possible that the metal ions involved may have been playing an important structural role in allowing the catalytically active

structure to form. For instance, Sero1C is rich in guanines (35% of the total number of bases), and UV1C, which was composed of 37.5% guanines, was shown to form guanine quadruplexes (G-quartets), I postulated that Sero1C may also form G-quartets, and that these may be important to thymine dimer repair.

Guanine quadruplexes are cyclical arrangements than can form in a single strand, or strands, of DNA when the DNA is rich in guanine bases, and subjected to the proper buffer conditions (Henderson *et al.* 1987; Sen and Gilbert 1988; Williamson *et al.* 1989; Sen and Gilbert 1990; Williamson 1994). Typically, G-quartets are stabilized by sodium or potassium ions, and are formed through Hoogsteen base-pairing (Figure 2-14). Several different forms of G-quartets may form depending on how many DNA strands are present. In the case of Sero1C, the most feasible possibility was for intramolecular G-quartets stacked upon on another, as was the case for UV1C.

I tested the activity of Sero1C when folded in buffers containing either lithium, sodium, or potassium. The results, shown in Figure 2-11, indicated that Sero1C worked optimally in the presence of sodium, rather than of lithium or potassium. Interestingly, Sero1C was still able to catalyze repair of the thymine dimer, at a slightly greater rate than that of the background, in the presence of lithium. This could suggest that Sero1C was not forming G-quartets to absorb the light energy, and also that the salt ions may have a role in catalysis as well as a structural role. In contrast, UV1C did form G-quartets, and it was shown that the G-quartets were actually part of the mode of catalysis. In UV1C it was the G-quartet structure that was responsible for harnessing the light energy, and transferring an electron to the thymine dimer for repair (Chinnapen and Sen 2004). In Sero1C, serotonin harnesses the light and likely contributes the electron. The

data presented in Figure 2-11 supports these significant differences between the two deoxyribozymes. The DNAzymes were obtained from the same *in vitro* selection, to catalyze the same reaction.

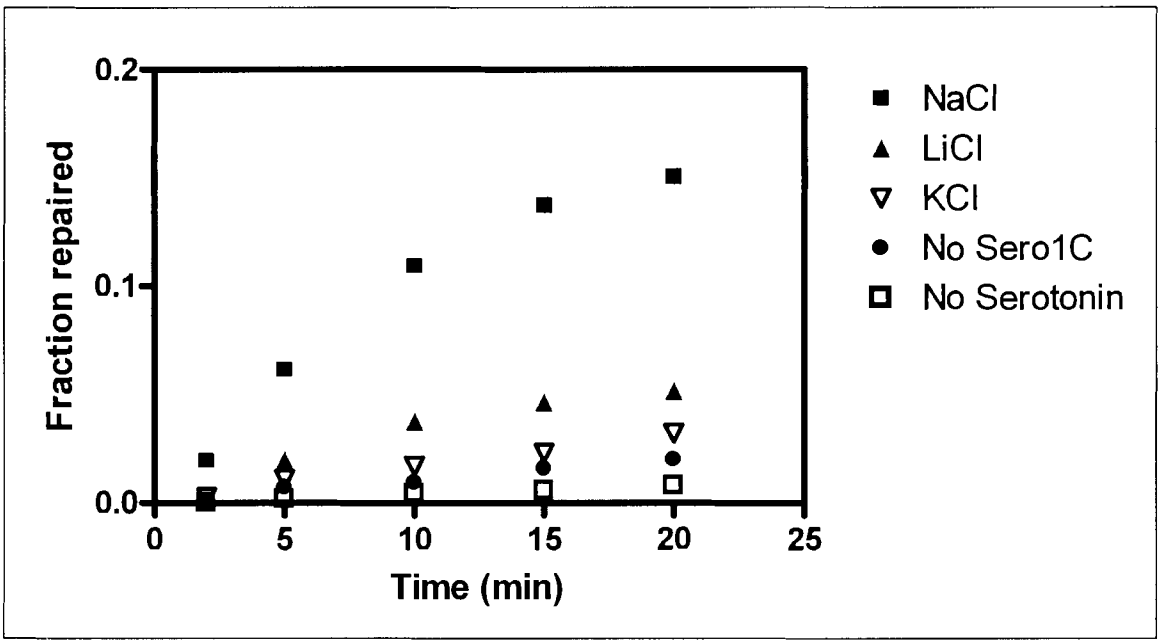


Figure 2-11 Salt dependence analysis.

To investigate these differences in more detail, and to further test whether Sero1C forms G-quartets, experiments involving methylation protection and spectrophotometric analyses were performed. These experiments were performed to confirm the results of the data regarding the salt dependence of Sero1C. The salt dependence experiments indicated that Sero1C was not forming G-quartets due to its ability to correctly fold and catalyze photorepair of the thymine dimers in the presence of LiCl, even though it was to a lesser extent.

Mergny et al. (Mergny *et al.* 1998) showed that oligonucleotides forming G-quartets, when folded, give a characteristic absorption spectra compared to that of when they are not folded. In absorbance spectra, where G-quartets are present, one expects some absorbance at wavelengths greater than 300 nm, whereas an oligonucleotide not forming G-quartets should have little or no absorption above 300 nm. Therefore, the difference spectra between the folded and unfolded enzyme normally gives a characteristic "tail" of positive absorption in the 290-305 nm region. The necessary spectrophotometric analysis for Sero1C therefore involved creating a difference spectra in which I recorded absorbance spectra of Sero1C folded in NaCl, and subtracted the absorbance spectra of Sero1C folded in LiCl. These results are shown in Figure 2-12. The shape of the difference spectrum was not consistent with the formation of G-quartets. This difference spectrum lacked the characteristic "tail" region (at a value above zero) between 290 nm and 310 nm. Therefore, these findings were consistent with the above

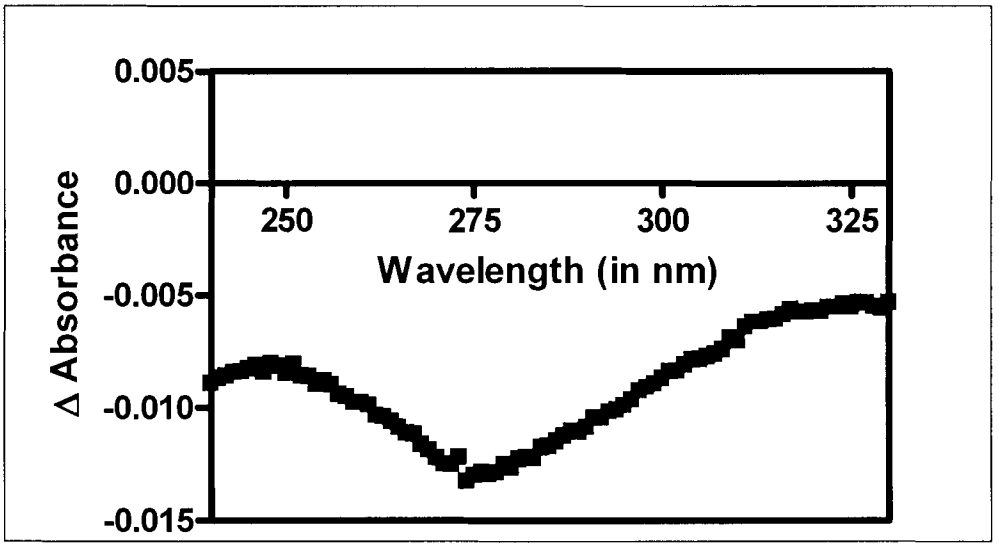


Figure 2-12 Difference spectrum of Sero1C; from absorbance spectra of Sero1C folded in sodium or lithium buffers.

mentioned salt dependence in which G-quartets were not thought to be part of the secondary structure of Sero1C. This was also confirmed by comparing my results to Dan Chinnapen's, shown in Figure 2-13, where he was able to conclude that G-quartets were forming.

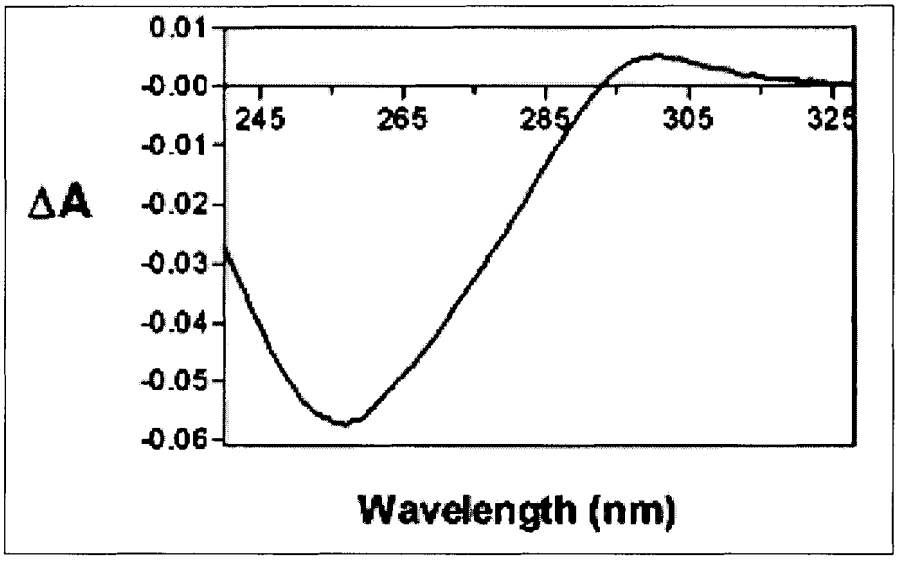


Figure 2-13 Dan Chinnapen's difference spectrum of UV1C.

Finally, to ensure that Sero1C was not forming G-quartets, I performed methylation protection assays, with dimethyl sulfate (DMS), to see whether or not the N7 positions of the guanines were being protected or not. As shown in Figure 2-14 it is the N7 position of guanines that are involved in the hydrogen bonds forming G-quartets. DMS methylates the N7 position of guanines if they are available, in other words, not occupied in a specific secondary structure involving the N7 position. If the N7 position is being occupied, by some hydrogen bonding, it will not be modified by DMS. Further piperidine treatment cleaves the DNA at the guanines that have been modified by the

DMS. If G-quartets form in Sero1C, we would expect to see protection of at least 4 guanines.

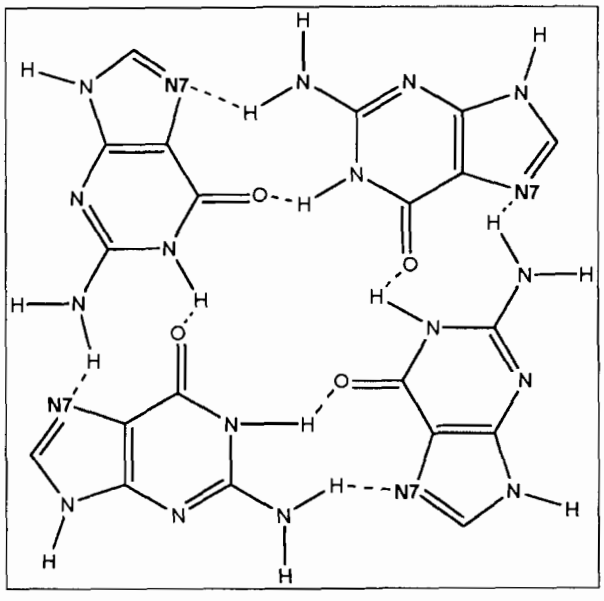


Figure 2-14 Guanine quartet structure.

To increase the stabilization of the putative G-quartet, the DMS reactions were performed on ice, under varying conditions with serotonin and LDP. As seen in Figure 2-15 little protection was seen under these conditions. Dan Chinnapen's DMS reactions showed significant protection of all of the grouped guanines (Chinnapen and Sen 2004). Therefore, there was insufficient protection seen at enough guanine positions to indicate G-quartets forming within Sero1C.

It was interesting to note that two deoxyribozymes, discovered in the same *in vitro* selection, have distinctively different sequences and apparently very different structures, and therefore possibly different mechanisms of photoreversing the thymine

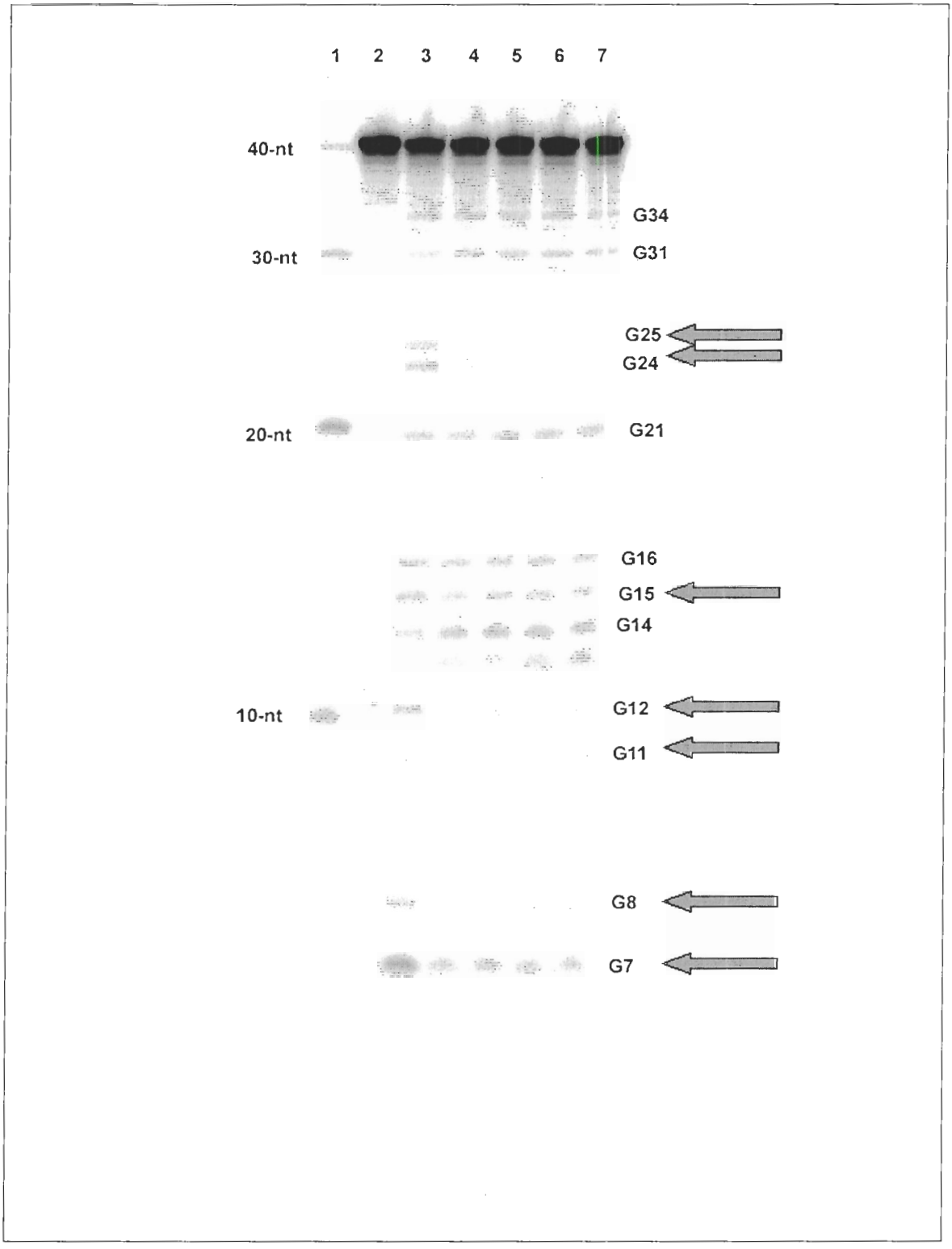


Figure 2-15 **Gel of DMS chemical probing reactions on Sero1C.**
Lane 1 = 10 base-pair ladder, lane 2 = non-DMS treated Sero1C, lane 3 = G-ladder, lane 4 = No serotonin present when DMS treated, lane 5 = 100μM serotonin present with LDP, lane 6 = 500μM serotonin present, lane 7 = 1mM serotonin present.

dimer substrate. These results are attributable to the addition of a cofactor, serotonin, as opposed to UV1C, the cofactor-independent deoxyribozyme.

