STUDENT SABBATICAL PROPOSAL
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Background of Research/Studies at U of M

Alkylating agents constantly attack the reactive nucleobases in DNA, creating a large amount of damaged lesions. Most of these lesions are repaired via the base excision repair (BER) pathway or a direct reversal pathway. My current research focuses on the first step of the BER pathway, in which a glycosylase flips out a damaged base and excises it from the DNA backbone, leaving an abasic site. With such a great variety of base adducts produced, multiple glycosylases are present in cells to recognize and repair the damage, in order to retain genome integrity.[1]

3-methyladenine DNA glycosylase II (AlkA) is a bacterial glycosylase responsible for excising damaged bases, most notably alkylated purines, in *E. coli*. As part of the Adaptive (Ada) response in *E. coli,* the expression of AlkA is upregulated when genomes are under alkylating stress. AlkA has a very broad substrate range, enabling it to catalyze the excision of many base adducts.[2] However, since the rate enhancement for all excisions is the same, the alkylated purines with delocalized positive charges are better substrates, due to the already weakened N-glycosidic bond.[3] Such broad substrate specificity leads to the long standing question as to how AlkA, and glycosylases in general, distinguish damaged and non-damaged DNA. My work hopes to investigate the specificity of AlkA as regards to its broad substrate range.

Steady state kinetics and structural work has been done with AlkA but transient kinetic studies have not been accomplished. The nature of AlkA interactions with DNA is also somewhat ambiguous due to the non-specific binding of the protein to DNA ends.[4] There have been no structures solved with a damaged base bound in the active site of AlkA, but it is known that AlkA flips the base out of duplex DNA, similar to other glycosylases.[5] The goal of my work with AlkA is to characterize AlkA's interactions with various base adduct lesions as well as determine its base flipping rates using transient kinetic studies.

In the past year I have been working to characterize the interactions between AlkA and different base adduct lesions. Previous work in the lab had demonstrated that high concentrations of WT AlkA showed decreased activity under single turnover conditions. This work proposed that multiple AlkA molecules bind a single DNA substrate as the enzyme concentration increases. Due to previous end-binding observations, it was proposed that AlkA binds first and most tightly to the ends of the DNA substrate and subsequently binds the base lesion. As the concentration of AlkA increased, the proteins would stack up along the DNA from the end, preventing AlkA from correctly aligning on the lesion. This would explain why some substrates (an unsymmetrical 19-mer with an inosine lesion) are not inhibited while others (a symmetrical 25-mer with an inosine lesion) are at single turnover conditions.[6]

The start of my work with this issue was to further understand why AlkA is inhibited at the higher conditions, and prevent such inhibition so that future work would not have this complexity. Most notably, we thought that AlkA was binding the ends of DNA, and we wanted to discover which end was favored, why it was favored, and how to prevent such binding. A 1:1 AlkA:DNA complex is needed in which AlkA binds the lesion only (and not an end) to do accurate transient kinetic studies. I thus repeated the single turnover assay to observe the inhibition myself, and discovered that the inhibition was not only dependent on the substrate, but also the ionic strength of the reaction, and the protein preparation.

More specifically, I used a WT AlkA that was purified with a His-tag and cleaved with TEV protease, leaving an extra eight amino acids on the C-terminus of the protein. The C-terminus is far from the active site and DNA binding region and was not expected to be important for DNA binding.

Interestingly, I discovered that the tagged protein showed similar maximal rate constants compared to WT, but greatly reduced inhibition on the 25-mer substrate (Figure 1). Overall, the WT AlkA with an extended C-terminus is much better behaved at single turnover conditions due to weakened end-binding or altered protein-protein interactions. To distinguish these possibilities and determine the binding stoichiometry we continued our work using a fluorescence anisotropy method.

The complex concentration suggests that multiple AlkA proteins can bind to a single oligonucleotide. We used fluorescence anisotropy to try to determine the stoichiometry for AlkA binding to DNA. Using a pyrrolidine (py) containing DNA that mimics the transition state and has a low Kd, a tight binding curve was observed by anisotropy (Figure 2). However, using an ethenoadenine (εA) containing strand, a binding curve was seen that was identical to the binding of non-damaged (nd) DNA (Figure 2). It was assumed, based on previous single turnover assays, that εA would be a tight binding lesion to AlkA. Due to its natural fluorescence, εA is convenient to work with as a fluorescent reporter of protein binding and base flipping. My research suggests that εA does not bind tightly to AlkA and may not be a useful reporter in the study of AlkA:DNA interactions. Further study must be done to compare the specificities between different lesions as discussed below.

