Examining the Mechanics of Chiral Recognition in Phosphotriesterase and Molecular Micelles

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Abstract:

Chiral recognition plays an important role in the many places including chiral chromatography and the biological world where many enzymes are enantioselective. Understanding how molecular micelles and enzymes like phosphotriesterase are able to detect and distinguish isomers can help explain how chiral drugs or compounds are consumed within the body. The binding of 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate to a molecular micelle containing a dipeptide head group was explored using NMR spectroscopy. Similarly, enzyme kinetics were used to study how site-target mutagenesis affected the enzymatic site of phosphotriesterase. In both cases, it was found that a macromolecules ability to detect chirality changed when the residues within the binding pocket of each changed.

Introduction:

Studying the way in which macromolecules interact with small chiral molecules is important in order to better understand the interactions which take place within a chiral pocket. Chiral recognition plays an important role in biochemistry and physiology. In many cases, one chiral enantiomer of a drug can lead to the alleviation of the desired symptoms, while the other may cause damage to the body. For example, a racemate mixture of the drug Citalopram works as an anti-depressant with mild side effects, while a dosage of just the (S) enantiomer shows fewer side effects. This result suggests that the (R) enantiomer is the cause of the majority of the side effects.[1] Other examples of biochemical chiral recognition include enzymatic recognition, where an enzyme will only bind one enantiomer.[2] Examining how these chiral interactions take place can shed light onto how drugs work in the body.

Enzymes and molecular micelles are examples of macromolecules which interact with chiral analytes. Organophosphates and 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (BNP) are examples of small chiral molecules. Two different methods were utilized in order to study the affect of chiral molecules binding. The first of these methods looked at the interaction of polymerized surfactants, in the form of molecular micelles, with BNP. Molecular micelles are similar to a typical micelle, except that in this case, roughly 40 surfactants have had their tails covalently bonded by irradiating the molecules with γ-radiation[3]. A typical micelle is made up of surfactants that are in solution but not bound to each other. These surfactants will aggregate around non-polar molecules, like organic compounds, and form a spherical shape that allows the non-polar compound to be washed away in solution. The reason that molecular micelles were chosen instead is that these surfactant micelles require a certain concentration (which varies depending on the micelle) before they will aggregate together and form the sphere. Attached to the other end of these tails is a polar head group. The head groups used were dipeptides and were comprised of gylcine (G), leucine (L), or valine (V). Each molecular micelle was giving the designation of Sodium Undecyl XY (SUXY), where X relates to the amino acid situated at N-terminal and Y relates to the amino acid at the C-terminal. The hydrocarbon chain made up of 11 carbons (undecyl) is attached to the N-terminus of the dipeptide headgroup, while the sodium ion acts to counter the C-terminus carboxylic acid anion. Six different dipeptides were used as head groups; GL, LG, LL, LV, VL and the achiral GG. These can be seen below.

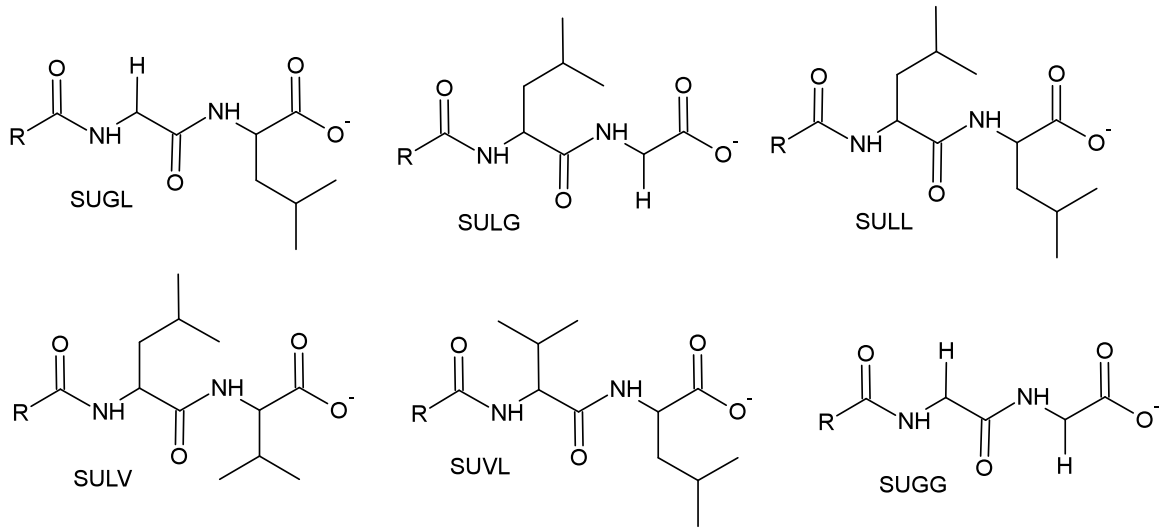


Figure 1. Chemical structures of each dipeptide group that make up the six different molecular micelles tested. Each head group has a carbon chain (R) that is 11 atoms long attached to the N-terminus of the dipeptide head group. The head groups contain glycine, leucine or valine. The naming scheme for each molecular micelle is Sodium Undecyl XY (SUXY) where X and Y represent an amino acid.

It is important to note that the amino acid glycine is an achiral molecule, containing no chiral center. In order to detect different enantiomers, the micelle itself must also be chiral.

The molecule BNP comes in the both (*R*)- and (*S*)-enantiomers. However, BNP does not contain a true chiral center. Instead the enantiomers are considered atropisomers and labeled as having axial chirality or a chiral plane.[4] Atropisomers are stereoisomers with restricted rotation around a single bond that would normally allow for a conformational change between enantiomers. By definition the half life ($t_{1/2}$) of antropisomers must be greater then 1000 seconds at a given temperature.[5] Axial chirality is generally implied when a molecule is described as having antroisomers, i.e. molecules without a chiral center, but possess an axis of chirality, or a chiral plane[5]. Atropisomers have no chiral center and non-super imposable mirror images. The phosphate group that links the naphthyl groups in BNP provides the hindrance that stops the interconversion

from happening rapidly. Three dimensional representations of the two enantiomers of BNP are shown in Figure 2.

