CHARACTERIZATION OF VARIANT FORMS OF ORGANOPHOSPHORUS HYDROLASE

A Senior Honors Thesis

by

CLAIRE E. ROWE

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2003

Group: Life Sciences 1

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James R. Wild
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(Executive Director)

April 2003

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ABSTRACT

Characterization of Variant Forms of
Organophosphorus Hydrolase. (April 2003)

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Department of Biochemistry and Biophysics

Organophosphorus hydrolase (OPH) is an enzyme capable of degrading toxic organophosphorus chemicals including the chemical warfare agents, sarin and VX, as well as many insecticides. This project seeks to understand how structure contributes to the enzyme's activity and stability by identifying regions of the enzyme necessary for each. PCR mutagenesis was used to create 67 variant forms of the gene that encodes OPH. Eight variant genes were then sequenced to locate the mutations in the enzymes' structure. Two of the eight variants contained only silent mutations and expressed wild-type OPH protein. The mutations made in two of the variants prevented the protein from being expressed. Characterization of the enzymes relative to two substrates, paraoxon and demeton-S, indicated that six variants had severely decreased activity. Five of these variants contained mutations in regions that have previously been shown to affect the enzyme's activity. Only one variant, 12, contained a mutation in a novel region of the enzyme.

ACKNOWLEDGMENTS

I would like to thank Dr. James R. Wild, Dr. Melinda E. Wales, Tony Reeves and Dr. Janet K. Grimsley for their guidance on this project and their help in putting together this thesis.

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INTRODUCTION

In March of 1995, the Aum Shinrikyo cult released sarin in the Tokyo subway system killing 11 people and injuring nearly 3,800 others[1]. This attack highlighted the danger of chemical weapons and the need to develop technology to detect and decontaminate them. Sarin is a member of the organophosphorus family of neurotoxins, which irreversibly inhibit acetylcholinesterase[2]. In addition to being used as chemical warfare agents, organophosphorus compounds are also used as agricultural pesticides and herbicides. The use of organophosphorus compounds as pesticides has increased crop yields and decreased the incidence of insect borne disease. However, in 2001 their toxic effects caused 68 cases of life-threatening poisoning in United States [3]. Organophosphorus compounds pose a major threat to human health and safety through both terrorism and accidental abuse.

Given the dangerous nature of organophosphorus compounds, finding ways to detoxify them is of vital importance to public health and safety. The toxicity of organophosphorus compounds is due to the reactive nature of their phosphoryl center. Organophosphorus compounds can be detoxified by breaking the bond between the phosphoryl center and the leaving group through a hydrolysis reaction. Enzymes from a variety of sources have been shown to degrade organophosphorus compounds. One of these enzymes, organophosphorus hydrolase (OPH), has been isolated from plasmids of *Pseudomonas diminuta* and *Flavobacterium sp.*[4]. OPH has been the subject of a great deal of research. Its gene, *opd*, has been

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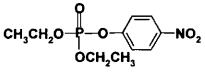
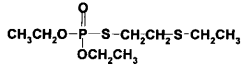
This thesis follows the style and format of Nature

sequenced and plasmids have been constructed to allow its expression in a variety of biological systems including *E. coli*. Using the *E. coli* expression system, more than 20 mg of purified protein can be recovered per liter of culture[5].

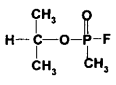
OPH is well suited for a variety of practical applications for a several reasons. The enzyme can be produced in large quantities and is relatively stable. OPH is capable of degrading a wide range of organophosphorus compounds including many pesticides and chemical weapons (Figure 1). OPH is the only organophosphorus degrading enzyme that has been shown to detoxify compounds containing P-S bonds, such as VX, a very potent chemical warfare agent[6].



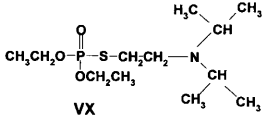
Paraoxon



Demeton-S



Sarin



VX

Figure 1: Substrates of Organophosphorus Hydrolase

There are many practical applications for the OPH enzyme. OPH could be used to detoxify concentrated stockpiles of organophosphorus chemicals. It could also be used as a sensor to detect low levels of organophosphorus compounds.