I continued to study the interaction between AlkA and an εA substrate by performing electrophoretic mobility shift assays (EMSAs) or native gel shifts. AlkA was shown to shift a pyrrolidine substrate which is known to bind tightly as mentioned previously. However, even with a 10:1 AlkA:DNA ratio, no clear shift of an εA substrate could be observed. The same εA was shifted effectively by AAG, a human functional homolog of AlkA, which has been shown to bind εA tightly and specifically (Figure 3). These experiments gave more evidence that AlkA, while able to bind εA and excise the lesion, is not binding specifically and tightly enough to the lesion and so the complexes fall apart in the gel matrix.

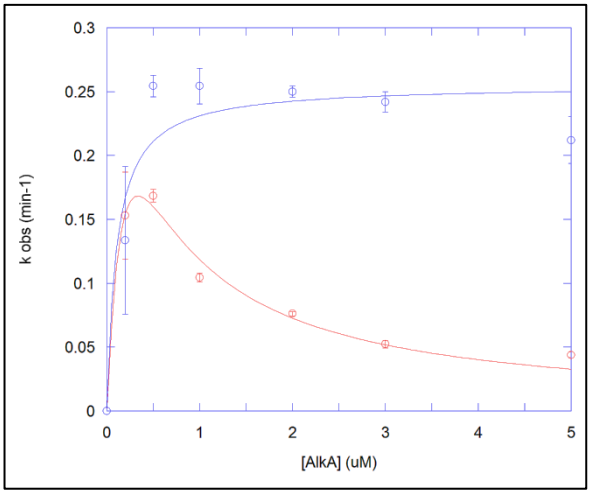

Figure 1. Single Turnover Glycosylase Assay with WT AlkA (red) and WT AlkA* (+C-term tag, blue) on symmetric 25-mer substrate containing inosine lesion. Inhibition at higher [AlkA] is only seen with WT AlkA (fit to noncompetitive inhibition model) and not with AlkA* (fit to Michaelis-Menten model).

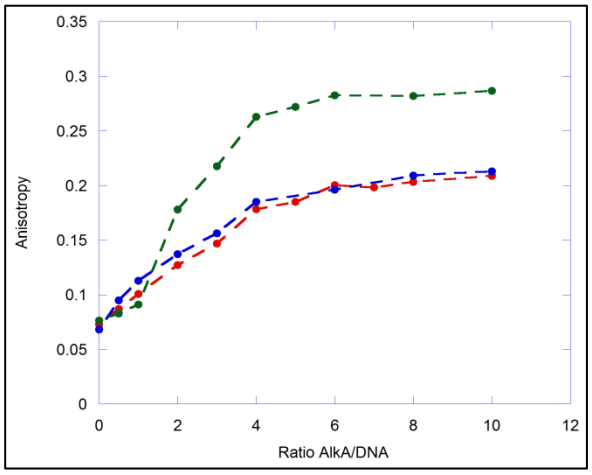

Figure 2. Fluorescence Anisotropy Assay with WT AlkA*, py substrate (green), εA substrate (red), and nd substrate (blue). εA and nd DNA show identical weak binding curves when compared to the tight binding pyrrolidine DNA.

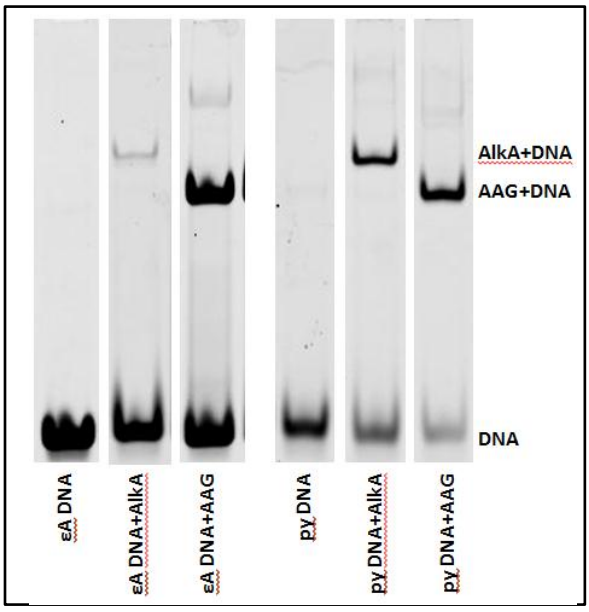

Figure 3. EMSA using 25-mer DNA containing either εA or py lesion. WT AlkA and WT AAG used in 1:1 ratios with DNA. AlkA does not significantly shift εA DNA when compared to py DNA.