2.3.1.5 Investigation of serotonin as the cofactor

As demonstrated from all of the above mentioned experiments, Sero1C did not seem to be harnessing light energy through a G-quartet structure, and could not be excited without serotonin present. Therefore, we hypothesized that it was the cofactor, serotonin, harnessing the light energy. The serotonin could be working in a number of ways once excited: It could either be passing an electron, after absorbing the light energy, directly to the thymine dimer as Sero1C holds it in position, or, serotonin absorbs the light energy and transfers an electron to Sero1C, and subsequently to the thymine dimer. Serotonin is an indole-containing compound, and very electron rich.

First we tested the ability of other indole containing compounds to absorb light energy and catalyze the repair of the thymine dimers as a cofactor for Sero1C. One problem was discovered when trying to find a suitable wavelength to excite these compounds since most of them did not absorb light at our wavelength of interest (greater than 300 nm). We tested compounds such as tryptophan, tryptophol, tryptamine, a tryptophan-glycine dipeptide, and finally a lysine-tryptophan-lysine tripeptide. Figure 2-16 shows the structures of all these different indole containing compounds compared to the structure of serotonin. Figure 2-17 shows that none of these indole-containing compounds were able to catalyze repair of the thymine dimers when irradiated at >300 nm. Interestingly, when I excited tryptophan at its maximum excitation, of 279 nm, as opposed to 300 nm where it can barely absorb any light, tryptophan was able to repair the thymine dimers better than any of the other possible cofactors, except not at a rate

greater than that of the background reactions (these were the tryptophan values shown in Figure 2-17). There were two problems with this result: 1) It was most likely the direct photoreversal of the thymine dimers that we were detecting, since the background rates are higher at 279 nm, and 2) The wavelength was too low, therefore we could be producing more thymine dimers at approximately the same rate of repair, which defeats the purpose of using a cofactor that can absorb light at longer wavelengths. One last cofactor we tested was 5-hydroxyindole, which lacks the amino-alkyl chain of serotonin. Overall, no other indole containing compounds were able to act as a cofactor for Sero1C. Therefore, since these compounds did not catalyze repair of the thymine dimer, we

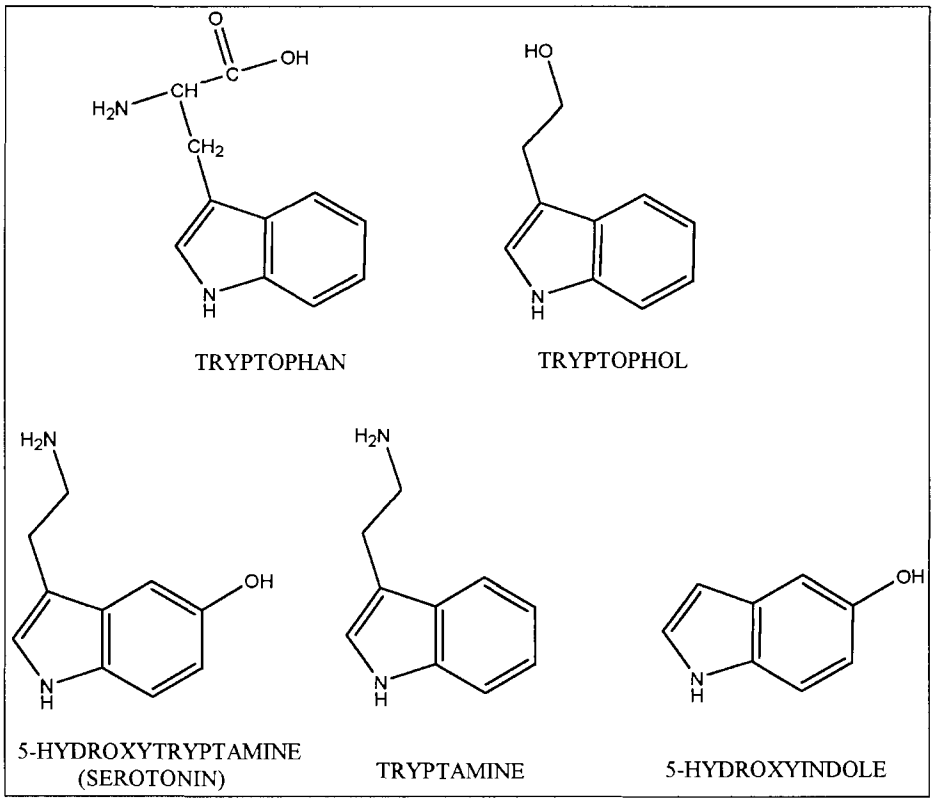


Figure 2-16 Structures of other indole-containing compounds tested for ability to act as cofactor for Sero1C.

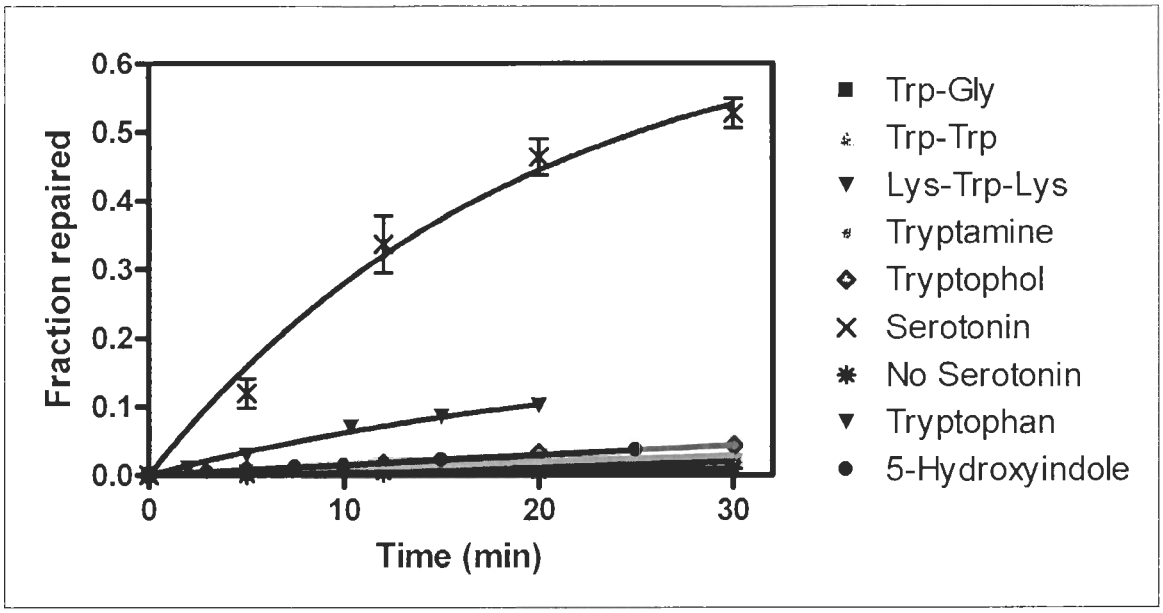


Figure 2-17 Analysis of assaying different indole containing compounds as possible cofactors for Sero1C.

hypothesized that the amino-alkyl group, of the serotonin, may be playing an important role in binding or intercalating within the secondary structure of Sero1C.

2.3.1.6 Active form of serotonin during catalysis

Serotonin can have an unionized and an ionized form, and only one of these forms may be the active form during catalysis. To further elucidate the role of serotonin with Sero1C in this thymine dimer repair reaction, I performed a pH dependence where I monitored k_{init} of Sero1C in buffers of different pH. To determine whether the ionization state of serotonin was important in catalysis these reactions were all carried out at subsaturating levels of serotonin (50 μM), and at varying pH. Since the literature value of the pK_a of serotonin is approximately 9.8 (Newton and Kluza 1978) I chose to monitor k_{init} of Sero1C between pH 7.4 and pH 10.2 (in 0.4 increments).

The analysis of this experiment involved determining the logarithm values of each k_{init} observed, and plotting these values against the pH levels at which each k_{init} was determined. A linear plot that generates a slope of 1, in this format, suggests that a single deprotonation event was occurring during catalysis (Fersht 1985). As shown in Figure 2-18, a linear plot was achieved through my analysis, with a slope of –0.46. This suggests that a deprotonation event of serotonin was not occurring during catalysis, therefore the deprotonation of serotonin was not required in order for optimal catalysis to occur.

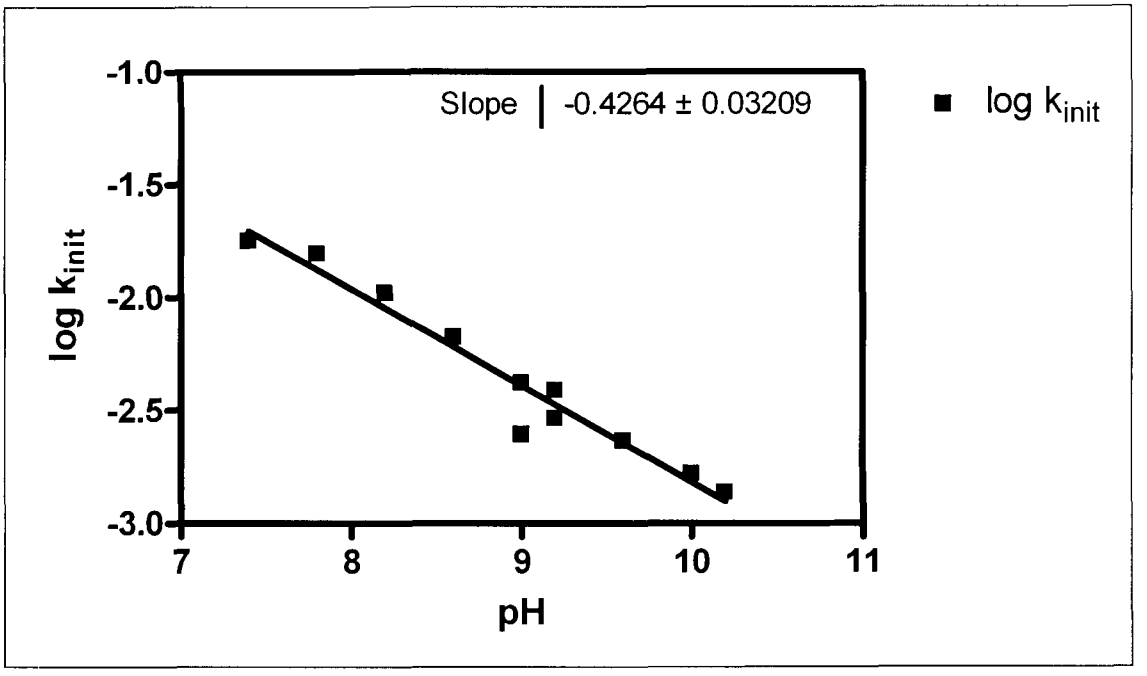


Figure 2-18 pH dependence, at pH values greater than 7.0, to determine catalytic role of serotonin.

2.3.1.7 Location of serotonin

I also wished to determine whether the role of serotonin, in these thymine dimer repair reactions, was solely catalytic or structural as well. I wished to determine where serotonin was actually binding. Is it binding to either Sero1C, TDP, or parts of both? For

this I utilized the fluorescent properties of serotonin. The fluorescence emission of an excited molecule, such as serotonin, can be quenched by specific binding interactions. Therefore, I performed the following binding assays under the assumption that the fluorescence from serotonin would be quenched upon binding to DNA. I had hoped to see fluorescence quenching when a constant concentration of excited serotonin was binding to either increasing amounts of Sero1C or the pseudosubstrate, LDP. I performed separate assays for Sero1C and LDP so that if quenching was observed with one oligomer and not the other, this would indicate whether serotonin was binding Sero1C or LDP specifically. I also performed experiments where the concentration of Sero1C was gradually increased in the presence of a constant amount of LDP, and where the concentration of LDP was gradually increased in the presence of a constant amount of Sero1C: These experiments test whether both the substrate and enzyme are necessary to form a tertiary structure that binds serotonin. Finally, I also monitored serotonin fluorescence while increasing the amounts of both Sero1C and LDP. Figure 2-19 shows the results for increasing concentrations of Sero1C in the presence of the pseudosubstrate, LDP. Some fluorescence quenching was seen as well as a red shift in the wavelength value where emissions were at a maximum. The maximum emission peak shifted from 335 nm to 350 nm. The same results were also seen when LDP was not present in the reaction mixture. Therefore, there was some form of change occurring with Sero1C and serotonin together.

It was difficult to make any substantial conclusions from these quenching data, as well as the fact that the peak in emissions shifted approximately 15 nm. Recent literature research revealed two possible explanations for the red-shifting, although more

experiments would be needed in order to confirm either hypothesis. First Stuart *et al.* revealed that while purifying endogenous serotonin, from a mammalian system, and

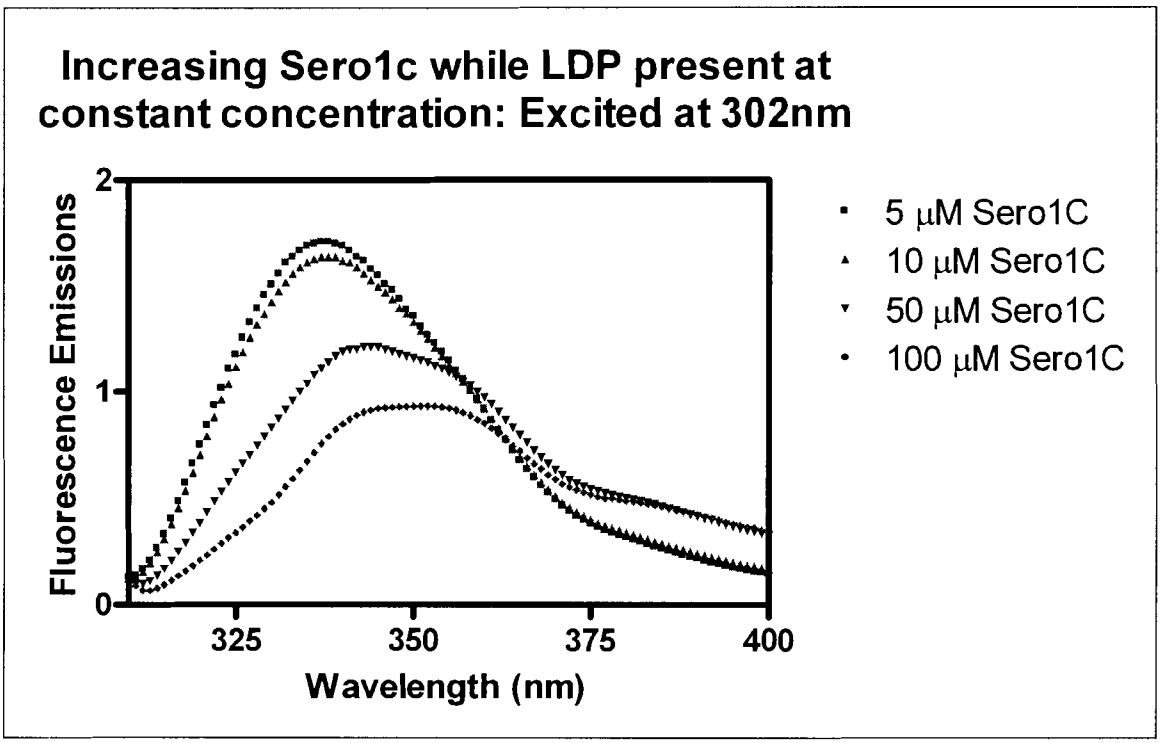


Figure 2-19 Monitoring fluorescence emissions of serotonin while increasing Sero1C concentrations. 5 μM UDP, and 5 μM serotonin were present at constant concentrations.

using electrokinetic injection system with capillary electrophoresis to analyze their samples, they detected a red-shift in the emissions peak of serotonin. Through further studies they determined that it was due to a serotonin dimer forming. Granted, these experiments were done with endogenous serotonin, and nitric oxide was also needed to form the serotonin dimers. Another possibility could be that the serotonin being detected was ionized, since Chattopadhyay *et al.* (Chattopadhyay *et al.* 1996) demonstrated a red-shift in the emissions peak, but only when pH was equal to the pK_a. Since my pH

dependence did not reveal an ionization event, this latter explanation was probably less likely.

Figure 2-20 shows the results of increasing LDP concentrations in the presence of constant concentrations of Sero1C and serotonin (the same results were also seen without Sero1C present). Fluorescence quenching was also seen under these conditions, almost to the same extent, although no red-shifting of the maximum emissions peaks was being detected. These experiments did not lead to any definite conclusions, other than serotonin may be binding Sero1C, possibly as a dimer, and more significant change (red shifting of emissions peak) was noted in the presence of increasing concentrations of Sero1C.