Medical applications of OPH are also being developed. For example, injections of red blood cells containing OPH could be used to treat people exposed to pesticides or chemical warfare agents[6]. Using modified enzymes, which have increased affinity for important organophosphorus substrates, could improve the usefulness of these technologies.

Although OPH is an extremely stable enzyme with broad substrate specificity it will be necessary to improve the enzyme's characteristics in order to use it in many practical applications. The first step in improving the enzyme is to understand how the natural enzyme functions. To better understand the source of the enzyme's stability, studies of the structure and folding of OPH have been conducted in the laboratory of Dr. J.R. Wild[7]. In these projects, mutations have been made to regions of the enzyme known to be important to catalytic activity or structure. In the project reported here, the function of other regions of the enzyme was probed by making random mutations throughout the enzyme. The goal of the project is to identify new regions that are important to enzyme function. By examining the effect of mutations on the activity of the enzyme, new insight into the enzyme's structure can be obtained.

METHODS

PCR Mutagenesis:

The mutagenesis reactions were preformed using the Diversify PCR Random Mutagenesis Kit (Clonetech). The forward PCR primer, 5' AAC AGC TAT GAC CAT GAT TA 3', anneals at –102 bp in the *opd* pUC19 plasmid. The reverse primer, 5' TTG TAA AAC GAC GGC CAG GT 3', anneals 38 bp downstream from the stop codon. Buffer condition 3 (Table 1), which is designed to produce 3 base pair changes per 1,000 bases, was used.

Component	Volume (μL)
Water	36.5
10 x Taq Buffer	5.0
$MnSO_4$ (8 mM)	2.0
dGTP (2 mM)	1.0
50 x Diversify dNTP mix	1.0
Forward Primer (1×10^{-6} μmol)	1.25
Reverse Primer (1×10^{-6} μmol)	1.25
Template DNA (1 ng)	1.0
Taq	1.0

Table 1: PCR Mutagenesis Reaction Buffer Conditions

Modifications were made to the recommended PCR program in order to obtain an adequate amount of product. The PCR primers designed for the *opd* gene did not have a high enough T_m to work with the PCR protocol recommended by the kit's manufacturer. Instead, the program shown in Figure 2 was used.

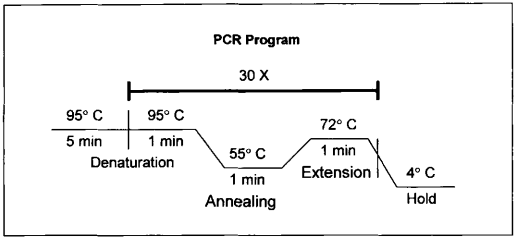


Figure 2: Mutagenesis PCR Program

Cloning and Transformation:

pUC19 plasmid (Invitrogen) was used to clone the variant *opd* genes. Both the vector and the PCR product were digested with *HindIII* and *BamHI*. Following digestion, the vector fragment and PCR product were gel purified. A five to one ratio of insert to vector molecules was used in the ligation reaction. The amount of vector and insert DNA needed to achieve this ratio was calculated using the following equation.

$$\frac{\text{Length of the Insert}}{\text{Length of the Vector}} * \frac{5}{1} = \frac{\text{ng Insert Needed}}{\text{ng Vector Needed}} \qquad (1)$$

The ligation reaction was performed according to the manufacturer's protocol and allowed to proceed overnight at 16° C. Competent DH5α *E. coli* cells were

transformed with 1 μL of the resulting ligation mixture. The cells were plated on LB plates that contained 50 μg/mL ampicillin and grown overnight at 37° C. The resulting colonies were then replica plated on to two LB^{Amp} plates and glycerol stocks were made for long term storage.

The variants were originally named WTRM or Wild Type Random Mutants because they were variants based on the wild type *opd* gene. They were assigned numbers based on their position in the grid on the replica plates.

Sequencing

Plasmid DNA for sequencing reactions was prepared using Quiagen's QIAprep Spin Miniprep Kit. Cells were grown in 5 mL LB cultures containing 50 mg/mL ampicillin overnight at 37° C. The reaction conditions used for the sequencing reactions are given in Table 2. Four primers were used to sequence the entire *opd* gene, AA98, AA194, AA298 and 008 (Table 3). These primers sequence the antisense strand of the *opd* gene.