Ongoing Work

My research has shed some light on the interactions between AlkA and lesions such as inosine and ethenoadenine. However, there is still evidence that AlkA is binding to εA tightly, even though it is not enough to shift the protein/DNA complex in a gel. To further my studies of AlkA, we decided to directly compete an inosine and an εA substrate in a multiple turnover experiment. By directly competing different substrates of various sizes, each intermediate could be

directly observed in a denaturing gel. The ratio of initial rates of excision between the two substrates could lead us to compare their kcat/Kms, or catalytic efficiencies. My current work hopes to optimize this assay and use it to compare many different substrates. Such comparisons could include substrates with different end identities, including hairpins, blunt ends, and PEG linkers to determine whether AlkA is indeed binding to the ends over a lesion. We have also planned on synthesizing a DNA containing a 3-methyladenine (3meA) adduct, 3-deaza-3meA that could mimic the substrate under acidic pH conditions. 3meA has been shown to be a better substrate for AlkA because of its delocalized positive charge, making it a good leaving group. Using this 3meA adduct will allow us to compare a 3meA-like substrate to the non-charged inosine and εA substrates more prevalently used in *in vitro* studies. These experiments will also allow us to explore how AlkA binds to a specific alkyl group.

Once a tight DNA lesion:AlkA interaction is observed, I wish to delve into the kinetic mechanism of AlkA enzymatic activity and N-glycosidic bond cleavage by utilizing stopped flow methodology. By using transient kinetic methods, the rates of previously unobservable mechanistic steps can be viewed and analyzed. The glycosylase reaction requires a base flipping step and a subsequent N-glycosidic bond cleavage step. Previous work in the O'Brien lab has provided the rate constants of these steps for the human AAG protein, and thus this methodology can be optimized for the bacterial AlkA. These studies will be used to investigate the DNA characteristics and AlkA residues that are required for AlkA recognition of a base.

Sabbatical Work Proposal

While I have been studying the DNA glycosylase, AlkA, for my current thesis work in the O'Brien lab, I hope to branch out and study another bacterial DNA repair protein, AlkB during my sabbatical in Leona Samson's lab at the Massachusetts Institute of Technology. AlkB is a direct reversal enzyme that repairs 3-methylcytosine (3meC) and 1-methyladenine (1meA) lesions in *E. coli* by directly removing the alkyl group from the base. AlkB is a member of the iron(II)/α-ketoglutarate dioxygenase superfamily and has a similar proposed catalytic mechanism to other family members who work on alkylated proteins.[7] AlkB has at least nine different human homologs, two of which (ABH2 and ABH3) are active on similar substrates using the same cofactors as AlkB *in vitro*.[8]

Leona Samson is the Director of the Center for Environmental Health Sciences at MIT and her research focuses on how living systems respond to alkylating agent exposure. More specifically, the Samson group has been studying the global transcriptional response of mammalian cells and tissues to alkylation and how the AlkB proteins work in alkylation repair.

I am proposing to complete my CBI sabbatical work in the Samson lab during the summer months of 2013, after completing my third year in the Biological Chemistry Department at the University of Michigan. While the specific goals of my sabbatical work are not yet concrete, I wish to perform *in vivo* studies on the role of AlkB and its human homologs in DNA repair during alkylation stress. For instance, I could perform knock down studies of WT AlkB in which I express mutant versions of the protein. Residues that affect the repair activity of AlkB can thus be studied *in vivo* at the Samson lab.

I will learn new techniques and ways of thinking during this sabbatical since I do not work with cell systems in the O'Brien lab. This opportunity will also provide me with *in vivo* data that I can take back to my home lab and use as further support of my thesis. We anticipate this sabbatical will lead to a productive collaboration regarding the chemistry and biology of the direct repair of DNA alkylation damage.

Relation of Sabbatical Work to Thesis

AlkA and AlkB are part of the same bacterial operon and are both upregulated as part of the adaptive response to alkylating agents. Although the two proteins act in different mechanisms, AlkA participating in the base excision repair pathway and AlkB directly repairing damage, they both function

as bacterial DNA repair enzymes. I hope to study the role of AlkB in *in vivo* DNA damage repair as part of my sabbatical at MIT and thus expand the focus of my thesis work and learn new techniques not performed in the O'Brien lab. I will use the sabbatical to forge a collaboration with the Samson lab that can continue on in my home lab at the University of Michigan. With the kinetic tools at my disposal, I will continue my study of AlkB by studying its kinetic mechanism, similar to how I studied AlkA. The same mutant AlkB proteins mentioned in the knockdown studies above can be used in the O'Brien lab for *in vitro* studies to observe their effects on the rates of alkylation repair. AlkB shares a substrate in εA with AlkA and also recognizes alkylated purines,[9] and thus similar methods could be used in the studies of both enzymes. There has even been a new crystal structure solved of AlkB binding to an εA containing substrate which would give information to which residues are essential for catalysis and binding. This sabbatical will thus allow me to expand my thesis study and gain insight into the mechanistic and biological role of cellular pathways for repair of alkylation damage.

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