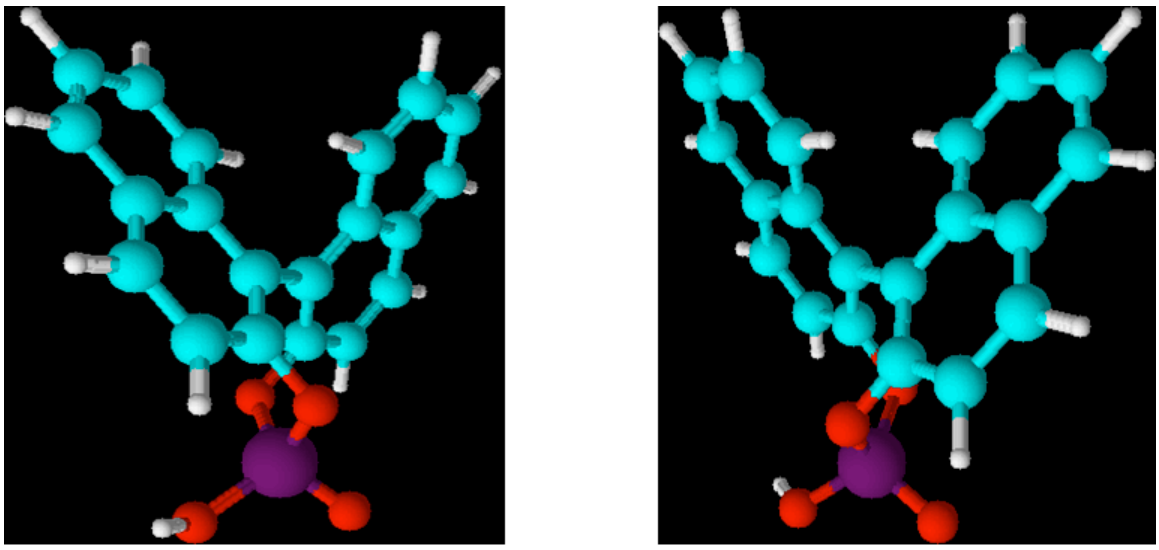


Figure 2. Three dimensional representation of the the (*R*)- and (*S*)- enantiomers of 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (BNP). Structures were modeled in ChemSketch.

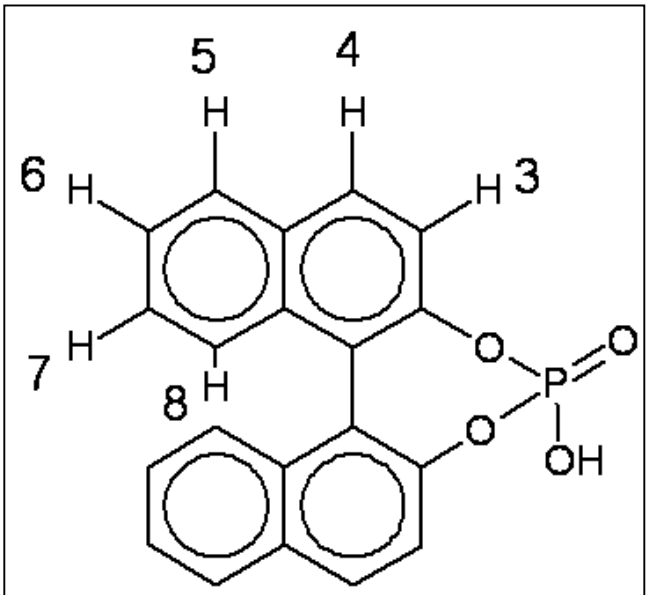


Figure 3. Chemical structure of 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (BNP) showing the six protons that were analyzed for their interactions with various molecular micelles. The second naphthyl ring also has six protons but they are identical to those shown above and were therefore not labeled.

There are six non-equivalent protons of interest on this molecule that interact with the chiral pocket and hydrocarbon chain of the molecular micelle.[3] These six protons are those located on the naphthyl groups and labeled in Figure 3. Each of these protons is represented twice on the molecule, as there are two naphthyl rings per BNP molecule. However, the two BNP rings are equivalent in NMR

spectroscopy. A chemical structure of BNP with the labeled protons is shown in figure 3.

When looking at molecular micelles and their ability to bind BNP enantiomers, a technique involving NMR spectroscopy was utilized. Solutions were prepared following the preparation developed by Kingsbury et al.[3] NMR spectroscopy can be used to measure two different relaxation times, T1 and T2, through well-established techniques.[6] These two values relate two different relaxation times of a molecule. The T1 value (or spin-lattice time) of a proton pertains to the rate at which the spin system returns its energy to the surroundings. While the T2 (or spin-spin time) of a proton pertains to the rate at which the M_{xy}, or magnetic field in the xy plane, decays to zero.

These two values, T1 and T2 times, can then be used to calculate a molecule's correlation time (τ_c).[7,8] The τ_c of a proton or molecule relates to the amount of time it spends in a particular position, before moving or rotating to another. This property of a molecule is calculated for molecules in solution. The equation used to calculate this value can be seen below (Equation 1);[9]

$$(1) \qquad \tau_c = -0.240395 + 0.394030(T1/T2) - 0.030491(T1/T2)^2 + 0.001324(T1/T2)^3 + 0.000021(T1/T2)^4$$

The relaxation of molecules depends on these correlation times because unlike most types of spectroscopy, where the difference between two energy levels is large, the difference between energy levels in NMR spectroscopy is small. Molecules require outside energy to transition from an excited to a ground state. This energy comes when the molecules interacts with a magnetic field oscillating at frequencies determined by the correlation time, i.e. when the molecules interact with the strong magnetic fields inside an

NMR spectrometer, the molecule gains enough energy to transition from its excited state, to its ground state.

Using the correlation times obtained for BNP bound to the various molecular micelles, Hamerly *et al*. set out to gain insight into why some polymers had high α values and other had low α values.[4] The α value of a polymer relates to how well it can distinguish between or separate enantiomers of BNP in column chromatography. If a polymer posses a high α value, then its ability to select one enantiomer over the other is better, or it does a better job separating the (*R*)- and (*S*)-enantiomers of BNP. If a polymer posses a low α value, or a value close to 1.00, then the polymer does not recognize differences in enantiomers as well, and does not separate them. Earlier work with chiral recognition showed that as the chiral selectivity increased, the fluorescence anisotropy of a molecule changed as well.[10,11,12] In fluorescence anisotropy, light that is emitted from an excited molecule is detected in both the parallel and perpendicular directions.[10] A molecule that is moving isotropically, or has the same movements among its entire structure, has a small fluorescence anisotropy while a molecule moving in an anisotropic fashion has a large fluorescence anisotropy. Anisotropic movements are the opposite of isotropic movements so that the molecule does not move uniformly. Furthermore, large fluorescence anisotropy correlates linearly with high chiral selectivity.[11] These results should correlate with the measurements taken via NMR spectroscopy.

A second system that can be examined is the world of biologically active proteins, otherwise known as enzymes. Generally, enzymes are not stereospecific, despite the fact that they themselves are made up of chiral molecules. There are however, enzymes are enantioselective and it is these enzymes that have caught the attention of labs across the

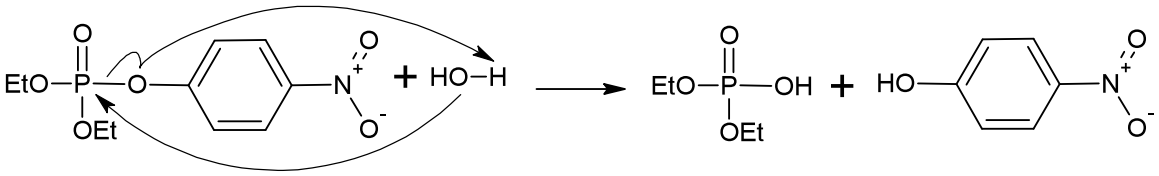
country. In some cases, having a stereospecific enzyme is not needed; the body can use both forms of an enantiomer. In other cases, enzymes in the body are stereoselective, forming or degrading only one enantiomer. One example of this is the enzyme cytosolic thymidine kinase (TK1). TK1 is one of four deoxyribonucleoside kinases in human cells.[13,14,15] TK1 functions as a nucleoside salvage enzyme, i.e. it functions to breakdown DNA from extracellular sources to make nucleosides for the body to use[14]. New drugs are being developed to combat viruses like HIV and hepatitis B virus (HBV) in which only the $_{L}$- form of the drugs are produced[16]. This is because while both the $_{L}$- and $_{D}$- form of the drugs are effective, TK1 will only degrade the $_{D}$- form of the drug. Therefore in the body the $_{L}$- form will not be broken down and will circulate throughout the system to help fight the viruses.[14,16,17] In order to better understand why this effect takes place in the body, kinetics studies can be done. These studies would shed light unto the subject of why enzymes are sterioselective and more importantly how they are able to differentiate enantiomers.