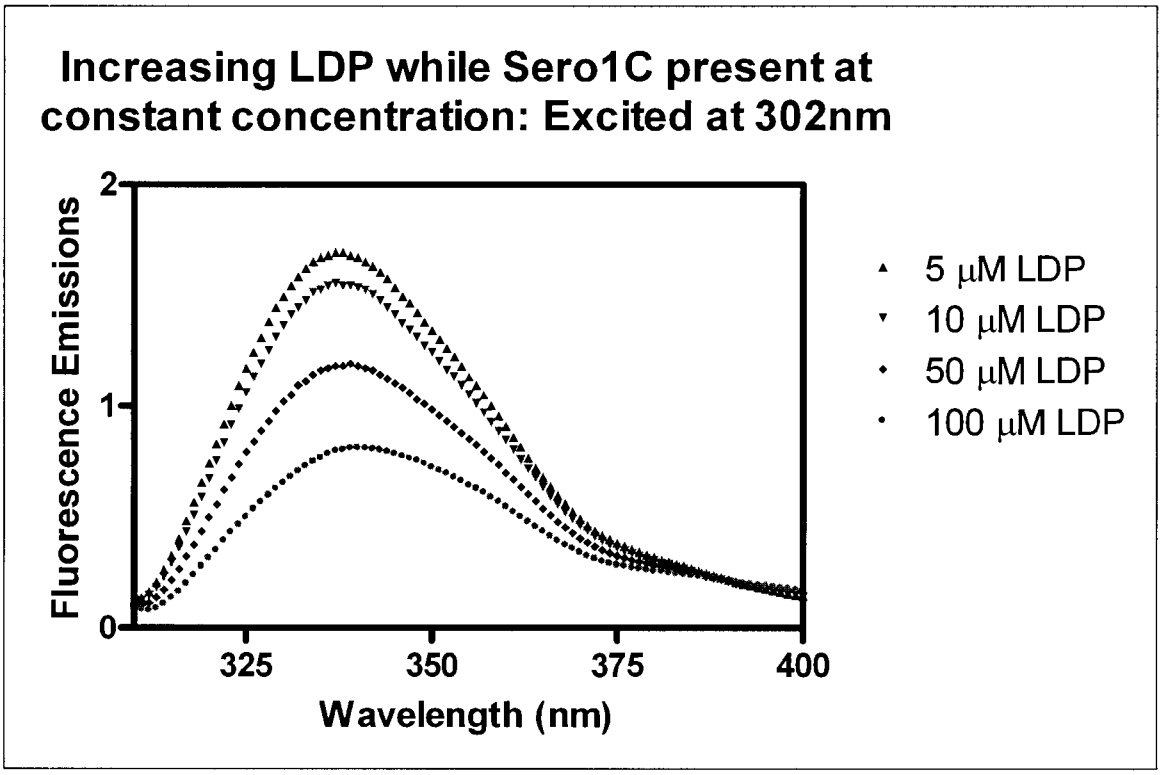


Figure 2-20 Monitoring fluorescence emissions of serotonin while increasing the concentration of the pseudosubstrate, LDP. 5 μM Sero1C and 5 μM serotonin were present at constant concentrations.

Further attempts at determining a more detailed secondary structure and relationship between Sero1C, TDP, and serotonin, are discussed in the next section (section 2.3.2). I also attempted these experiments with 5-HI as well, and I did not see any red shift of the emissions peak occurring, but did see fluorescence quenching (to the same extent as with serotonin).

2.3.2 Structure and Folding of Sero1C

2.3.2.1 Mutant deoxyribozyme and substrate constructs

To hopefully aid in the determination of a catalytic domain within the 40 bases of Sero1C, and probe the secondary structure, I created deletion mutants of Sero1C. These mutants were created to determine which bases were critical for catalysis. Many different deletion mutants were created by removing one base at a time from, either the 3'-end, the 5'-end, or both simultaneously. These shortened, deletion mutants, were then tested for their ability to catalyze photorepair of the thymine dimer with the aid of serotonin. I first tested the mutants in which bases had been removed from the 5'-end (Figure 2-21). There was a noticeable drop in the ability of these shortened Sero1C constructs to catalyze repair of the thymine dimers after the removal of only 1 or 2 bases (Figure 2-22). E10 and E11 showed a decrease in their initial rate of repair to approximately 50% compared to that of full length Sero1C. Also, after the removal of only 3 bases from the 5' end of Sero1C, E12, there was another noticeable drop in efficiency of repair down to 25% of that of wild type. The activity of E13 and E14 remained around 25% of that of wild type Sero1C. These results indicate that the 5' most bases having a significant effect on the folding of Sero1C necessary for efficient catalysis of thymine dimer repair.

```
      5'                                                    3'
Sero1C TAAGTCGGATGGAGGGATCCGTTGGCACATGTAGTCACGT
E10     AAGTCGGATGGAGGGATCCGTTGGCACATGTAGTCACGT
E11      AGTCGGATGGAGGGATCCGTTGGCACATGTAGTCACGT
E12       GTCGGATGGAGGGATCCGTTGGCACATGTAGTCACGT
E13        TCGGATGGAGGGATCCGTTGGCACATGTAGTCACGT
E14         GGATGGAGGGATCCGTTGGCACATGTAGTCACGT
```

Figure 2-21 Deletion mutants of Sero1C: E10 through E14.

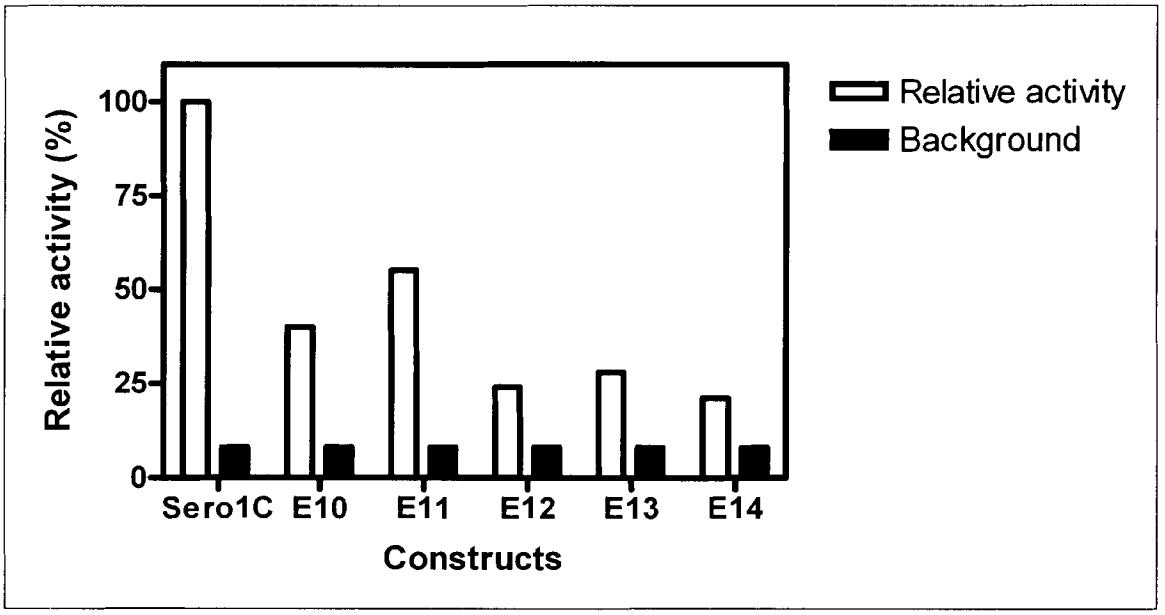


Figure 2-22 Analysis of activity of shortened constructs E10 through E14.

I also created shortened forms of Sero1C by removing bases, from the 3' end, one at a time. Once these constructs (shown in Figure 2-23) were tested for their ability to repair TDP, I noticed a different trend than for the above mentioned enzymes. The initial rate of activity of E5 dropped to approximately 50% of that of repair by Sero1C. Further removal of bases, constructs E6 through E9, resulted in initial activity levels which remained at approximately 50% (Figure 2-24). Therefore, while the 3' most bases seemed

	5'	3'
Sero1C	TAAGTCGGATGGAGGGATCCGTTGGCACATGTAGTCACGT	
E5	TAAGTCGGATGGAGGGATCCGTTGGCACATGTAGTCACG	
E6	TAAGTCGGATGGAGGGATCCGTTGGCACATGTAGTCAC	
E7	TAAGTCGGATGGAGGGATCCGTTGGCACATGTAGTCA	
E8	TAAGTCGGATGGAGGGATCCGTTGGCACATGTAGTC	
E9	TAAGTCGGATGGAGGGATCCGTTGGCACATGTAGT	

Figure 2-23 Deletion mutants of Sero1C: E5 through E9.

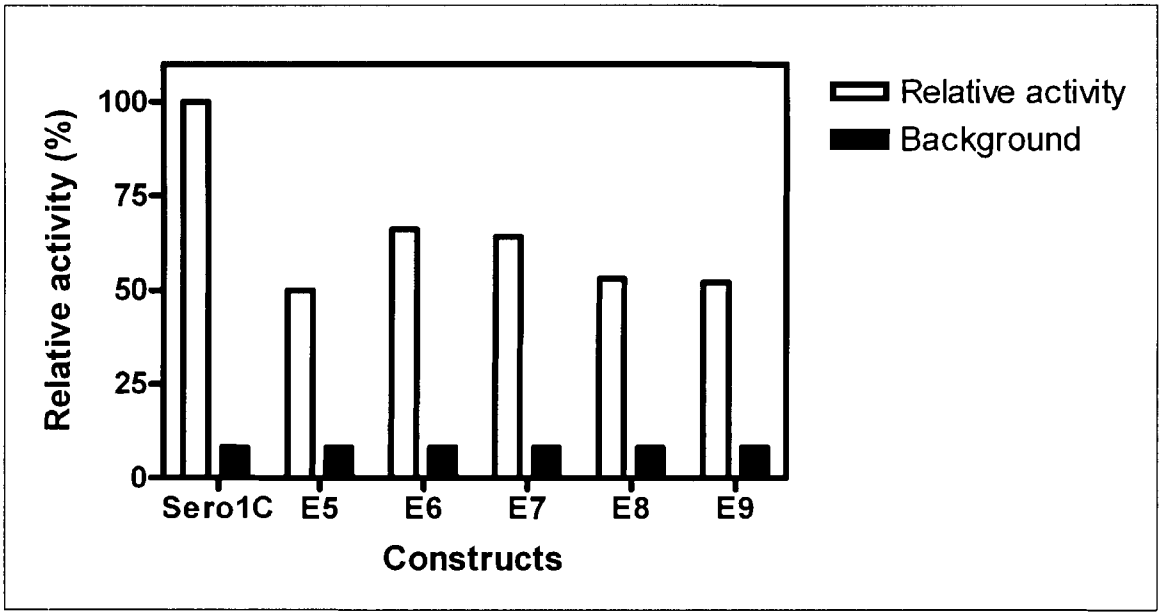


Figure 2-24 Analysis of activity of shortened constructs E5 through E9.

important for folding and catalysis, the 5' most bases appear to be even more significant.

Finally, I tested combinations of removing bases from both the 5' end and 3' end at the same time (constructs shown in Figure 2-25). E1 has a single base removed from each of the 5' and 3' ends, and seemed to be able to catalyze repair at approximately 70% of that of Sero1C (Figure 2-26). This was interesting since a single base removal individually from the 5' end or 3' end resulted in a decrease in the initial rate of repair to

50%, therefore E1 was able to compensate slightly for this loss. The remaining constructs, E2, E3, and E4, all had decreased initial rates down to between 30% and 40% of that of Sero1C, and were not able to compensate for the lost bases at both ends (Figure 2-26).

```
          5'                                                    3'
Sero1C TAAGTCGGATGGAGGGATCCGTTGGCACATGTAGTCACGT
E1       AAGTCGGATGGAGGGATCCGTTGGCACATGTAGTCACG
E2         AGTCGGATGGAGGGATCCGTTGGCACATGTAGTCAC
E3           GTCGGATGGAGGGATCCGTTGGCACATGTAGTCA
E4             TCGGATGGAGGGATCCGTTGGCACATGTAGTC
```

Figure 2-25 Deletion mutants of Sero1C: E1 through E4.

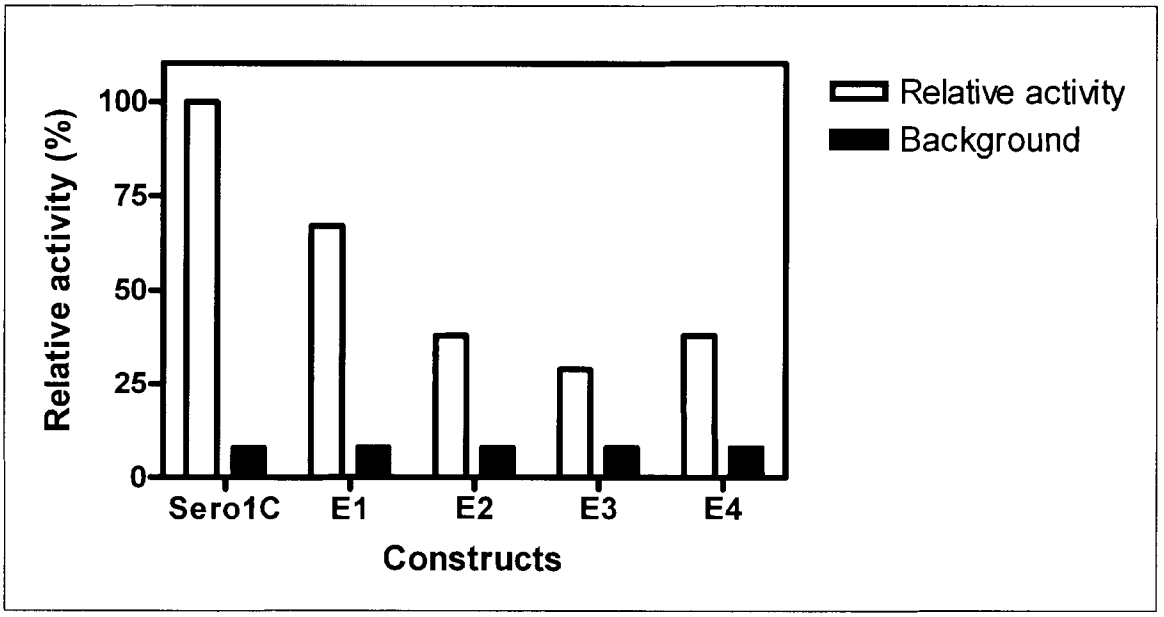


Figure 2-26 Analysis of activity of shortened constructs E1 through E4.

The only construct shorter than E4, that was tested for the ability to repair the thymine dimer within TDP, was E20 (Figure 2-27). This construct was designed to have complementary binding arms to TDP, assuming perhaps that Sero1C and TDP completely base-paired around a catalytic core of the deoxyribozyme. I made an assumption that the catalytic core may include all the guanines in pairs. This construct had no activity at all above the background rates, therefore I was either inhibiting recognition of the substrate, or, disrupting the catalytic core of Sero1C.

E20

5' 3'

CATATACTCGTACGCACAC**C**GGATGGAGGGATCCGTTGG**C**TACATGTAGATCCT

Figure 2-27 Sequence of E20 with complementary binding arms to TDP: Blue C's indicate a bulge nucleotide, and the other larger blue letters indicate an assumed catalytic region.

Altogether, the shortened Sero1C constructs were not substantially conclusive as to a minimal catalytic sequence, or minimal folding sequence required, shorter than the original 40 bases, since removal of single bases showed significant decreases in repair. Therefore, I next tested shortened substrate constructs. These constructs, shown in Figure 2-28, were shortened at least 3 bases at a time, since forming the dimer was time consuming and gives such a low yield. The same method used for creating the thymine dimer in TDP was applied in the shortened constructs.

Once these shortened substrates were created, we tested the ability of Sero1C to recognize and repair them in the presence of serotonin (results shown in Figure 2-29). Interestingly, Sero1C was not able to recognize or repair the construct T3T2, which only

	5'	3'
TDP	AGGATCTACATGTAT=TGTGTGCGTACGAGTATATG	
T3T2	ATCTACATGTAT=TGTGTGCGTACGAGTATATG	
T5T2	TACATGTAT=TGTGTGCGTACGAGTATATG	
T1T4	AGGATCTACATGTAT=TGTGTGCGTACGAGT	
T1T6	AGGATCTACATGTAT=TGTGTGCGT	
T3T4	ATCTACATGTAT=TGTGTGCGTACGAGT	
T5T6	TACATGTAT=TGTGTGCGT	

Figure 2-28 Deletion mutants of TDP.