One of these studies was performed on the enzyme Phosphotriesterase (PTE), which is another enzyme that favors one enantiomer over the other. In this case however, it would be ideal for it to catalyze the reaction of both enantiomers at an equal and relatively fast rate. PTE is an enzyme found in a soil bacteria called *Pseudomonas diminuta* and is known to break down many organophosphate compounds, with favoritism towards the (*S*)-enantiomer.[18] This favoritism is a direct result of the binding pocket found within PTE. Investigating this binding pocket could give insight as to why the enzyme favors one enantiomer and help produce an enzyme that is capable of dealing

with both enantiomers of many toxic organophosphate compounds that inhibit Acteylcholinesterase.

Acetylcholinesterase (AChE) is an enzyme which breaks down the neurotransmitter acetylcholine in the synaptic cleft of nerve cells.[19,20,21] If the acetylcholine was not broken down, then the nerves would not be ready to send/receive another impulse. Over the past four decades, extensive research has been done on this enzyme because it is the main target of organophosphates, which includes common insecticides as well as several toxic gases.[19,22] When any of these molecules react with AChE, the enzyme progresses via an SN1 reaction mechanism with a tetrahedral intermediate.[23] This reaction causes the inhibition of the AChE enzyme. Without this enzyme, muscles in body will not release and when the concentration of organophosphates is high enough, this can become fatal. A method for preventing this in the body is desirable and has brought forth a plethora of studies to identify a cure.

PTE was found to exist in soil dwelling bacterium several decades ago and since then the enzyme has been found to contain several different cofactors including; Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, and Mn^{2+} to name a few.[22,24] Focusing on just the cobalt based PTE, although all known varieties exhibit the same behavior, the enzyme heavily favors the breakdown of the (*S*)-enantiomer's of many organophosphates. Therefore, manipulating this enzyme so that it may function to breakdown both enantiomers of any given organophosphate has been the goal of many labs.[18,25] If PTE were able to breakdown many of these organophosphates it could be used to ensure that AChE is not inhibited. The mechanism for the reaction of PTE with diethyl *p*-nitrophenyl phosphate can be seen in Scheme 1.



Scheme 1. Mechanism for the breakdown of organophosphates (in this case diethyl *p*-nitrophenyl phosphate) by the enzyme phosphotriesterase (PTE). Proposed based on the work by Chen-Goodspeed *et al.*[18]

Scheme 1 presents just one of many organophosphates that were used in order to investigate this reaction. However, all of these compounds follow the same mechanism within the site. It is believed that this molecule proceeds by means of an SN2 reaction with a cleavage of the P–O bond.[22] There is one other mechanism which has been proposed in recent years in which a serine residue within the active site reacts with the orgonaphosphate and forms a trignonal bipyramidal transition state.[19,22] Table 1 shows the base structure of the organophosphate, as well as the different side chains used to modify the molecule.

Substrate	X	Y	Substrate	X	Y
I	CH_3	CH_3	R_P-VI	$CH(CH_3)_2$	CH_2CH_3
R_P-II	CH_2CH_3	CH_3	S_P-VI	CH_2CH_3	$CH(CH_3)_2$
S_P-II	CH_3	CH_2CH_3	R_P-VII	C_6H_5	CH_2CH_3
R_P-III	$CH(CH_3)_2$	CH_3	S_P-VII	CH_2CH_3	C_6H_5
S_P-III	CH_3	$CH(CH_3)_2$	VIII	$CH(CH_3)_2$	$CH(CH_3)_2$
R_P-IV	C_6H_5	CH_3	R_P-IX	C_6H_5	$CH(CH_3)_2$
S_P-IV	CH_3	C_6H_5	S_P-IX	$CH(CH_3)_2$	C_6H_5
V	CH_2CH_3	CH_2CH_3	X	C_6H_5	C_6H_5

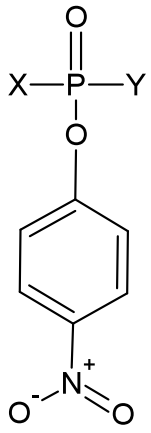


Table 1. On the right is the base structure of the organophosphate used, with a nitrophenyl leaving group. The X and Y refer to the side chains present on the various compounds. Note that compounds I, V, VIII and X are not chiral. Adopted from Chen-Goodspeed *et al.*[18]

Ten different compounds were used in the kinetic study of PTE. Four of these compounds are achiral. However, they still possess some importance because they can show which side chains in general, affect the speed of degradation. For instance,

compound X has two phenyl groups, both of which are quite large; therefore this compound should have a relatively low v_{max} when compared to compound I which contains only methyl substituent's. The large phenyl rings on compound X mean that few of the molecules can get into the active site, while the small methyl groups attached to compound I allow for easy and quick access into the pockt. The other compounds, those that are chiral, should help give data to suggest why this enzyme prefers certain enantiomers over others. Further studies to obtain x-ray crystal structures of the enzyme bound to these compounds will produce a better idea of how they bind and possibly give more insight as to why one enantiomer is favored.

Once the general structure of the enzyme bound with analyte has been found, the next step is to look at how to manipulate the enzyme so it does not favor one enantiomer. This can be done by site-targeted mutagenesis. However, this step must follow the x-ray crystallography because without a basic map of the enzyme, the binding site would be unknown and the specific residues in the site would not be known. The goal of this site targeted mutagenesis is to look at how changing different residues to alanine affects the k_{cat} ratios for the breakdown of the (*S*)-enantiomer compared to the (*R*)-enantiomer.

Here in the interaction between macromolecules and small chiral molecules was explored using two different techniques and two different types of macromolecules. The common idea behind both experiments was to explore the specific part(s) of the macromolecules that allow them to differentiate chirality. The first method used NMR spectroscopy to explore the binding of the analyte 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (BNP) to polymerized molecular micelles with varying dipeptide head groups. The second method involved the binding of various chiral organophosphates with

enzyme phosphotriesterase. This enzyme was also modified, by using site-targeted mutagenesis, to examine which residues are involved in the chiral selectivity. The basic premise is to alter the binding pocket in these macromolecules to try and find a correlation with the molecules ability to differentiate enantiomers. Examination of the binding pockets for both the macromoles will be discussed first, followed by analysis of the data attained from each experiment respectively.

Results and Discussion:

Probing the interactions between macro molecules and chiral analytes was performed using two different methods. The firs of these methods involved molecular micelles containing various dipeptide head groups binding to the molecule 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (BNP). NMR spectroscopy was used to examine the correlation times (τ_c) of the BNP while bound to the molecular micelles and compared the times to those of free solution BNP. From this comparison, a model for how these molecular micelles were able to differentiate enantiomers could be developed. The second of these experiments looked at a biological situation in which an enantioselective enzyme, phosphotriesterase (PTE), bound to various organophosphates. This was done using kinetics studies in which various amino acids were altered within the binding pocket to determine which residues were most significant in determining the enantioselective properties of the enzyme. From these results an application for how this enzyme could be used to stop the inhibition of acetylcholinesterase could be developed.

Previous research has shown that a binding pocket forms when the molecular micelles are placed in solution, and that BNP will bind to the molecular micelle in such a way as shown in Figure 4.

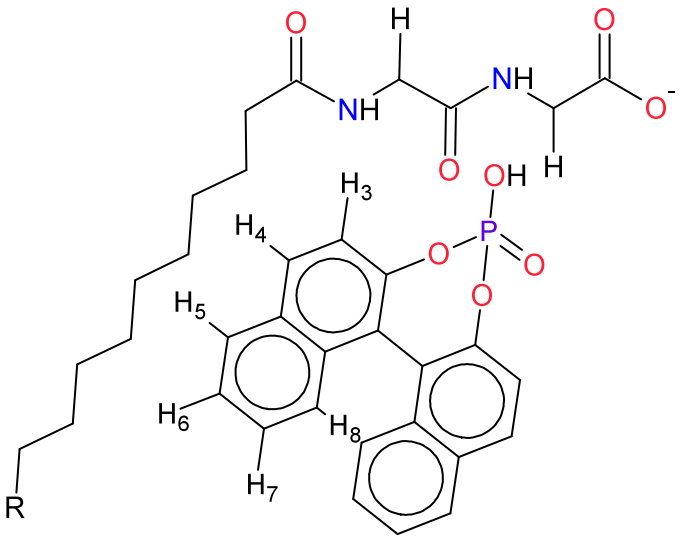


Figure 4. Graphical illustration of the 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (BNP) analyte bound in the pocket created by the sodium undecyl glycine glycine (SUGG) polymer. The complex is stabilized by hydrogen bonding of the phosphate group to the C-terminal end of the dipeptide.

Examination of this binding pocket gave the results shown in Figure 1 where the polymer SUGG has bound the BNP molecule. It is important to note that this same pocket is formed for all of the other molecular micelles that were tested as well. This pocket was determined using an NMR experiment called a NOESY (Nuclear Overhauser Effect Spectroscopy), which shows protons that are not coupled but are within 5 angstroms in distance. The number 8 proton is situated close to the hydrocarbon chain where it meets the head group. It is buried in the pocket so that its movement is heavily restricted. This proton would not normally bind in this area because there are several oxygen and nitrogen atoms close by, and these would push the proton away due to hydrostatic interactions. However, the rest of the BNP molecule is perfectly positioned within the pocket. It has its polar phosphate group forming hydrogen bonds to the hydrophilic atoms in the dipeptide and protons H5, H6, and H7 are pointing towards the molecular micelle's hydrocarbon chain. This means that within the pocket there are regions that are stabilized by interactions and there are regions that are actually

destabilized by unfavorable interactions. This effect is often associated with anisotropy, or the amount of variability in the correlation times (τ_c) of the BNP's protons.

When the BNP binds to the different polymers, there will also be a certain amount of steric hindrance involved for the molecule as a whole. For example, with the SULL polymer shown in Figure 4, where there are two leucine residues with large carbon R-groups jetting into the pocket, there is a lot of steric hindrance; where the steric hindrance is coming from these large R-groups interacting with the BNP molecule. This should result in less anisotropy in the BNP's protons because the molecule cannot get certain protons, like H8, buried deep in the pocket while H3 is exposed. This means that the H8 proton is in an area where its movement is severely constricted by steric hindrance in the molecular micelle's pocket. The H3 proton is located outside of the pocket, where its rotation is hardly restricted at all. Conversely, with the SUGG polymer, there are only glycine residues with single protons attached to the α-carbon, which results in essentially no steric hindrance. This means that SUGG is achiral and cannot differentiate between enantiomers. It also means that there should be an even ground, a molecular micelle with residues that give more definition to the pocket without providing a lot more steric hindrance i.e. residues that also don't decrease the polymer's ability to differentiate bound enantiomers through the idea of anisotropy.

Enzymes can also use chiral recognition as a means to control the stereospecificity of a reaction. Sometimes enzymes are used to transform one enantiomer to another, and other enzymes will only deal with one enantiomer through the whole reaction. There is a yet another group of enzymes, ones including phosphotriesterase (PTE), which bind both enantiomers, but breaks down one enantiomer much more

quickly than the other. The problem with this, as mentioned in the introduction, is that when dealing with chiral organophosphates, both enantiomers devastate the nervous system equally and so an enzyme that breaks down both enantiomers equally fast is desired.[18] To determine why PTE breaks down enantiomers at such vastly different rates, site-directed mutagenesis was used to modify the binding site. The binding site of the wild type can be seen below in Figure 5.

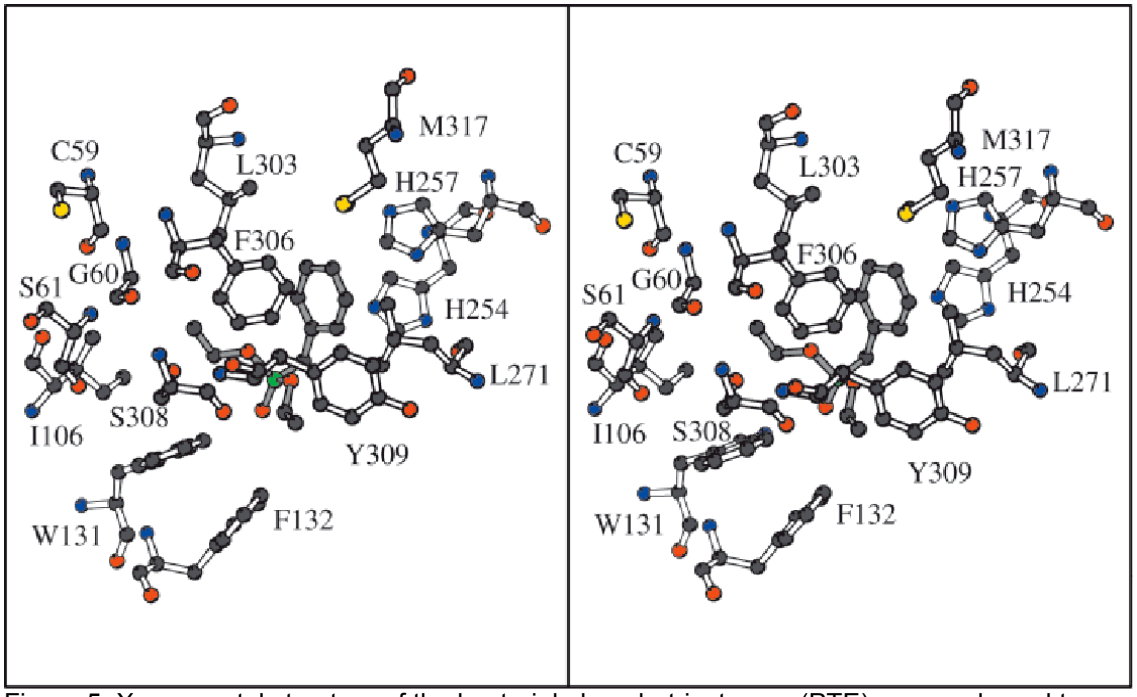


Figure 5. X-ray crystal structure of the bacterial phosphotriesterase (PTE) enzyme bound to a substrate analogue. There are three distinct regions located within the pocket that help the enzyme distinguish between enantiomers and are designated the small, large and leaving group subsites. Each picture shows three-dimensional structure of diethyl 4-methylbenzylphosphonate bound to PTE. On the left is the (S)-enantiomer and on the right is the (R)-enantiomer. Taken from Chen-Goodsped *et al*.[18]