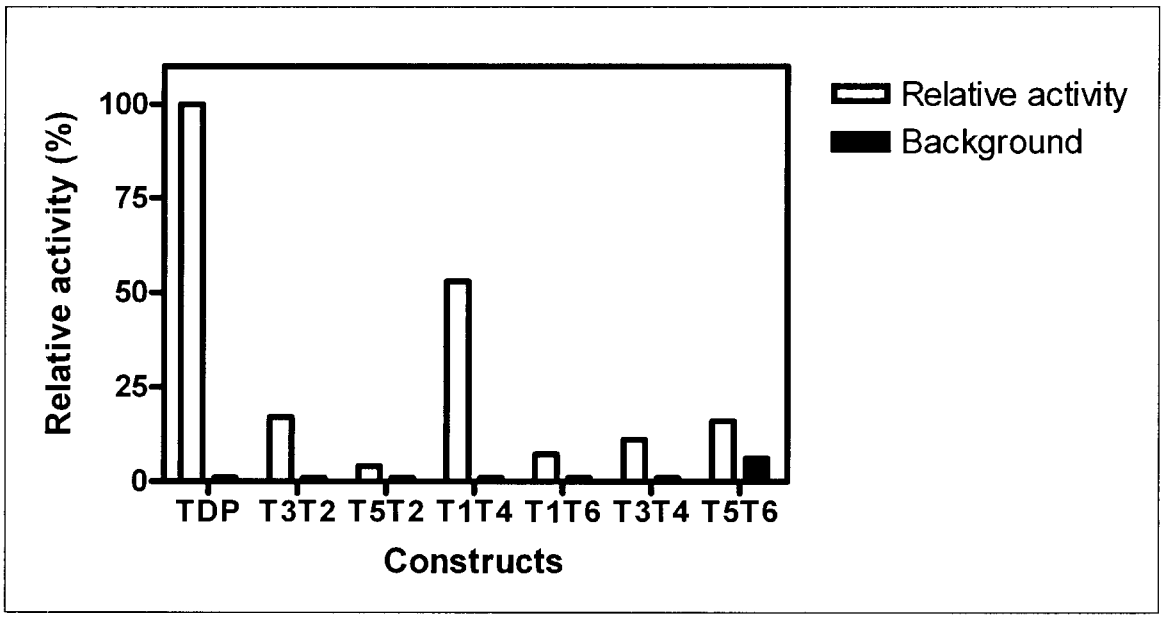


Figure 2-29 Shortened substrate constructs

had three of the 5' most bases removed. The initial rate of repair of this substrate was approximately 17% compared to wild type repair. On the other hand, the T1T4 construct has 5 bases removed from the 3' end of the original substrate, and was able to be recognized and repaired by Sero1C and serotonin, at an initial rate of approximately 50% of that of wild type. The different results with these two constructs suggests that the 5' most bases of TDP seem to be critical in recognizing either Sero1C, possibly through

base pairing, or recognizing serotonin. On the other hand a decent amount of repair was still obtained when at least 5 3' most bases of TDP are removed. However, removing another 6 bases from the 3' end of the original substrate, as in construct T1T6, nearly abolished all repair by Sero1C and serotonin. Finally, I attempted to determine whether some repair activity could be regained by testing the constructs, T3T4 and T5T6, which were shortened at both the 5' and 3' ends. Sero1C, together with serotonin, was not able to significantly increase the rate of repair of T3T4 and T5T6. Therefore, these shortened substrates (T3T4 and T5T6) were not able to compensate for the missing bases, also indicating that the missing bases were critical in recognition of Sero1C, or serotonin, or both.

2.3.2.2 Contact cross-linking experiments

Another effort to elucidate a secondary structure between Sero1C, TDP, and serotonin involved the use of 5-iodouracil (Figure 2-30). 5-iodouracil has been shown to be a photocross-linking agent when substituted into a DNA molecule (Willis *et al.* 1993). (Willis *et al.* 1993) studied the effects of substituting a 5-iodouracil in place of a thymine base in duplex DNA, and did not notice any significant change in structure. They also discovered exceptionally high yields of cross-linked species from a 5-iodoracil substituted DNA molecule to a nearby protein. 5-Iodouracil was also beneficial to our experiment due to the longer range of excitation wavelengths (308 nm to 325 nm) by which 5-iodouracil can be excited to initiate photocross-linking to other species. We attempted to use 5-iodouracil to induce cross-links between our deoxyribozyme and our DNA substrate. We incorporated the 5-iodouracil at two different thymine positions in the DNA substrate, creating two different substrate cross-links (shown in Figure 2-31).

We chose to substitute the 5-iodouracil at these thymine positions (which normally comprise the thymine dimer) to aid in determining which bases in Sero1C were in close proximity to the thymine dimers during catalysis. I also expected that these results would help confirm our assumptions from the deletion substrate construct results, and possibly with out DEPC protection experiments for a possible secondary structure.

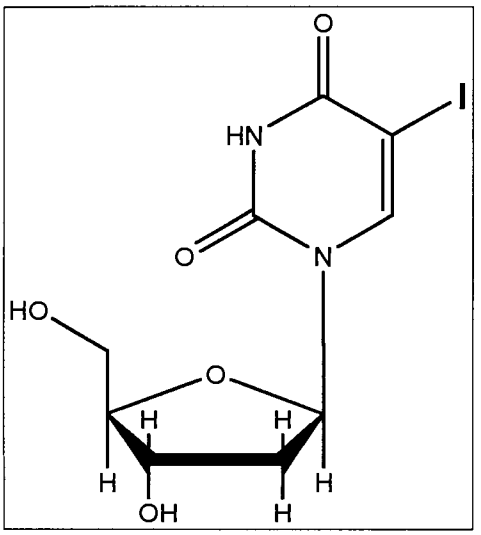


Figure 2-30 Diagram of 5-Iodouracil structure.

	5'	3'
T15IdU	AGGATCTACATGTA**IdU=T**GTGTGCGTACGAGTATATG	
T16IdU	AGGATCTACATGTA**T=IdU**GTGTGCGTACGAGTATATG	

Figure 2-31 Sequence of T15IdU and T16IdU constructs, indicating location of 5-IdU: No actual dimer is present between the thymine and IdU bases.

I also wanted to assay whether or not serotonin was necessary in order for a secondary or catalytic structure to form. Therefore, I performed these cross-linking experiments with ^{32}P-radiolabeled Sero1C in the absence of serotonin, and in the

presence of either 50 μM or 500 μM serotonin. The results, shown in Figure 2-32, indicated that a small amount of a cross-linked species was formed upon irradiation, and can be formed either in the presence or absence of serotonin. Although, especially with the T16IdU construct, I noticed that at a serotonin concentration of 500 μM, there was a slight inhibition of forming the cross-linked species. It appears that both 5-iodouracil substituted constructs form the same cross-linked species. In order to confirm that the cross-linked species were the same, and to determine which bases the 5-iodouracil was photocross-linking to, I further eluted the cross-linked species out of the gel, and subjected them to piperidine treatment.

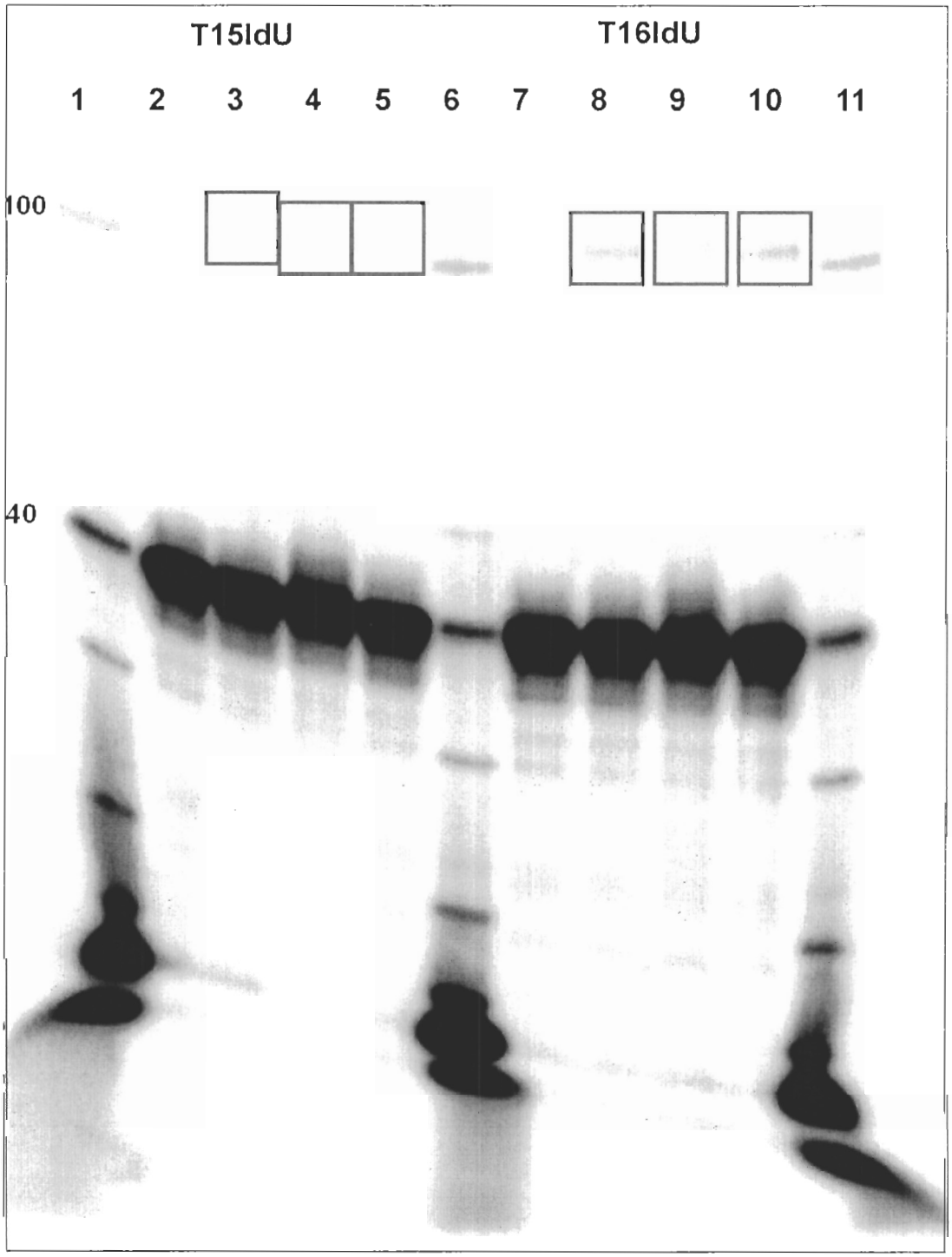
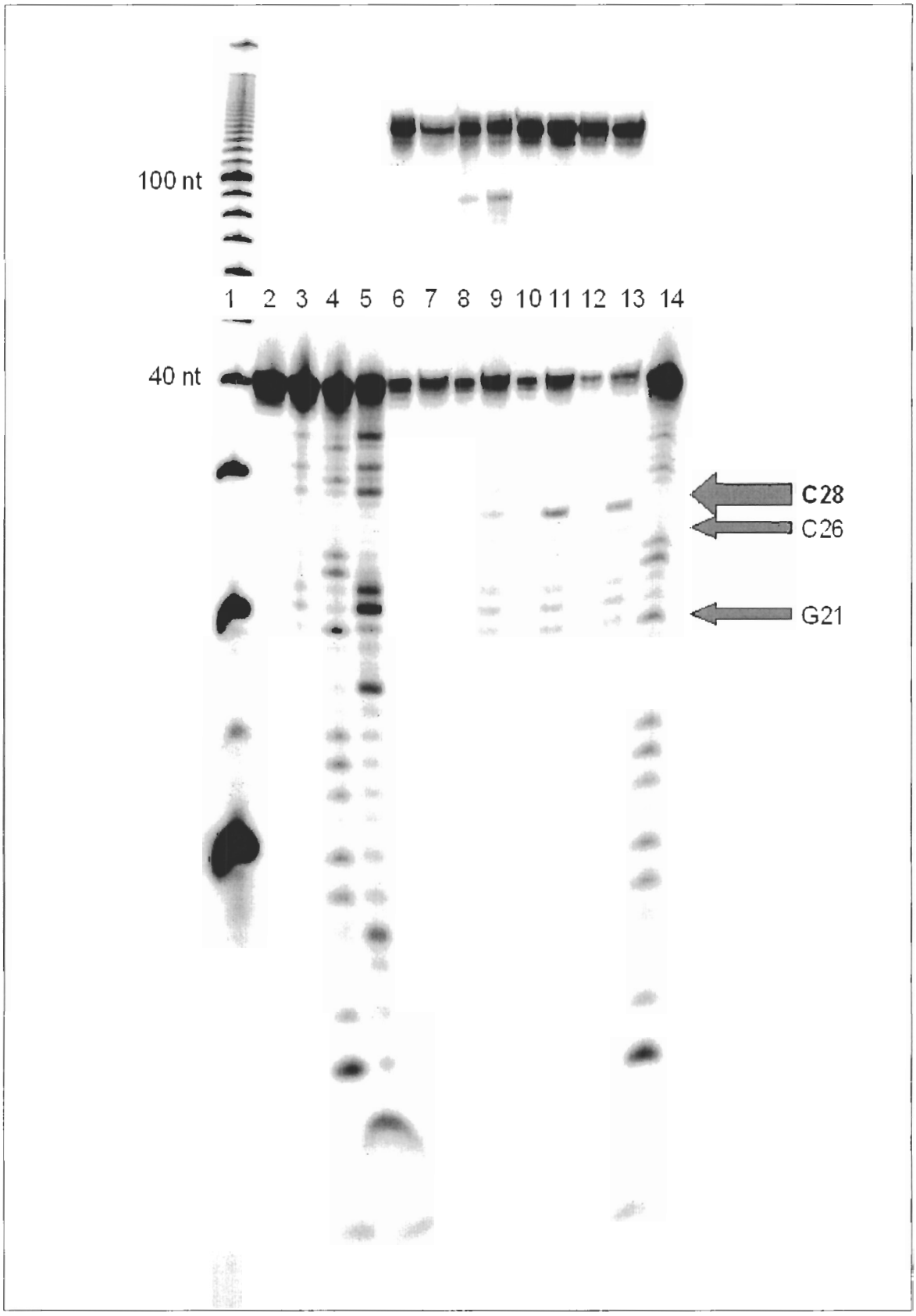


Figure 2-32 Cross-linking gel analysis: Red boxes indicate detection of a cross-linked species. Lane 1 = 10 bp ladder; Lane 2 = Non-irradiated sample; Lane 3 = Sero1C, T15IdU and 50μM serotonin; Lane 4 = Sero1C, T15IdU, and 500μM serotonin; Lane 5 = Sero1C, T15IdU, with no serotonin; Lane 6 = 10 bp ladder; Lane 7 = Non-irradiated sample; Lane 8 = Sero1C, T16IdU, and 50μM serotonin; Lane 9 = Sero1C, T16IdU, and 500μM serotonin; Lane 10 = Sero1C, T16IdU, with no serotonin; Lane 11 = 10 bp ladder.

Piperidine will cleave the DNA at bases that have been damaged or modified, including where a cross-linked species has been formed. Figure 2-33 shows the piperidine treatments of the different cross-linked species in hopes of mapping where the cross-linking was occurring. For this piperidine experiment Sero1C was 5' radioactively labelled with ^{32}P, and I created a G-ladder and a T-ladder as markers. Our assumption was that if a base in Sero1C has been damaged or modified by the 5-iodouracil cross-linking, it will be more susceptible to piperidine treatment, and will produce a signal on the PAGE gel. The PAGE analysis of the piperidine treated cross-linked sample does agree with the above cross-linking gel: Both 5-iodouracil substituted constructs did appear to be forming the same cross-linked species. The arrows in Figure 2-33 indicate the bases which showed damage, above that seen in the negative control lane. The negative control lane contains radiolabeled Sero1C that was subjected to piperidine treatment alone (no cross-linking treatment) to show bases that were perhaps susceptible to piperidine cleavage even without being cross-linked. Figure 2-34 shows where these bases were located within Sero1C, in relation to where the 5-iodouracil substitutions were made, and illustrates a possible secondary structure of Sero1C with TDP and serotonin. Of note in Figure 2-34, T16IdU seemed to be able to cross-link to C28 easier than T15IdU. Interestingly, Figure 2-33 shows that T22 and T23 in Sero1C seemed especially piperidine labile, and Figure 2-34 shows these thymines exposed to solution, hence agreeing with the mapping.

Figure 2-33 **Mapping damage within Sero1C via piperidine treatment: Larger arrow indicates greater damage.**
Lane 1 = 10 bp ladder; Lane 2 = Sero1C alone; Lane 3 = Piperidine treated Sero1C alone; Lane 4 = G-ladder; Lane 5 = T-ladder; Lane 6 = non-piperidine treated cross-link of T15IdU without serotonin; Lane 7 = piperidine treated cross-link of T15IdU without serotonin; Lane 8 = non-piperidine treated cross-link of T15IdU with 50μM serotonin; Lane 9 = piperidine treated cross-link of T15IdU with 50μM serotonin; Lane 10 = non-piperidine treated cross-link of T16IdU without serotonin; Lane 11 = piperidine treated cross-link of T16IdU without serotonin; Lane 12 = non-piperidine treated cross-link of T16IdU with 50μM serotonin; Lane 13 = piperidine treated cross-link of T16IdU with 50μM serotonin.


100 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14
40 nt
C28
C26
G21

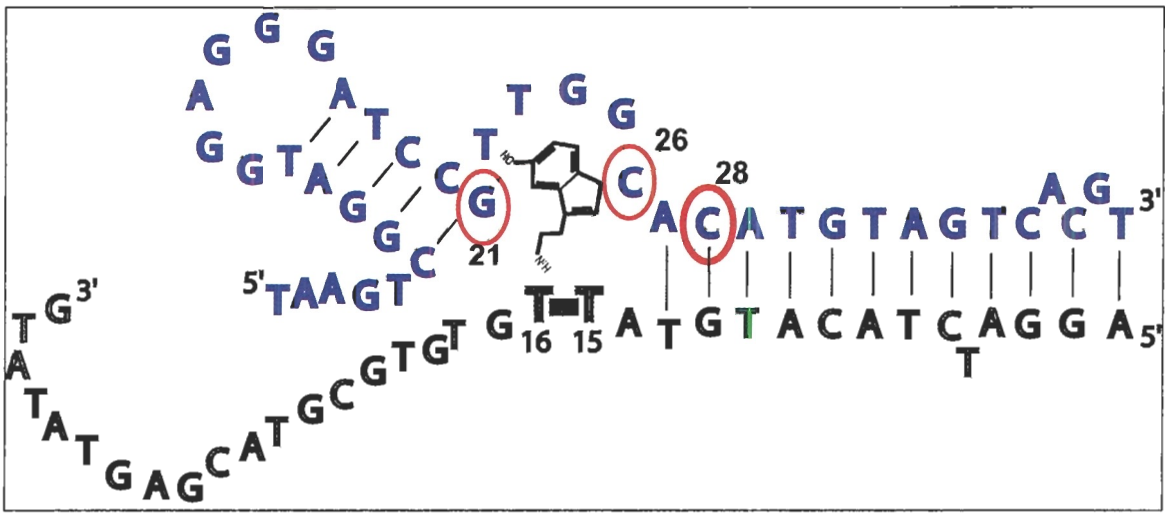


Figure 2-34 Diagram of possible secondary structure formed between Sero1C, TDP, and serotonin. Red circles indicate to which bases T15IdU and T16IdU were able to cross-link to (the thicker the circle the greater the cross-link signal); outlined green bases are from TDP substrate; solid blue bases are from Sero1C.

2.3.2.3 Chemical probing of Sero1C for secondary structure proposal

I have already performed DMS protection reactions, which modify the available guanines, in Sero1C, followed by piperidine treatment and PAGE analysis. This was done to determine if any guanines in Sero1C were being protected and participating in a secondary structure. As mentioned previously, I was not able to detect any significant protection of any guanines in Sero1C. Yet, DMS is not able to distinguish between double-stranded and single-stranded DNA, such that guanines participating in any base pairing will still get modified.

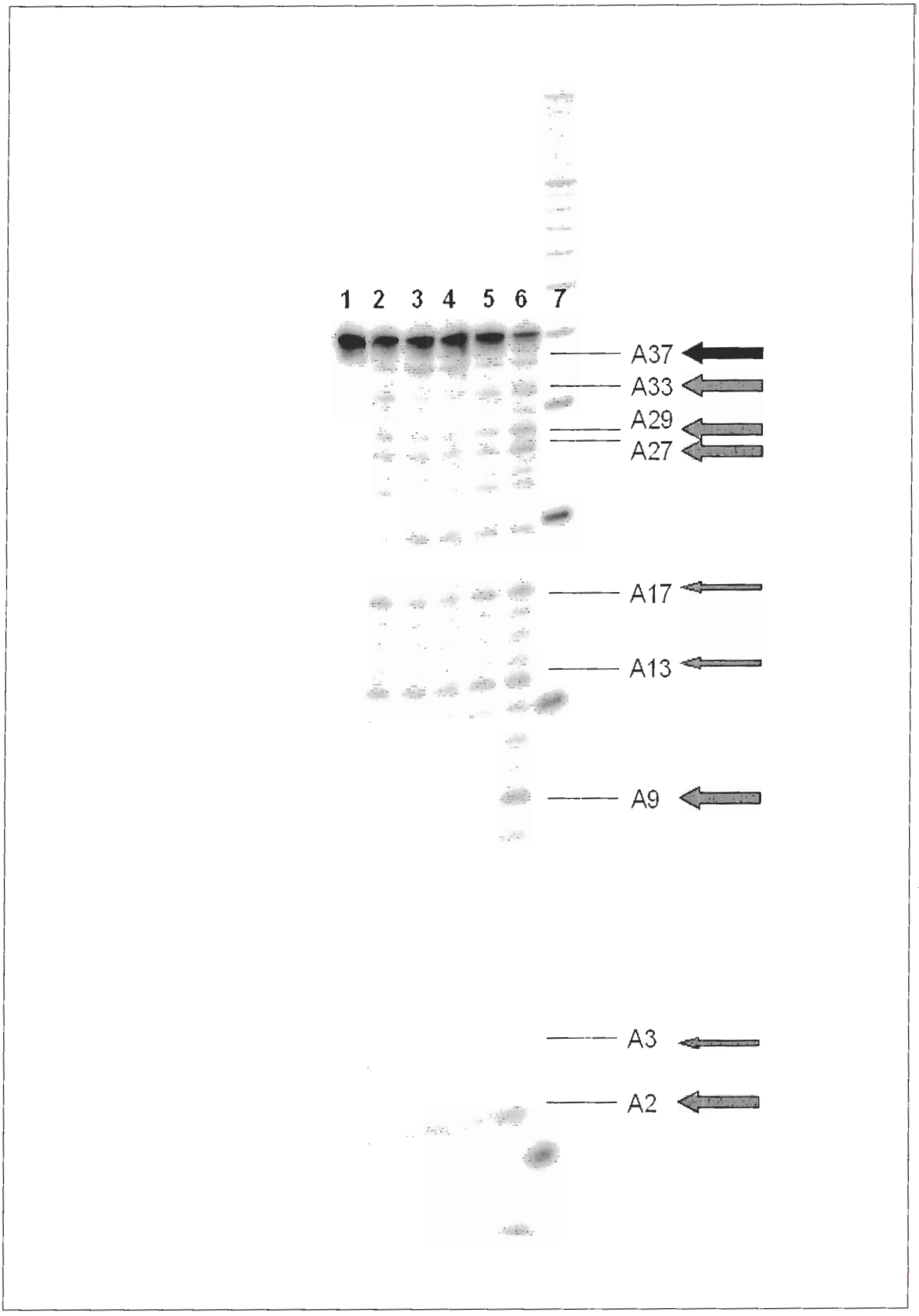
In order to create a more accurate model of a possible secondary structure involving Sero1C, serotonin, and TDP, and confirm the model illustrated in Figure 2-34, I decided to perform more chemical probing experiments using diethylpyrocarbonate (DEPC) and potassium permanganate ($KMnO_4$). Both DEPC and $KMnO_4$ can be used in protection assay experiments to modify bases in a DNA strand that are not being

occupied in a secondary structure. The main difference with DEPC and $KMnO_4$, compared to DMS, is that they can also distinguish between double-stranded and single-stranded DNA, such that they do not tend to modify DNA bases in a double-stranded structure. Therefore, I used these chemicals to determine whether there was any simple base pairing occurring between TDP and Sero1C for recognition of the substrate, and stabilizing the complex for the catalytic event of repair of the thymine dimer.

$KMnO_4$ modifies thymines that are not participating in a secondary structure by oxidizing the double bond between the C5 carbon and the C6 carbon. If thymines in the strand of DNA of interest were within a double-stranded helix, base stacking would prevent $KMnO_4$ from reacting with the C5-C6 double bond of the thymines (Hayatsu and Ukita 1967). In our experiments, involving reacting $KMnO_4$ with folded and unfolded Sero1C, with and without TDP and serotonin, we noticed a tendency of $KMnO_4$ to react strongly with Sero1C. One explanation could be the fact that Sero1C has a 5' most thymine since it has been shown that the presence of a thymine base at the most 5' position of an oligonucleotide creates problems for the $KMnO_4$. It is more difficult for $KMnO_4$ to react with internal thymines, since the thymine at the 5' end is the most easily accessible (McCarthy *et al.* 1990).We also noticed that upon addition of serotonin to some reactions, all reactivity of $KMnO_4$ with the thymines was abolished. This could have been due to $KMnO_4$ reacting with a double bond in serotonin once it was added to the reactions, which closely resembles the target double bond in the thymine bases. Therefore, Sero1C was not a suitable candidate for $KMnO_4$ structure probing, even at low temperatures.

I then turned my attention to probing the secondary structure of Sero1C using DEPC, which also differentiates between single-stranded and double-stranded DNA. DEPC reacts with both adenine and guanine bases, yet preferentially with single-stranded or unstructured adenines. We tested again whether serotonin and, or TDP, were necessary to aid in forming the secondary structure of Sero1C. We employed several different conditions such as Sero1C alone with NaCl, Sero1C with and without the substrate TDP, Sero1C with and without serotonin, and combinations of all of the above. The DEPC reactions were all performed on ice to aid in stabilizing a secondary structure. The results, shown in Figure 2-35, indicated that there were some adenine bases that were being protected in a secondary structure. The light coloured arrows indicate areas of protection in comparison to the A-ladder (Sero1C reacted alone). I noticed that upon addition of NaCl to Sero1C, there was already some protection observed at certain adenine positions. Interestingly, after addition of the pseudosubstrate, LDP, as compared to reactions with only Sero1C and NaCl, I noticed a couple of differences. First, the adenine bases that were showing some protection with Sero1C and NaCl were now being even more protected. And second, the 3' most adenine in Sero1C, A37, was being more damaged (indicated by a black arrow in Figure 2-35), indicating that this base was most likely more exposed to the solvent once Sero1C has folded with LDP present.

Figure 2-35 **DEPC experiment (performed on ice for 3 hours) run on 12% denaturing PAGE. Lane 1 = piperidine treated Sero1C alone, lane 2 = Sero1C, NaCl, and serotonin, lane 3 = Sero1C, NaCl, serotonin, and LDP, lane 4 = Sero1C, NaCl, and LDP, lane 5 = Sero1C and NaCl, lane 6 = A-ladder, and lane 7 = 10 bp ladder. Black arrow indicates increased damage; red arrows indicate protection; small arrows indicate less protection than large arrows.**


1 2 3 4 5 6 7
A37
A33
A29
A27
A17
A13
A9
A3
A2

2.3.2.4 Structural Model

All of the results from these experiments were taken together to create a structural model (Figure 2-36). One area of possible discrepancy is that the 5'-end of Sero1C, and

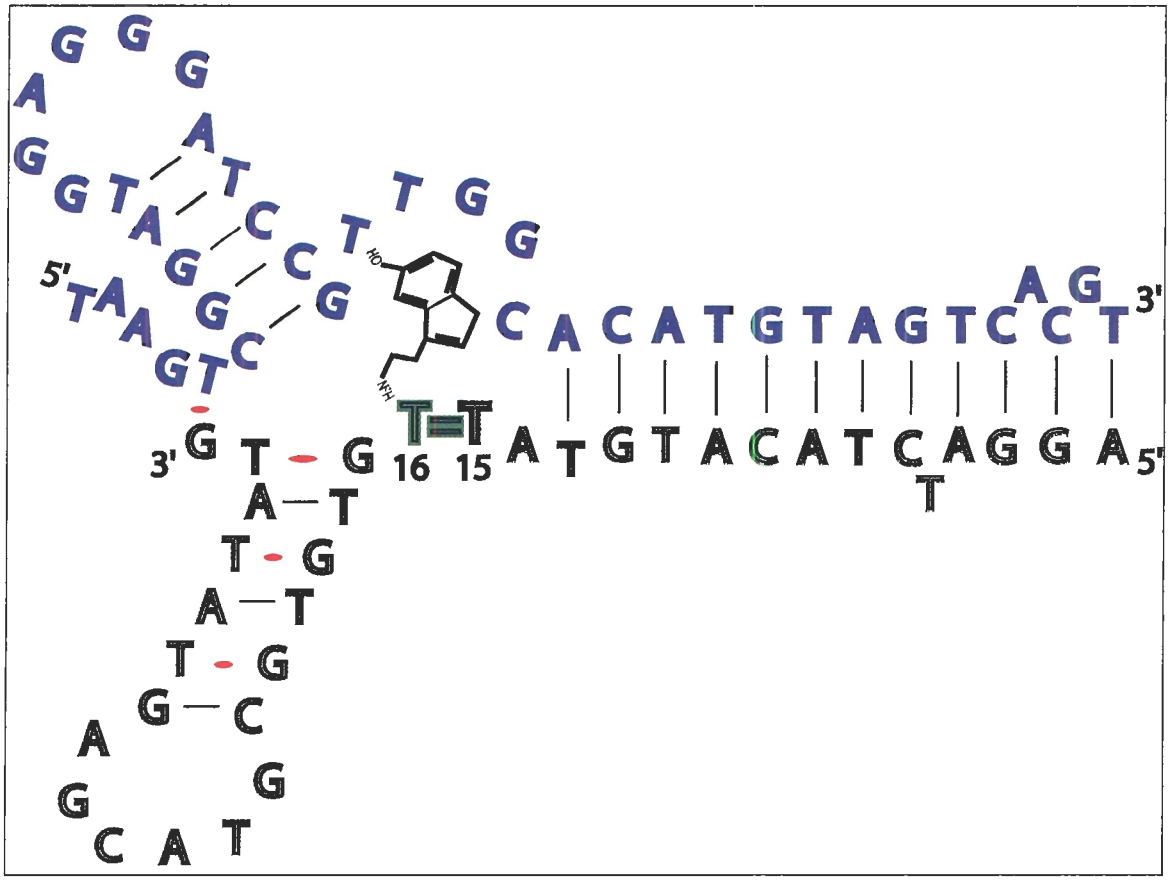


Figure 2-36 Final structural model of Sero1C with serotonin and TDP
Solid blue bases are from Sero1C, outlined green bases are from TDP substrate; black lines indicate hypothetical base pairs.

the 3'-end of TDP are not shown to participate much in the secondary structure of the complex. However, removal of these bases did decrease the activity of repair, therefore, it was likely that there is a more complicated relationship between these regions that I have not portrayed in this model. It is also possible that many G-T wobble base pairs are

present (as shown in Figure 2-36), which may be stabilizing the overall structure and chemistry of this complex. Most of these G-T wobble pairs were being depicted to occur in the substrate, which I have not yet monitored with DEPC or $KMnO_4$ chemical probing (protection) assays to date.