X-ray crystallography was performed in order to determine the three-dimensional crystal structure of diethyl 4-methylbenzylphosphonate bound to PTE[18]. There are three distinct binding regions that have been designated as follows; small, large and leaving group subsites. These three regions are made up of several residues each. The small subsite is defined by the side chains of Gly-60, Ile-106, Leu-303 and Ser-108. Two

additional residues, Cys-59 and Ser-61, oriented away from the binding subsite and may be important for catalysis. For this reason there is some discrepancy as to how significant they are. The large subsite is defined by the residues His-254, His-257, Leu-271 and Met-317. The final subsite, the leaving group subsite, is defined by the residues Trp-131, Phe-132, Phe-306 and Tyr-309.

The names designated to each site are based on what piece of the organophosphate binds in that general area in the binding site. For example, the leaving group subsite is where the enzyme prefers to position the leaving group of the analyte, a nitrophenyl group. The same is said for the small and large subsites, the enzyme makes more room for the larger piece of the organophosphate to be placed in the large subsite. This simple idea is what allows for the chiral differentiation of enantiomers and also explains why the enzyme deals with one enantiomer so much faster. The S-enantiomer is structurally built to fit into the wild type pocket perfectly, while the R-enantiomer would be able to match with only one of the subsites. The other two subsites would not have the correct side chain oriented in their pockets.

This also explains why with certain organophosphates, and certain mutations of the enzyme, the reaction occurs close to a 1:1 ratio: S_p:R_p breakdown. If the enantiomers have relatively small side chains attached to the X and Y positions, like compound II in Table 1, then they are closely related and would act nearly the same within the pocket. When the size of the side chains on the analyte differ greatly, like compound IV in Table 1, with a phenyl ring and a methyl group, then only one enantiomer will fit very well into the pocket. The other enantiomer would have to continuously associate and dissociate with the enzyme before it found a conformation where the reaction could take place. This

time is what composes the difference in k_{cat} values between certain organophosphates or mutations.

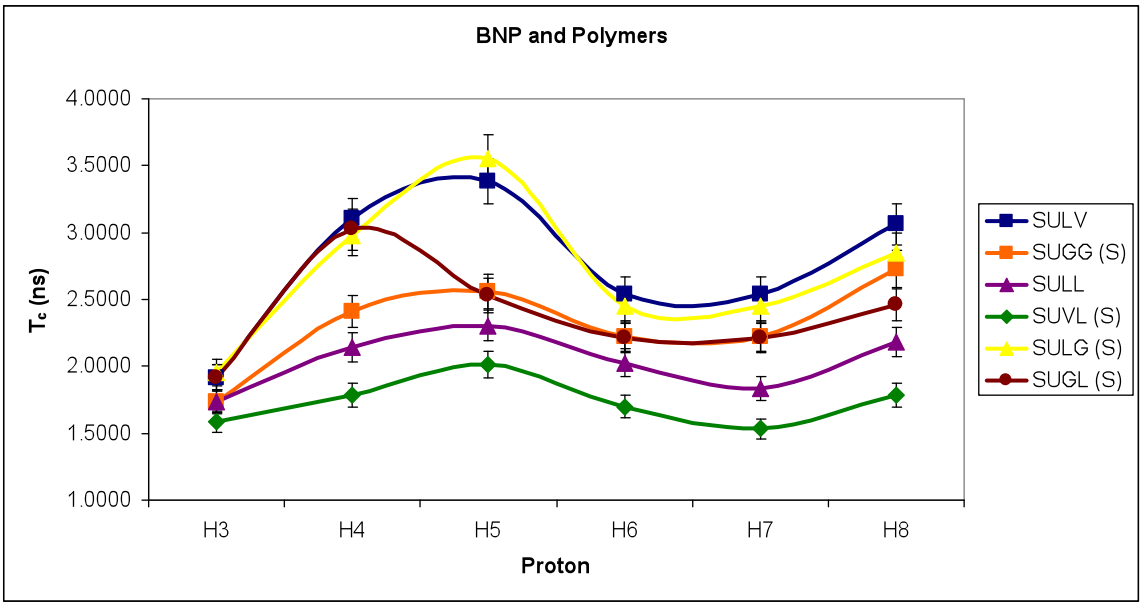


Figure 6. Correlation times of each proton for the six polymer micelles studied, sodium undecyl (glycine, leucine or valine). The polymers with the greatest amount of variation in their correlations times also have the most anisotropic movements.

The data obtained from the interactions of the various polymers and BNP via NMR spectroscopy is shown in Figure 6. Figure 6 begins to explain in a broad sense how chiral recognition takes place within the polymers. The basic trend is that certain protons of the BNP molecule have longer τ_c's within the same polymer, while others have shorter τ_c's. The bigger the difference between the longer and shorter τ_c's, the more anisotropic the motion of the molecule, i.e. the larger the range in τ_c's, the more random movement the molecule has. The random movement relates to the fact that each proton has a different τ_c and will move different and independently from the others.

A larger range in τ_c's is characteristic of longer τ_c's in general for the molecule bound to the polymer as seen in Figure 6. For example, the polymers SULV, SULG and SUGL (shown in blue, yellow and brown respectively in Figure 6) have overall longer

correlation times and their correlation times vary over the widest range. Conversely, SUGG, SULL and SUVL (shown in orange, purple and green respectively in Figure 6) have smaller overall correlation times and their correlation times vary over a narrower range. This property in keeping with the idea that as you increase the amount of anisotropy, something else in the molecule will also change to promote these motions. In this case, the τ_c's are actually physically longer.

This effect however, is not directly related to the binding affinity of the BNP to the polymers. In other words, the binding constant does not relate to the range of correlation times. For example, SULL has a small BNP binding constant (46) but has larger correlation times as well as a larger range of BNP correlation times (purple curve in Figure 6). However, SUVL has a larger BNP binding constant (73) but has smaller correlations times as well as a smaller range of BNP correlation times(green curve in Figure 6).[4] An explanation of this effect would be due to the size of the pocket that is formed by the different amino acids. Another factor would be steric hindrance between the BNP and the amino acid side chains within the chiral pocket which would not allow for longer correlation times, despite a high binding constant. For example, SULL has a leucine in the N-terminal position and a leucine in the C-terminal position. The leucine does not allow the BNP to get in tight within the pocket, while the leucine, with its long side chain, helps to better define the pocket. While this gives a larger pocket over all, the N-terminal leucine, which helps define the pocket a little, keeps the BNP from getting deep into the pocket and slowing down its τ_c's by a larger amount.