2.3.2.5 Mapping the position of serotonin.

Another aspect of the secondary structure of Sero1C, other than how it recognizes the thymine dimers, involves where serotonin is located. Is serotonin physically binding Sero1C, or is it binding the substrate? Or is serotonin simply located close enough to the thymine dimer, being held by Sero1C, to directly donate electrons? Hence, we also wished to determine whether or not serotonin was actually binding, or whether the excited serotonin was donating electrons via a collision reaction. Our attempt to determine whether or not serotonin was actually binding, and where, involved using a tritium (^3H)-radiolabeled serotonin. Also in these reactions I used one of the 5-IdU containing constructs, T15IdU, in attempt to be able to cross-link to the radiolabeled serotonin. I first tried the cross-linking experiments with radiolabeled Sero1C to see if I could detect a cross-link species between serotonin, Sero1C, and the T15IdU substrate. Unfortunately I was unable to detect any such cross-link. Also, as mentioned above, in section 2.3.2.1, I noticed that the substrate constructs containing 5-IdU used in the cross-linking experiments, cross-linked best to a cytosine. In contrast, Dan Chinnapen's cross-linking results (Chinnapen 2005) demonstrated that the same substrate constructs cross-linked best to guanine residues, which were participating in the guanine quadruplexes. With Sero1C, repair of the thymine dimers did not occur without serotonin present, and the 5-IdU containing substrates did not cross-link as well to guanines, therefore in

Sero1C it did not appear that the guanines are donating electrons. This also suggests that serotonin is donating the electrons for repair. Therefore, I tried the same experiment using radiolabeled T15IdU to determine whether the serotonin was close enough to the thymines, in the dimer, in the substrate, in order to be able to directly donate electrons for repair and therefore cross-link to T15IdU. Unfortunately, even when I used a sensitive tritium screen to detect the tritium signal from the serotonin, I was still unable to detect any cross-linked species at all. This could have been due to the design of the experiment, or the low concentration of the ^{3}H-radiolabeled serotonin. It was also possible that the serotonin was being somewhat 'trapped', but not cross-linked, within the cross-linked species of Sero1C and T15IdU together. Thus it may have been lost during the ethanol precipitation steps, even though steps were taken to try and prevent and monitor this.

All of the above mentioned data supports and agrees with this structural model, therefore I wished to move on, and further elucidate how the actual repair reaction was taking place, and to determine the full capabilities of Sero1C. In order to examine the full potential of Sero1C, I started testing whether Sero1C was able to repair other types of dimers, as the *E. coli* photolyase was capable of.

2.3.3 Substrate Specificity

The *E. coli* photolyase is able to repair cyclobutane thymine dimers, but was not limited to repair of only thymine dimers. It is also able to catalyze the repair of other pyrimidine dimers (Kim and Sancar 1991). Other such pyrimidine dimers include uracil dimers, cytosine dimers, and chimeric uracil-thymine dimers. Since we were attempting to create a DNA mimic of the *E. coli* photolyase protein enzyme, I wished to determine whether Sero1C had the same potential to repair different types of dimers. This would be

especially interesting since it was only selected to be able to repair cyclobutane thymine dimers. I tested uracil dimers, cytosine dimers, chimeric uracil-thymine dimers, and I also investigated the role of the type of sugar, within the nucleotide, necessary for catalysis. Finally, I also wanted to determine whether Sero1C could also act on an all RNA substrate. In testing Sero1C's ability to repair all these different types of dimers, I hoped to dissect the substrate specificity of Sero1C in a hierarchical manner.

We first created an all RNA, uracil dimer-containing, substrate, named RDP (Figure 2-37 for structure), which Sero1C was unable to repair efficiently (Figure 2-38).

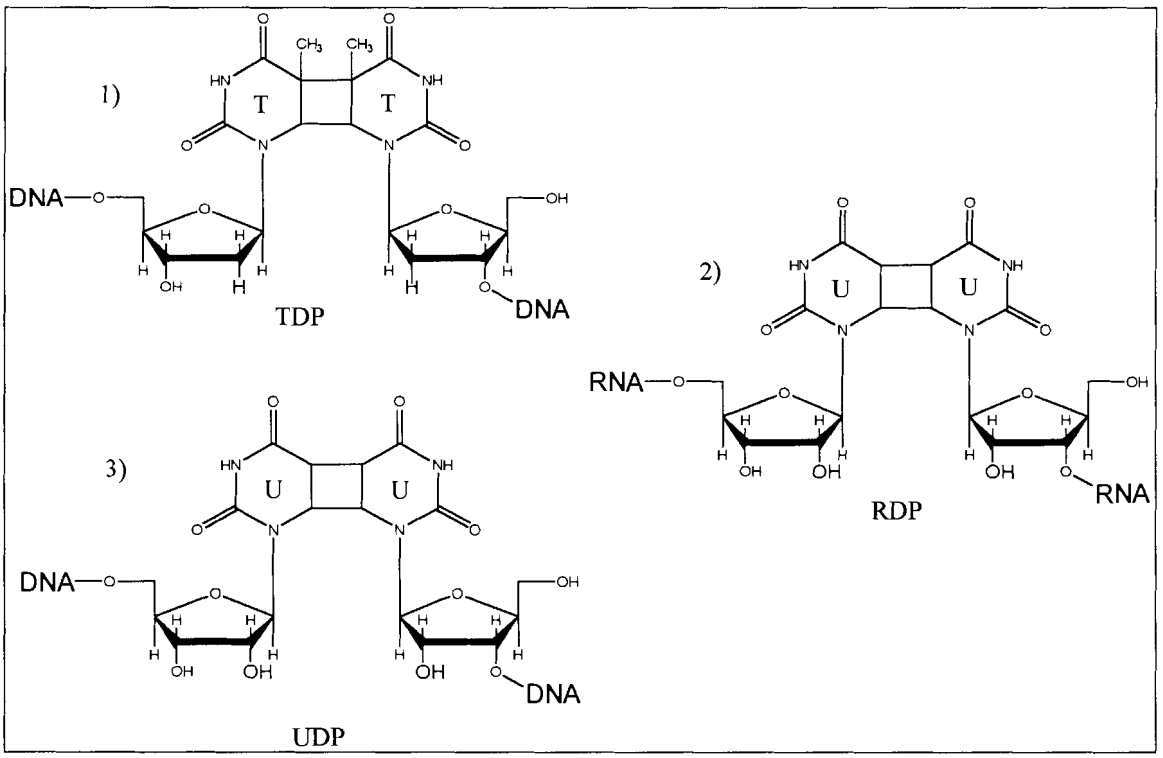


Figure 2-37 Structures of TDP, an all RNA-containing substrate with a uracil dimer, named RDP, and a substrate containing a uracil dimer yet with DNA flanking strands, named UDP.

We also created a substrate that was all DNA, except for the actual dimer, which was a uracil dimer, named UDP (Figure 2-37). This would give us an indication of whether or not just the flanking strands had to be DNA, or if Sero1C preferred an all DNA substrate. Interestingly, Sero1C was able to repair these uracil dimers, with flanking DNA sequences, yet only at an initial rate approximately equal to a third of that of wild type.

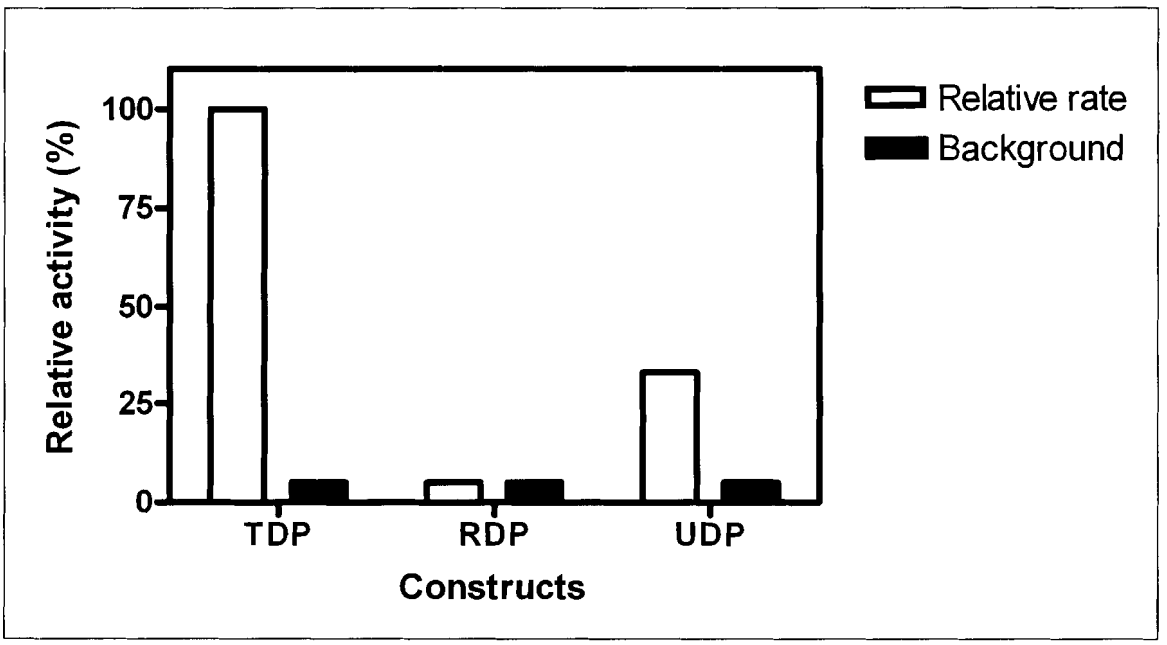


Figure 2-38 Analysis of TDP, RDP, and UDP repair by Sero1C.

There were other components differing between the RDP, UDP, and TDP constructs, therefore I further dissected the substrate preferences of Sero1C by testing some uracil-thymine chimeric substrates, again also testing all RNA flanking strands, and RNA-DNA chimeric flanking sequences. Figure 2-39 shows the different substrate constructs tested, and Figure 2-40 depicts their relative activities of repair by Sero1C, as

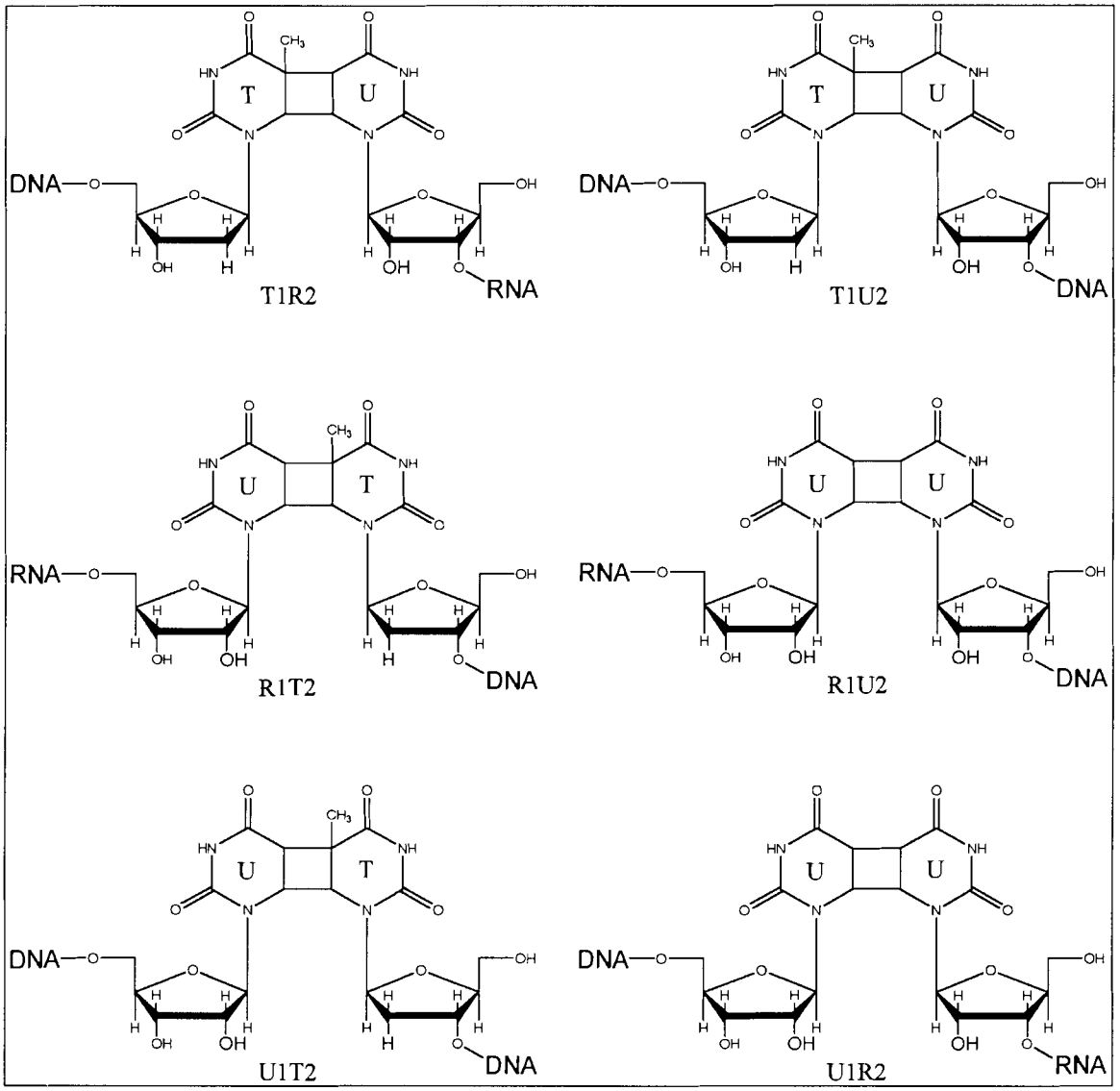


Figure 2-39 All chimeric U-T structures.

compared to repair of the wild-type substrate TDP. As was seen in the relative rates of repair, Sero1C seemed to prefer a uracil at the 5'-end of the dimer and a thymine at the 3'-end of the dimer (U-T), as in construct U1T2, and had a substantial rate of repair as compared to wild type. Yet this was only seen with DNA flanking strands on both sides of the dimer since R1T2 had the same dimer but had a 5' RNA flanking sequence. Interestingly, the reverse dimer, 5'-thymine-uracil-3' (T-U), as in T1U2, was not as

efficiently repaired. In fact, Sero1C was only able to repair this dimer at a fifth the rate of wild type. These results are indicative of a preference of directionality of either electron donation, repair, or recognition of the substrate at the 5' end of the dimer.

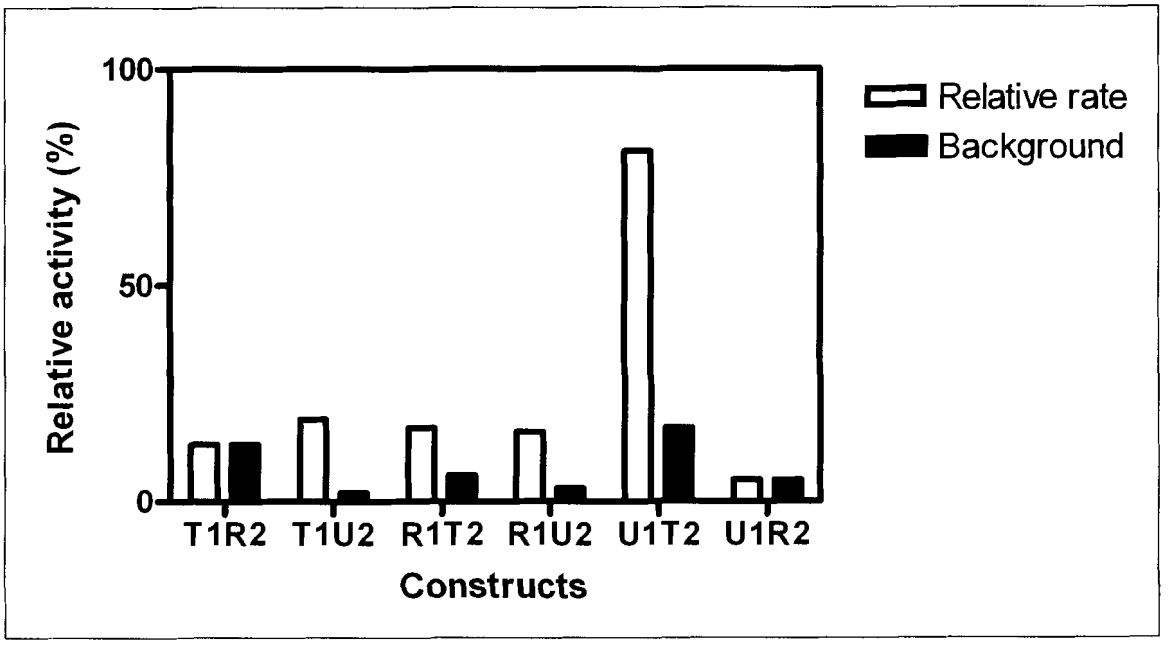


Figure 2-40 Analysis of chimeras.