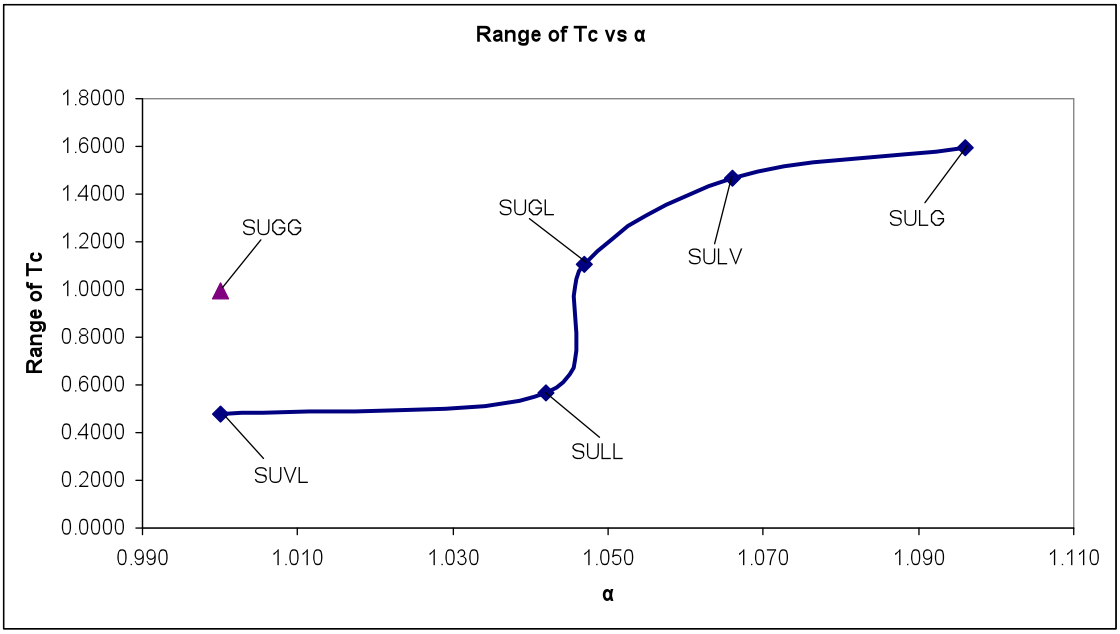


Figure 7. Graph showing the trend of increasing selectivity (α) relating to an increased range in correlation times for 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (BNP) analyte bound to each polymer, sodium undecyl (glycine, leucine or valine).

Figure 7 shows a plot of the chiral selectivity (α) values for each polymer against the range in correlations times. This plot shows that a wide range of correlation times is characteristic of large α value and high chiral selectivity and vice versa. In other words, as the α value or selectivity increases for a given polymer, so does the range in correlation times for the bound BNP. For example, SUVL has small range in correlations times according to Figure 7, which correlates to an α value of 1. Conversely, SULG has a large range in correlation times according to Figure 7, which correlates to an α value of 1.096. A polymer that exibits a high chiral selectivity must posses some form of chrial recognition. The data suggests that this recognition is most pronounced when there are large differences in the τ_c's for BNP's protons. In cases where the range in times is small, the selectivity is also low.

The protons on BNP are all moving at a comparable rate, so this makes it hard for the polymer to detect differences in chirality and suggests that all protons on BNP are in

similar environments in the bound state. However, when the protons are moving at different rates, then the polymer must be interacting with BNP in such a way to promote this effect. When BNP binds to these polymers, regardless of how favorable this interaction is, the protons are experiencing varying amounts of motional freedom. These different interactions, allowing for various freedoms of motion of the protons, promote chiral recognition. If the polymer has a way of sensing differences in these protons then the polymer, according to the above data, will also be more capable of sensing different enantiomers.

Table 2 shows the k_{cat} values for the hydrolysis of the (*R*)- and (*S*)-enantiomers of the respective organophophates by PTE with the varying mutations to the enzymes sequence. The results for compounds I, V, VIII and X are insignificant in this study because these compounds are not chiral. Therefore the enzyme will break down each organophosphate enantiomer at an equal rate.

Table 2. k_{cat}/K_a ratios for the Hydrolysis of Chiral Substrates by Co/Co-Phosphotriesterase[a]. Adapted from Chen-Goodspeed *et al.*[18]

Mutant	II	III	IV	VI	VII	IX
WT	1	32	90	10	21	35
C59A	1	15	110	13	21	93
G60A	12	410	13000	24	11000	15000
S61A	1	11	43	6	11	13
I106A	1	9	3	3	2	3
W131A	2	40	5	21	14	10
F132A	1	12	5	6	3	5
H254A	1	25	48	29	4	7
H257A	1	23	86	15	17	37
L271A	1	7	45	17	11	27
L303A	1	25	25	17	18	27
F306A	1	42	160	14	18	100
S308A	1	59	140	11	5	14
Y309A	2	13	10	13	8	35
M317A	2	9	120	13	6	24

[a] This ratio was determined according to the equation S_p/R_p $(k_{cat}/K_a)_S/(k_{cat}/K_a)_R$

As mentioned in the introduction, compounds II, III and VI contain side chains of only methyl, ethyl or propyl groups while compounds IV, VII and IX contain one phenyl group side chain as well as one of the above groups. The compounds containing a phenyl group have a distinctly larger side chain compared to the other side chain. This difference correlates to the enzyme's ability to break down one enantiomer faster then the other, as seen in compound IV. The reason this makes such a dynamic difference relates back to the previously mentioned binding sites within the enzyme pocket. When looking at the (*S*)-enantiomer, which seems to be the preferred enantiomer for the enzyme, the phenyl group fits perfectly into the large subsite shown in Figure 5. The smaller methyl, ethyl, or propyl groups fit well into the small subsite. Therefore the (*R*)-enantiomer cannot possibly have its side chains in this same orientation and will therefore have fewer molecules that can bind 'well enough' for the breakdown to take place.

The reason why the ratio of k_{cat} values for organophosphate enantiomers with side chains consisting of just methyl, ethyl, or propyl groups are nearly always equal to 1 is that there is not a large difference between the side chains size. In other words, the enzyme can place either side chain in the small and large subsites and breakdown the molecule. To the enzyme, the (*R*)- and the (*S*)-enantiomers are basically the same. For example, molecule II has a methyl and an ethyl side chain. Normally, when an enantiomer begins to interact with the enzyme, the enzyme will direct the large and small side chains into their appropriate subsite. However, because methyl and ethyl side chains are similar to one another in size, and they are also small, the enzyme may direct the side chains into either subsite. It may put the methyl in either the small or large subsite, depending on which it is nearest to when it begins to interact. This has no negative effect

on the speed at which the enzyme breaks down the molecule, but it does affect the enzymes ability to distinguish between enantiomers. This is why the ratio of the k_{cat} values for the non-phenyl containing organophophates, like compound I, is nearly always 1. The enzyme can take in both enantiomers at the same rate, and in turn gives very similar k_{cat} values.

Certain mutations to the enzyme also have an effect on its ability to differentiate enantiomers. Looking at compound IV in Table 2, which contains a phenyl group and a methyl group, most of the k_{cat} ratios are under 100. There are several instances where the ratio goes above 100, most notably when Gly-60 is mutated to alanine. In this instance, the ratio between (*S*)- and (*R*)-enantiomers is 13,000. This ratio shows that the (*S*)-enantiomer is overwhelmingly preferred over the (*R*)-enantiomer. For all other mutations however, the ratio is much smaller. This means that there is something significant about this mutation and its relationship to compound IV. Both glycine and alanine are small side chains, and this residue is part of the make up the small subsite pocket. When the wild type interacts with the molecule, the (*S*)-enantiomer is only slightly favored, when Gly-60 is mutated to alanine the enzyme is able to differentiate enantiomers more effectively.

The raw data for compound IV shows that the wild type enzyme breaks down both enantiomers at fairly similar rates. The k_{cat}/K_a values for the (*S*-) and (*R*)-enantiomers with the wild type enzyme are 9.3×10^7 and 1.0×10^6 respectively. This means that the (*S*)-enantiomer is only favored by a factor of 93. Examining the mutation of Gly-60 the k_{cat}/K_a values for each enantiomer change to 2.2×10^8 and 1.7×10^4 for the (*S*)- and (*R*)-enantiomers respectively. Two very important conclusions can be drawn from this

information. The first is that certain modifications of the enzyme can result in drastic changes to the enzymes ability to differentiate chiral molecules. The second is that as expected, the k_{cat} value for the (*R*)-enantiomer drop, and the (*S*)-enantiomers k_{cat} value goes up. The latter statement is of more significance in this research. It shows that the breakdown of the (*S*)-enantiomer is actually sped up by the mutation, something that was not expected. The simplest explanation of this is that glycine is an achiral molecule, i.e. not a chiral molecule. When the glycine is mutated to alanine, a chiral residue now replaces the achiral one and the small subsite now has another chiral molecule. The addition of this one chiral residue could increase the chiral selectivity of the enzyme greatly. This would explain why the k_{cat} value went up for the (*S*)-enantiomer compared to the wild type.

Still examining compound IV, there are many mutations that actually cause a decrease in the ratio for the breakdown of the (*S*)- and (*R*)-enantiomers. For example, residues that are normally large like L271 and F132 were replaced with smaller alanines. Although this molecule still possesses a chiral center, it is much smaller then the residues it is replacing. These mutations leave the specific subsite in which the mutation occurred to lose one of the residues which help with chiral differentiation. The mutations in the large subsite residues lose the most recognition, with ratios of 10 and under. This means that having these larger residues in the large subsite is important in helping the enzyme in differentiating enantiomers. These results are to be expected, replacing a residue with a smaller one should alter the subsite enough so that both enantiomers will have similar k_{cat} values.

There are, however, two mutations where the replacement of a residue by an alanine residue increases the k_{cat} ratios for the breakdown of the (*S*)- and (*R*)-enantiomers. One of these mutations takes place in the large subsite and the other mutation in the leaving group subsite. The former is a mutation of the Met-317 and the latter is of Phe-306. The mutation in the leaving group subsite, F306A, increased the ratio from 90 to 160 when compared with the wild type. This is a increase in chiral differentiation makes intuitive sense. The leaving group on the ananlyte is a nitro phenyl group, which is fairly big. This phenylalanine, however, does not play a major role in the differentiation of the enantiomers because the k_{cat} only increases by a factor of 2. Therefore, replacing the phenylalanine with an alanine just makes more room for the nitro phenyl group. Phenylalanine and alanine in the pocket act the same way, with a methyl group to direct the leaving group, with the only difference being that the phenyl ring projects into the pocket more and may disrupt the binding of the organophosphate.

The increased ratio resulting from the mutation of Met-317 to an alanine residue was not expected, but may have an explanation similar to that for the F306A mutation. This mutation takes place in the large subsite. Methionine is not as large as some other residues, but it does contain a chain of 4 atoms: 3 carbons and a sulfur. Two factors may explain why the alanine residue increases the ratio of k_{cat} value from 90 to 120. The first is the size difference between residues, although this is minor as mention before. The bigger factor that explains this difference is that the methionine contains a sulfur atom, a polar atom, while alanine does not. The organophosphate side chain that would fit into the large subsite is purly organic, containing no polar groups. Therefore, when this sulfur is removed from the pocket, the organic side chain of the organophosphate will insert

more readily into the pocket. This effect also explains why the (*R*)-enantionmer does not have an equal increase in its k_{cat} value. The altering of one of two sites that direct and dictate chiral differentiation should have a positive effect on the (*S*)-enantiomer, and a negative effect on the the (*R*)-enantiomer.

The mutations C59A and S61A both gave unexpected results that raised several questions. Neither of these residues side chains are oriented towards the binding pocket, which raises the question of how then were they able to affect the binding of the various organophosphate enantiomers. A theory has been proposed to explain this effect. The side chains are not oriented into the pocket, but they are oriented in such a way to cause some amount of rigidity or stiffness. This may help the small binding site, where these residues are located, in precluding larger side chains on the organophosphate from entering this binding site. This would help the enzyme in discriminating the two enantiomers. This effect is supported by one of the mutations, S61A, where there is a decrease in chiral selectivity across all compounds. However, the mutation of C59A contradicts this explanation. There was an increase in most compounds, with only compound III producing a decrease in selectivity. The fact that this result is not consistent across all the organophosphates tested and that it had the opposite affect as the S61A mutation suggests that another explanation is needed. However, a theory for this cannot be developed without further research into the structure of the binding site with this mutation.

The explanation of chiral differentiation by macromolecules such as enzymes and molecular micelles was explored using enzyme kinetics and NMR spectroscopy respectively. In each case, an alteration to the macromolecule was performed, in hopes of seeing significant changes in the binding of a specific analyte. The results of these

alterations were compared to other alterations, as well as the wild type for the enzyme. The conclusions that can be drawn from these experiments point towards the idea that macromolecules need a certain amount of flexibility within the binding pocket to allow for the recognition of one enantiomer and the hindrance of another.

When a macromolecule such as a molecular micelle binds to a small analyte like BNP, there are several factors that help differentiate enantiomers. The first of these is the amount of steric hindrance from the side chains of the dipeptide headgroup. When the chains were both too large, the analyte could not bind in the pocket and therefore little differentiation took place. Likewise, when the dipeptide headgroup consisted of residues with small side chains, e.g. SUGG, there was no chiral differentiation. It was only when the dipeptide headgroup contained residues with side chains that produced little steric hindrance but gave the most definition to the pocket that the greatest amount of chiral differentiation took place.

The enzyme kinetics data showed that slight alterations of a residue, e.g. G60A, can make large differences in the ratio of k_{cat} values for the (*S*)- and (*R*)-enantiomers. It also showed that alterations of larger residues, e.g. W131A, had a negative effect on the enzyme's ability to differentiate enantiomers. These mutations made the small or large subsites less defined and made it easier for the (*R*)-enantiomer, which normally wouldn't fit into the pocket, to bind to the enzyme and be broken down. In general, dramatic mutations like the one previously described, had either little effect or actually increased the enzyme's ability to differentiate enantiomers.