To further distinguish and characterize the substrate preferences and directionality preferences in repair by Sero1C, I tested the effects of deoxyribose sugars versus ribose sugars, within the nucleotide, in combination with either a uracil base or a thymine base, in other words the lack or presence of a methyl group respectively. The nucleotides used to make the dimers were ribothymidine and deoxyuridine. These substrate constructs (shown in Figure 2-41) were tested with DNA flanking sequences around the dimer, since I already showed that this was what Sero1C preferred. Since I have already shown how Sero1C responds to the U-T chimeric dimers, any variance in the response between these

constructs, and the other chimeras, will be attributable to the sugar moiety. The results of these experiments were shown in bar graphs (Figure 2-42) as a percent of activity compared to repair of the wild type substrate. Both the ribothymidine (rT) dimer and deoxyuridine (dU) dimer substrates were only repaired at a rate approximately 50% of that of wild type TDP. Sero1C was demonstrating significant sensitivity in its catalytic repair abilities when either a methyl group on the base or hydroxyl group on the sugar was removed or added. Comparing the UDP and dUdU constructs, the removal of the hydroxyl group increased Sero1C's ability to repair uracil dimers. This indicates that the lack of methyl groups, on the uridine bases, impairs Sero1C's catalytic abilities. Conversely, adding the hydroxyl groups, on the sugars, to the thymine dimers decreased Sero1C's ability to repair the dimers.

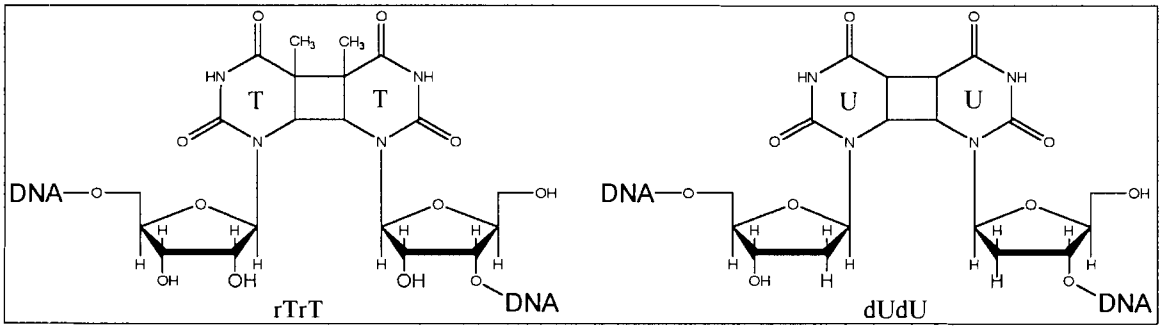


Figure 2-41 Structures of rT-rT and dU-dU dimers.

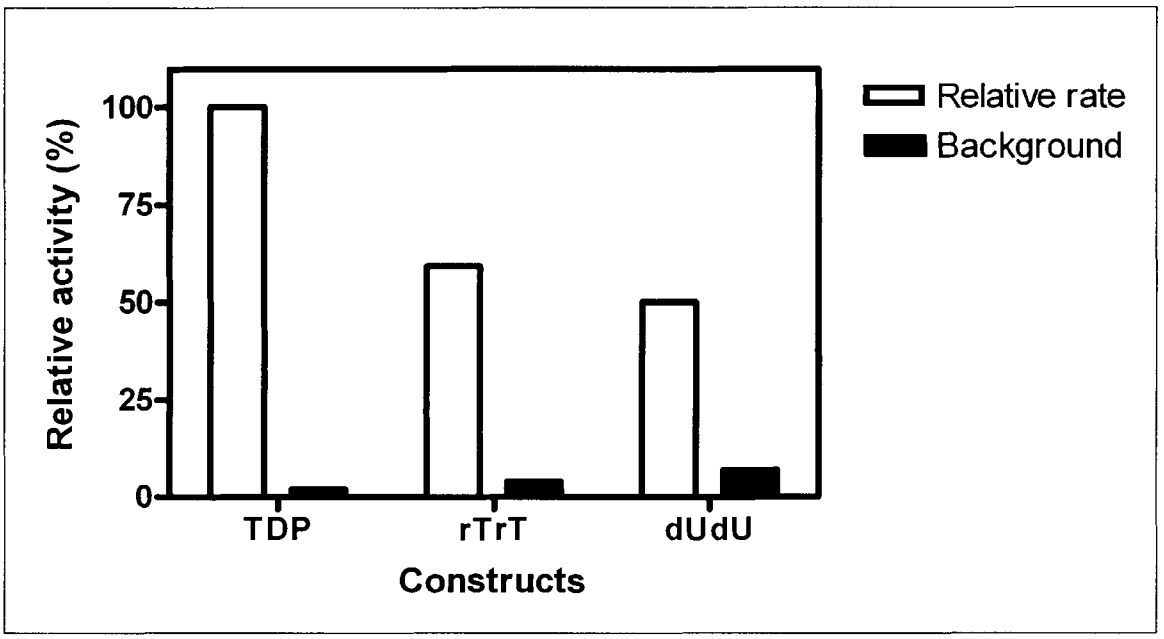


Figure 2-42 Analysis of repair of the rT-rT and dU-dU dimers compared to repair of TDP.

To investigate this sensitivity even further, the next set of dimer constructs tested for repair by Sero1C were chimeric dimers constructed with the rT and dU nucleotides, such that the nucleotide at the 5' end of the dimer would differ from the 3' nucleotide of the dimer (Figure 2-43). These results were the most interesting due to the ability of Sero1C to repair some of these substrates better than the wild type, TDP (Figure 2-44). Overall, it appeared that a lack of a methyl group on the base at the 3' end of the dimer was least preferred, as seen with the dT-dU construct, and this agrees with the repair results of T1U2. The only difference between this construct, dT-dU, and TDP, was the lack of a methyl group at the 3'end, and its repair was 68% of wild type. This was a higher rate than repair of T1U2, therefore the removal of the 2'-hydroxyl increased the rate of repair three-fold. All of the other constructs, rT-dT, dT-rT, and dU-dT, showed greater activity than that of repair of wild type substrate. The repair of dU-dT had the

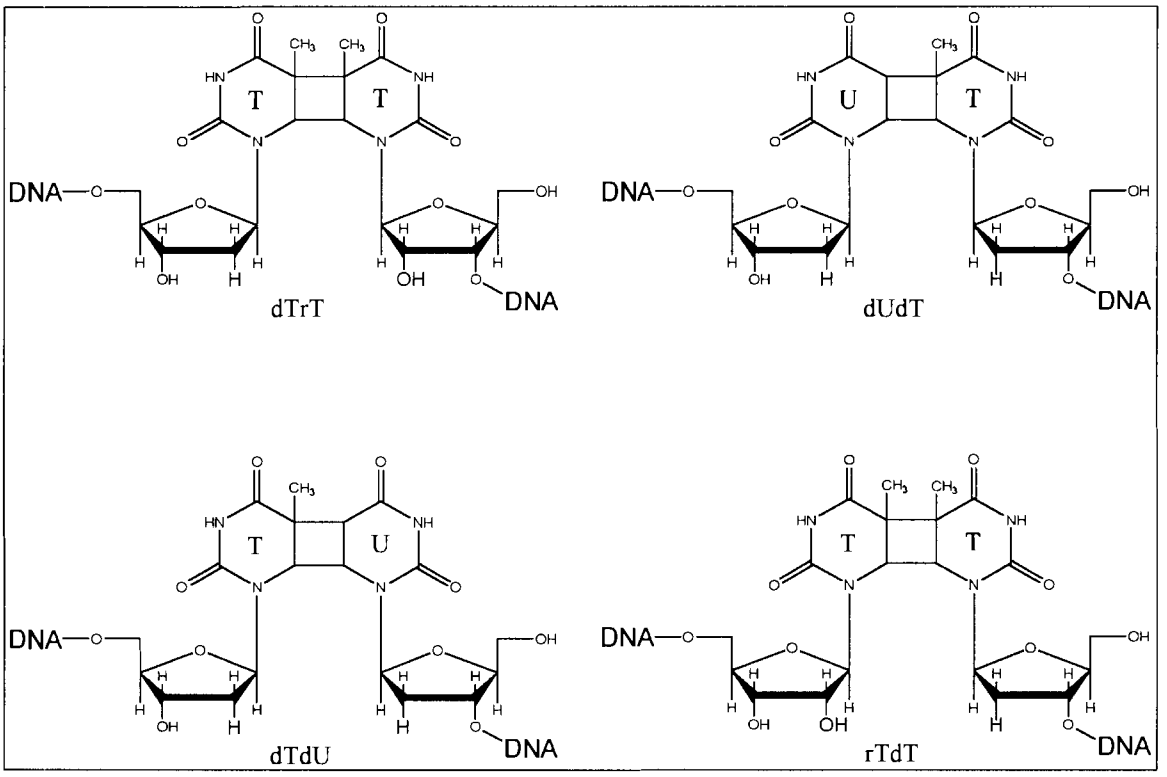


Figure 2-43 Structures of the rT, dU, and dT chimeric dimers.

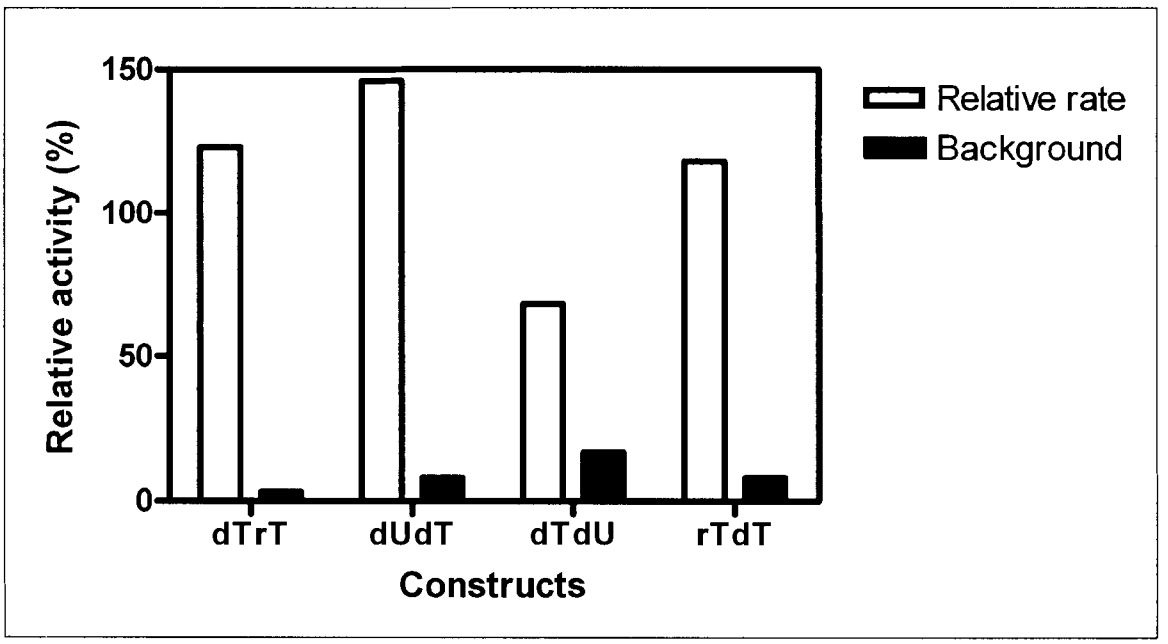


Figure 2-44 Analysis of repair of the rT and dU chimeric dimers.

highest activity at 150% of that of wild type, and these results also agree with the previously mentioned results of rate of repair of the U-T dimer. The only difference between these two constructs, U-T and dU-dT, was the lack of a 2'-hydroxyl on the sugar at the 5' base of the dimer in the dU-dT construct. The lack of this hydroxyl group almost doubled the rate of activity of Sero1C. Also, the only difference between TDP and dU-dT was the lack of a methyl group on dU. These results indicate that even the slightest changes at either position (especially at the 3' end) of the dimer can create a substantial difference in rate of repair. They also indicate a possible directionality preference of electron donation, possibly due to sterics. Overall, one ribose sugar as part of the dimer did not make a significant difference, yet two hydroxyl groups decreased the rate of repair by Sero1C

Finally, we tested the ability of Sero1C to repair another pyrimidine dimer; a cytosine dimer. The cytosine dimer, with flanking DNA sequences, turned out to be a very poor substrate for Sero1C.

Generally, all of these substrate specificity experiments indicated that at the 3'-end of the dimer, within the substrate, it is necessary to have a thymine base for efficient catalysis. Removal of a methyl group at the 3' position severely impairs Sero1C's ability to repair the dimer while the removal or addition of a 2' hydroxyl on the sugar of this nucleotide did not seem to have much effect. If serotonin is actually binding, for example intercalating, somewhere near the dimer, it may sterically fit better with a thymine base, since thymine bases contain a methyl group that uracil bases lack. It is also possible that it is the base at the 3'-end of the dimer which receives an electron to initiate reversal of the dimer, or that the 3'-end is more important in recognition. As for the 5'-position of the

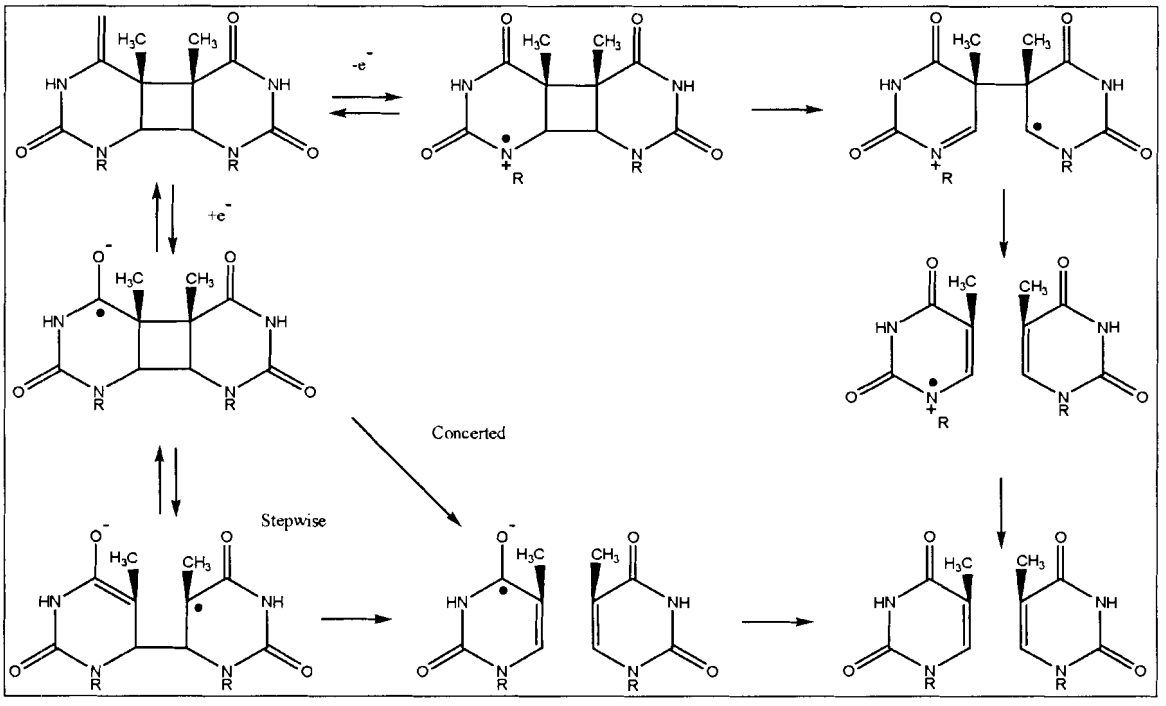
dimer, many substitutions, with bases and sugars, were performed with little effect on the ability of Sero1C to repair these dimers. Therefore Sero1C is not looking for specific chemical properties at the 5' end of the dimer, but is perhaps more stringent of the chemical properties at the 3' end of the dimer. This could indicate that the 3' end of the dimer is a deciding factor in repair or recognition by Sero1C.

Base-pairing would probably not appear to be a major contributing factor in these experiments to elucidate a photochemical mechanism of repair since DNA and RNA can base-pair together. Yet there was a great difference in the rates of activity of repair by Sero1C on T1R2 and T1U2, where the only difference was the RNA versus DNA flanking strands, respectively. Again, there was a noticeable difference between the activities of R1T2 and U1T2 where the only difference again was RNA versus DNA flanking strands. These conclusions led me to probe further into the photochemical reaction mechanism, as discussed in section 2.3.4.