In order to differentiate enantiomers, macromolecules must contain chiral pockets. This was best illustrated by the polymer SUGG binding to the enantiomers of

BNP and having an α value of 1. To increase the chiral differentiation by macromolecules a general trend of the construction of the pocket has risen. The basic structure of this pocket is to use residues whose side chains create a well-defined binding pocket that also has some flexibility to allow one enantiomer to quickly and easily enter. Large residues that protrude into the pocket push the analyte out and away and allow for little binding of either enantiomer. Similarly, small residues allow both enantiomers to interact with the pocket equally and allow for low recognition as well. There is a middle ground within these two extremes: one that allows a high amount of chiral recognition, but does not diminish the speed at which an enantiomer is broken down, in the case of an enzyme.

Conclusion:

The interactions of a molecule capable of differentiating enantiomers by various means was explored by two methods, NMR spectroscopy and enzyme kinetics. Through these techniques, several trends were noticed. The first was that the macromolecules capable of differentiating enantiomers were chiral themselves. They contained animo acids in the L orientation. This results in the pocket these residues form to be chiral, making the chiral differentiation by the macromolecules possible. The side chains of these residues played a critical role in this differentiation. Only one form of an enantiomer could fit well into the chiral pocket with these specific residues. When certain residues in the sequence were replaced by another residue, the chiral selectivity was altered. In some cases, a residue was altered and the chiral selectivity was decreased, in others, it was increased. The general conclusion was that if a larger residue was replaced, and it was responsible for restricting one enantiomer from entering the pocket, then the chiral selectivity would decrease. The macromolecule would not be able to keep one

enantiomer out of the pocket and this means that both enantiomers would bind at roughly even rates. The opposite of this effect, replacing a smaller residue with a larger one, often times increased chiral selectivity. This is explained with a similar reason as above, except that the macromolecule could restrict one enantiomer more. It should be noted that there were cases where an increase in selectivity decreased v_{max}. This effect was minor however, as most mutations resulted in an increased v_{max} regardless of how the chiral selectivity changed. There were also cases where replacing a small residue with a large one decreased selectivity because neither enantiomer bound well with the new macromolecule. There was too much steric hindrance introduced by this larger residue.

Several cases arose which were not explained, and further research to explore these results could be done. Two residues that were in the binding site, but which had their side chains oriented away from the pocket, changed the ratios for the breakdown of the enantiomers. The unexplained part of this is that the change of one residue decreased chiral selectivity while the change in another increased it. A change in the rigidity of the enzyme after these mutations was proposed to explain this effect. When an alanine replaces a larger residue, the pocket becomes less rigid and this may explain a decrease in chiral selectiviy. However, an explanation of the increase in chiral selectivity was not proposed. Researching the actual reason for this idea would benefit both projects. X-ray crystallography could be used in order to explore the structure of the mutant enzyme bound to the various organophosphate analytes.

The rigidity of the binding pockets within the macromolecules could play a crucial role in many applications. Specifically, in the breakdown of harmful organophosphates that contain chiral enantiomers by enzymes like phosphotriesterase.

This enzyme could be placed in humans to prevent the inhibition of actylcholinesterase by organophosphates, because the PTE would breakdown both enantiomers at rates approaching its v_{max}. This means that humans who have ingested deadly pesticides or chemical weapons in the form of organophosphates can have them removed safely and quickly.

References:

(1) McConathy, J.; Owens, M. J. *J. Clin. Psychiat.* **2003**, *5*, 70-73.
(2) Garrett, R. H.; Grisham, C. M. *Biochemistry*; 4th ed.; Brooks Cole, 2008.
(3) Kingsbury, S. A.; Ducommun, C. J.; Zahakaylo, B. M.; Dickinson, E. H.; Morris, K. F. *Magn. Reson. Chem* **2010**.
(4) Hamerly, T.; Morris, K. Measuring the Correlation Times of Molecules Bound to Chiral Molecular Micelles **2010**.
(5) Eliel, E. L.; Wilen, S. H. *Stereochemistry of Organic Compounds*; 1st ed.; Wiley-Interscience, 1994.
(6) Atkins, P.; Paula, J. D. *Physical Chemistry*; 8th ed.; W. H. Freeman: Oxford, 2006.
(7) Decatur, J. D.; Farrar, T. C. *The Journal of Physical Chemistry* **1990**, *94*, 7391-7394.
(8) Stringfellow, T. C.; Farrar, T. C. *The Journal of Physical Chemistry* **1995**, *99*, 3889-3891.
(9) Carper, W. R.; Keller, C. E. *The Journal of Physical Chemistry A* **1997**, *101*, 3246-3250.
(10) McCarroll, M. E.; Billiot, F. H.; Warner, I. M. *Journal of the American Chemical Society* **2001**, *123*, 3173-3174.
(11) Kimaru, I. W.; Xu, Y.; McCarroll, M. E. *Analytical Chemistry* **2006**, *78*, 8485-8490.
(12) Geng, L.; McGown, L. B. *Analytical Chemistry* **1994**, *66*, 3243-3246.
(13) Munch-Petersen, B.; Cloos, L.; Tyrsted, G.; Eriksson, S. *J. Biol. Chem.* **1991**, *266*, 9032-9038.
(14) Wang, J.; Choudhury, D.; Chattopadhyaya, J.; Eriksson, S. *Biochemistry* **1999**, *38*, 16993-16999.
(15) Alexandre, J. A.; Roy, B.; Topalis, D.; Pochet, S.; Périgaud, C.; Deville-Bonne, D. *Nucleic Acids Research* **2007**, *35*, 4895 -4904.
(16) Bryant, M. L.; Bridges, E. G.; Placidi, L.; Faraj, A.; Loi, A.; Pierra, C.; Dukhan, D.; Gosselin, G.; Imbach, J.; Hernandez, B.; Juodawlkis, A.; Tennant, B.; Korba, B.; Cote, P.; Marion, P.; Cretton-Scott, E.; Schinazi, R. F.; Sommadossi, J. *Antimicrob. Agents Chemother.* **2001**, *45*, 229-235.
(17) Jurovčík, M.; Holý, A. *Nucleic Acids Research* **1976**, *3*, 2143 -2154.
(18) Chen-Goodspeed, M.; Sogorb, M. A.; Wu, F.; Hong, S.; Raushel, F. M. *Biochemistry* **2001**, *40*, 1325-1331.
(19) Epstein, T. M.; Samanta, U.; Kirby, S. D.; Cerasoli, D. M.; Bahnson, B. J. *Biochemistry* **2009**, *48*, 3425-3435.
(20) O'Brien, R.; Hetnarski, B.; Tripathi, R.; Hart, G. In *Mechanism of Pesticide Action*; American Chemical Society, 1974; Vol. 2.

(21) Jennings, L. L.; Malecki, M.; Komives, E. A.; Taylor, P. *Biochemistry* **2003**, *42*, 11083-11091.
(22) Aubert, S. D.; Li, Y.; Raushel, F. M. *Biochemistry* **2004**, *43*, 5707-5715.
(23) Siegel, G. J.; Albers, R. W. In *Basic neurochemistry: molecular, cellular, and medical aspects*; Raven Press: New York, 1994; pp. 231-260.
(24) Benning, M. M.; Shim, H.; Raushel, F. M.; Holden, H. M. *Biochemistry* **2001**, *40*, 2712-2722.
(25) Vyas, N. K.; Nickitenko, A.; Rastogi, V. K.; Shah, S. S.; Quiocho, F. A. *Biochemistry* **2010**, *49*, 547-559.