2.3.4 Photochemical Mechanism Proposal

In this section I will describe some experiments performed that elude to a certain photochemical mechanism by Sero1C. From previous experiments, I have shown that Sero1C was unable to catalyze repair of the thymine dimer substrate without the presence of serotonin. Also, serotonin could not use another piece of DNA given that another unrelated oligonucleotide could not substitute for Sero1C: it had to be Sero1C to facilitate the electron donation to the thymine dimer. These results indicated that it was serotonin that was being excited, not Sero1C, to donate electrons to the thymine dimer to facilitate its repair. The action spectrum also suggested that it was the serotonin responsible for the catalysis, and not Sero1C. Two main questions regarding the photochemical mechanism

remained: Was the excited serotonin passing electrons to Sero1C to be subsequently donated to the thymine dimer, or was Sero1C simply 'holding' the serotonin in the correct place for direct electron donation to the thymine dimer? And, which excited state was serotonin using in the photoinduced electron transfer (singlet or triplet)? To address the former question, our assumption thus far, was that the electron was donated directly from serotonin to the thymine dimer, due to serotonin being so electron rich, and due to the fact that Sero1C can not be excited on its own. This assumption was made based on studies performed on the DNA photolyase and model systems (Lamola 1972; McMordie *et al.* 1993) from which (Jacobsen *et al.* 1995) created a possible mechanistic scheme for the photosensitized reaction (Figure 2-45). From Figure 2-45, the pathway in which an



Modified and reprinted with permission from (Jacobsen *et al.* 1995). Copyright 1995 American Chemical Society.

Figure 2-45 Scheme of hypothesized repair of the thymine dimer via oxidative (loss of electron) or reductive (gain of electron) pathways.

electron is donated to the dimer was a more likely route for serotonin since it is so electron rich and very capable of donating an electron to initiate the reversal of the dimers.

The following experiments address the latter question regarding the excited state of serotonin. Photoexcitation of serotonin from its ground state leads to the formation of excited states. Serotonin can be excited initially to a singlet state, in which an electron moves to a higher energy state. It may then emit energy as fluorescence when it moves back to its ground state or undergo another reaction: It can also undergo a spin flip and move to a lower energy triplet state. If the electron does change its orientation into a triplet state, it may then relax back to the ground state via emission of light as phosphorescence. In order to help determine whether serotonin uses, an excited singlet or triplet state, in our reactions, we used the fact that the presence of oxygen can quench an excited triplet state (Wilson *et al.* 1996). Therefore we performed repair reactions using Sero1C and serotonin, as well as both negative controls, and compared the reaction rates under regular oxygenated conditions and under deoxygenated conditions. If serotonin was utilizing an excited triplet state, we would have expected to see an increase in the reaction rates under deoxygenated reaction conditions, since we have removed the oxygen that would normally have quenched the reaction.

To obtain deoxygenated reaction conditions, all buffers and reaction solutions were deoxygenated under a freeze-vacuum thaw method followed by purging with nitrogen. These reaction conditions were used with the wild type substrate, TDP, and the all RNA substrate, RDP (containing a uracil dimer). All reactions were also carried out in duplicate. The results for repair of TDP, shown in Figure 2-46, showed a slight increase

in rate of repair under deoxygenated conditions, although perhaps not a significant amount. The initial rate observed under regular conditions was 0.067 min^{-1} compared to an initial rate of 0.074 min^{-1} under deoxygenated conditions. This was not a significant difference, although these results were duplicated, leading us to believe that they were indeed valid. Both negative controls of no serotonin present, and no Sero1C present (but control DNA present) were also monitored under both conditions. Interestingly, the initial rates for the control where no Sero1C was present doubled consistently under deoxygenated conditions. I concluded that during the catalysis reaction with Sero1C, a triplet state may have contributed slightly to repair, although the excited singlet state was more prevalent and acting as the primary source of electrons.

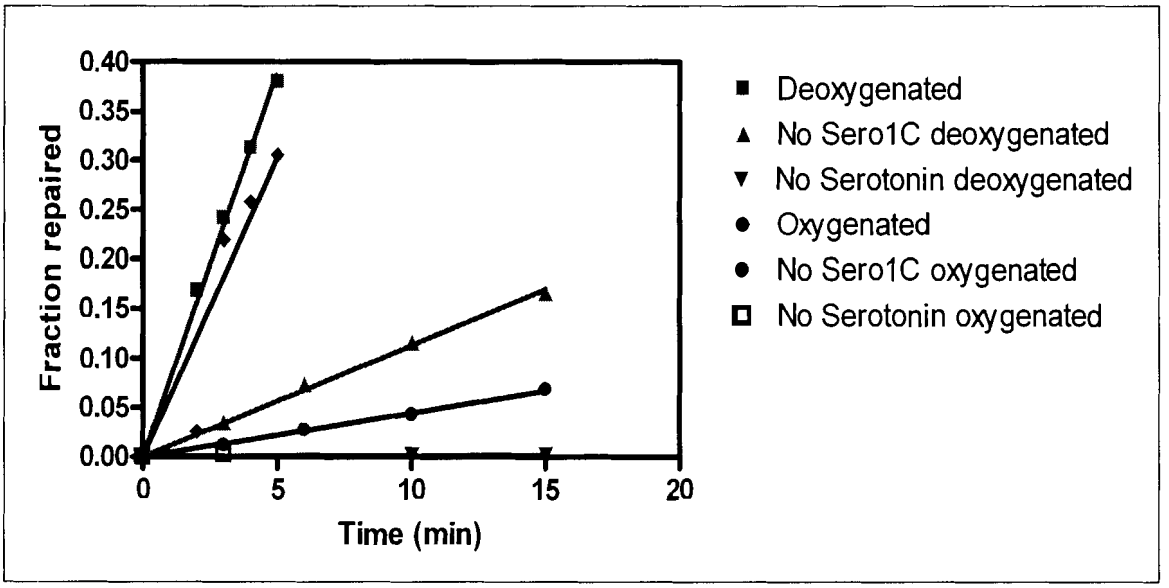


Figure 2-46 Analysis of the repair of TDP under deoxygenated conditions. (500 μM serotonin)

I also investigated the initial rates of repair of the RDP substrate under oxygenated and deoxygenated conditions. In the presence of oxygen, I had measured the

initial rates of repair of RDP, compared to repair of TDP, to be less than 10%, by Sero1C. Interestingly, under deoxygenated conditions, the rate of repair of RDP by Sero1C doubled (Figure 2-47). Also, the initial rate of repair, catalyzed by Sero1C, was almost equal to that of the negative control where no Sero1C was present. I also noticed that the initial rates of this negative control also doubled under deoxygenated conditions. This was indicative of serotonin being in a triplet state since the deoxygenated reaction conditions increased the rate of repair, except that the negative control rates also increased, and it was not the wild type substrate. These results indicated that serotonin alone (without Sero1C), in a triplet excited state, seemed capable of repairing these dimers.

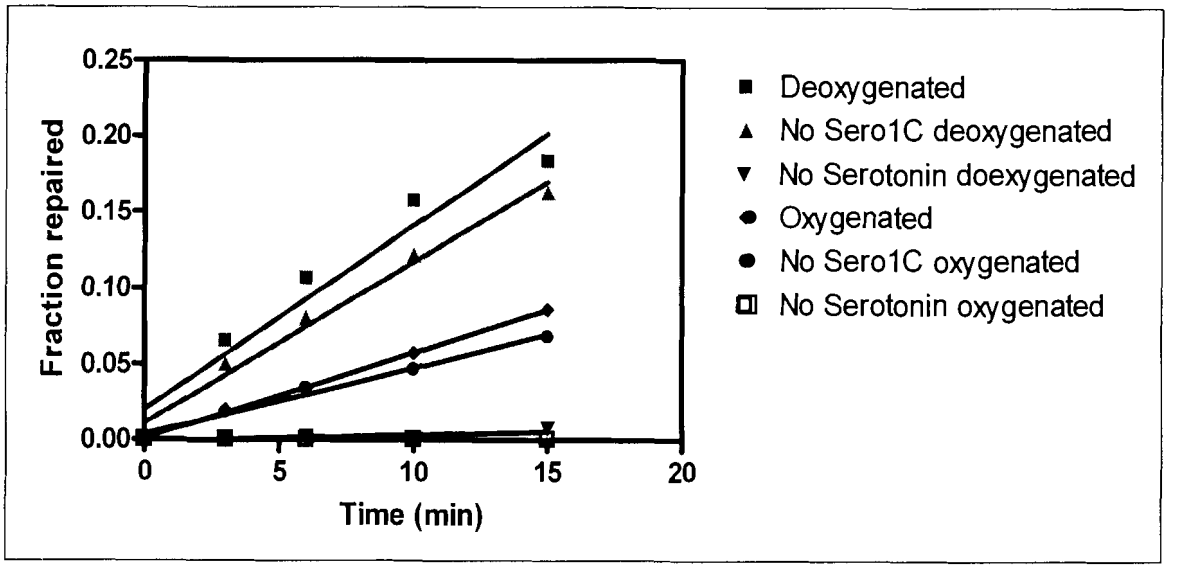


Figure 2-47 Analysis of the repair of RDP under deoxygenated conditions. (500 μM serotonin)

All together, the initial rates of repair of TDP did increase slightly, and reproducibly, under deoxygenated conditions, but not to an extent where I was able to

conclude that serotonin was mainly being excited to a triplet state. I believe that serotonin was effecting repair mostly from an excited singlet state, but also, to a lesser extent, utilizing a triplet state. Therefore, this experiment assisted in concluding that an excited state singlet mechanism was most likely responsible for repair of the thymine dimer substrate.

Hence, Sero1C was able to act quite similarly to *E. coli* photolyase, with a hypothetical electron donation mechanism (as depicted in Figure 2-45). Therefore, we concluded that DNA can function as a photolyase, with the aid of serotonin as a cofactor.

2.4 Summary

A *cis*-acting light activated deoxyribozyme was discovered that was able to use light, and serotonin as a cofactor, to repair thymine dimers in DNA. We were able to manipulate the original deoxyribozyme to create a multiple turnover catalyst that repairs an external substrate. Also we were able to monitor the wavelength dependence of Sero1C to determine a peak wavelength of activity and rate enhancement, as well as noticing that the shape of the curve matches the absorption spectra of the cofactor, serotonin. I was also able to determine a K_d for the cofactor, serotonin, by monitoring the activity of Sero1C at increasing serotonin concentrations. I was unable to find another cofactor that was able to catalyze the photoreversal of the thymine dimers, at a wavelength >300 nm, by testing other indole containing compounds.

In determining a possible secondary structure of Sero1C, in contact with serotonin and TDP, I was able to create a model that agrees with all of the data from chemical probing methods, deleterious mutants (of both Sero1C and TDP), and cross-linking studies. Although there were, most likely, some more complicated aspects, which I was not able to conclude specifically from my data alone. However, I was unable to definitively define a catalytic core within Sero1C. The removal of any bases, from either the 3'-end or the 5'-end, significantly affected the catalysis reaction.

Sero1C, similarly to the *E. coli* photolyase, was able to repair dimers other than thymine dimers. Sero1C was not able to recognize or repair an all RNA substrate, yet I was able to determine an order of substrate preference, which also aided in determining a photochemical mechanism. The proposed photochemical mechanism is an electron

transfer mechanism in which the excited serotonin donates an electron directly to the thymine dimer when bound by Sero1C.

Since serotonin is such a small molecule, I had difficulty determining where this molecule was binding with respect to Sero1C and TDP. Monitoring the fluorescence emissions indicated a specific relationship with Sero1C, but was not specific in a location. Attempts were also made to determine an exact location of serotonin, using ^{3}H-radiolabeled serotonin, to no success. This did not conclusively indicate that serotonin did or did not bind Sero1C. Although, we were able to conclude that the deprotonation event of serotonin was not the cause of catalysis.

In the next chapter I will discuss more thoroughly the 'key' results and the significance of these results. I will also discuss future experiments to solidify the conclusions made in this thesis, and to further our knowledge on this thymine dimer repair deoxyribozyme. Finally, I will mention how these results were significant to the 'RNA world hypothesis'.

CHAPTER 3:
CONCLUSION

3.1 Discussion

The goal of this thesis was to investigate the general properties and characteristics of a previously discovered, serotonin-dependent deoxyribozyme that uses light to repair thymine dimers in DNA. Characterization of this deoxyribozyme, Sero1C, and the specific role of the cofactor, serotonin, revealed that it was the electron rich serotonin molecule that was absorbing the light energy. Serotonin alone could not perform or catalyze a repair reaction of the thymine dimers, nor could it use just any sequence of DNA to achieve repair. There was a detailed relationship between Sero1C, serotonin, and TDP, although I was not able to determine specifically where serotonin was binding.

Deletion mutants were constructed in attempts to determine a minimal catalytic core, which we were unable to identify. It appears that removal of any bases posed some threat to catalysis, or, recognition and binding of the substrate or serotonin.

The chemical probing, assays using DMS, were not conclusive for a guanine quadruplex structure. Interestingly, slight protection, of most of the guanines, was detected even after the addition of NaCl, and before the addition of serotonin. However, DMS is not sensitive to double-stranded structures, therefore the DEPC reactions were more useful in determining base-pairs occurring between TDP and Sero1C.

Cross-linking studies agreed with, and supported, the structural model that was created, which was originally based on the chemical probing experiments.

3.2 Proteins versus Nucleic Acids as Catalysts

In attempt to discover a catalytic nucleic acid that could act like the *E. coli* photolyase, Dan Chinnapen discovered two separate deoxyribozymes with this potential (Chinnapen and Sen 2004). I have demonstrated, through characterization of one of these deoxyribozymes, Sero1C, that nucleic acids do indeed have the catalytic potential to act as a photolyase enzyme, with the aid of a cofactor. Sero1C was able to repair cyclobutane thymine dimers in DNA with the aid of serotonin. There are many interesting differences when comparing Sero1C to the *E. coli* photolyase. The *E. coli* photolyase is a protein enzyme composed of 471 amino acids, which uses two noncovalently attached cofactors, the FAD and MTHF chromophores. It is interesting to note that Sero1C is a photolyase deoxyribozyme, composed of 40 bases, which only needs one cofactor, serotonin, to catalyze and perform the same repair reaction. Both of these photolyase enzymes are specific for cyclobutane thymine dimers, with the ability to catalyze repair of other dimers. Again, another similarity between these photolyase enzymes seemed to be the actual mechanism of electron donation and repair of the thymine dimers. Overall, Sero1C is a photolyase deoxyribozyme with very similar properties and abilities, compared to the *E. coli* photolyase, yet it is a much smaller enzyme, and perhaps may be more suitable, especially sterically, *in vivo*.

3.3 Implications to the 'RNA world hypothesis'

This project has a significant relevance to the RNA world hypothesis and the conditions in early Earth. Since the early Earth was thought to have been susceptible to high levels of UV radiation, it was likely that nucleic acids would have sustained significant UV damage, such as thymine dimers. If RNA molecules were the functional,

catalytic molecules, they needed to be transcribed from the DNA, therefore any damaged DNA would have had to be repaired prior to the transcriptional event. The question was asked whether or not DNA molecules were capable of repairing such UV created lesions, and the answer was yes. Sero1C, a serotonin-dependent deoxyribozyme, which uses light to repair thymine dimers in DNA was previously discovered, and characterized throughout this thesis. This deoxyribozyme, Sero1C, may partially explain a possible transition from when RNA molecules performed catalytic functions to when proteins starting performing these functions: Since this deoxyribozyme was a DNA molecule with the ability to use an amino acid analogue as a cofactor in such a light-dependent reaction (since most protein enzyme use small molecules as cofactors). These results also confirm the possibility of nucleic acids, and earlier life forms, being able to survive such harsh conditions in early Earth.

3.4 Future Work

Further structural studies, using DEPC and $KMnO_4$, to monitor protected bases, and labelling and monitoring the damage on the substrate (as opposed to the enzyme), would reveal whether our or not our assumptions of the base pairs occurring between Sero1C and TDP were correct or not. Also, future structural work will concentrate on determining a more detailed and precise structure to explain all of the interactions occurring between Sero1C, TDP, and serotonin.

We have also proposed a photochemical mechanism, but additional studies to detect radical ion intermediates would confirm, and add further detail to our hypothesis. EPR (electron paramagnetic resonance) studies would detect radical intermediates formed on either serotonin, the thymine dimer, or Sero1C. EPR, in combination with a

spin trapping agent, since the radicals are so short lived, is an effective way of detecting radical species, if any were being formed.

A final aspect of this project would be to determine whether this deoxyribozyme can catalyze repair of thymine dimer substrates located within a double-stranded duplex of DNA, since all of these experiments have been performed with single-stranded DNA, to be more applicable *in vivo*. One feature to overcome would be to determine how to detect the repair of the dimers, within a double-stranded helix, in which both strands have phosphate backbones.

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