Component	Quantity
Plasmid DNA	250 – 300 ng
Primer (10 μM)	0.65 μL
Big Dye	2.00 μL
Sterile Water	Enough to bring the total volume to 7.05μL
Total Volume	7.05 μL

Table 2: Sequencing Reaction Conditions

Primer	Sequence
AA98	5' CCA CGA TAT GAA CGT CG
AA194	5' GGT TAC CGG AAC ACC GG
AA298	5' CCA GTC ATT CGA AAC GAG G
008	5' CCA GTC ACG ACG TTG TAA AAC G

Table 3: OPH Sequencing Primers

Colony Screening and Its Limitations

The proposed colony screening procedure was designed to take advantage of the fact that one of the products of enzymatic degradation of paraoxon, p-nitrophenol, has a yellow color. This allows for simple visual monitoring of the reaction by monitoring the appearance of p-nitrophenol's yellow color. The screening procedure was designed to identify variant forms of OPH with increased stability. Following transfer to a nitrocellulose membrane the enzyme could be subjected to a denaturant and then tested for activity. In this project, chemical denaturation with urea and guanidinium-HCL was tested, however, thermal denaturation is also a possibility.

The first step in the screening process is to attach OPH to a nitrocellulose membrane. Freshly grown *E. coli* colonies expressing OPH were transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences). The colonies were then lysed by placing them face up on filter paper saturated with 0.02 M NaOH for 30 minutes. The membrane was washed by placing it face up on filter paper saturated with 10mM Tris at pH 8.2 for 1 hour. The proteins were then denatured by placing them on filter paper soaked in the urea or guanidinium-HCL solution. After the membrane was treated with denaturant, the activity of enzyme was tested by monitoring paraoxonase activity. The membranes were placed on 1% agarose

plates containing 1 mM paraoxon. Yellow color developed under colonies that contained active enzyme.

Determining the correct concentration of denaturant is an essential step in the screening procedure. The urea solution should be just concentrated enough to denature the wild-type enzyme. Ideally the concentration would be determined to one tenth of a molar. Efforts to determine the concentration and the incubation time needed to exactly denature wild type OPH were unsuccessful. A potential explanation for this is that binding protein to a membrane stabilizes them. If the analysis had been performed in solution, the assay may have been more discriminatory. A titration of cell free extract into a dilution series of urea or guanidinium-HCL may have been more successful at identifying the critical denaturant concentration.

Cell Free Extract Preparation

The cell free extract used for activity assays was prepared in the following way. The cells were grown under the same conditions typically used for OPH expression. Terrific Broth (TB) media with 0.4 % glycerol, 1 mM $CoCl_2$ and 50 μg/mL ampicillin was used. Tubes containing 5 mL of TB were inoculated with single colonies and grown for 40 hours at 30° C. After 12-15 hours growth, an additional 5 μL of ampicillin (50 mg/mL stock) was added. The cells were harvested by centrifugation in 15 mL corex tubes at 5,000 rpm for 15 minutes. The media was discarded and the cell pellet re-suspended in 500 μL of 10 mM KPO_4 buffer, pH 6.5-6.9, 50 μM $CoCl_2$. This mixture was then placed in disposable 3 mL transfer pippet

and sonicated for 3 x 30 sec. The cellular debris was removed by centrifuging the cell lysate for 15 min at maximum speed in a chilled micro-centrifuge.

Western Blotting

The samples used in the western blot analysis were cell free extracts prepared according to the previous instructions. The samples were mixed will SDS dye and then denatured by the boiling for 2 minutes. The samples were then loaded on a gel with a prestained molecular weight marker and purified OPH protein as a positive control. After running the SDS-PAGE gel, the protein was transferred to a nitrocellulose membrane. Four liters of transfer buffer was prepared by dissolving 11.6 g glycine, 23.3 g Tris Base, 1.48 g SDS and 800 mL methanol in distilled deionized water. The protein was transferred to the membrane overnight using 46 V at room temperature.

After transferring the protein, the membrane was blocked with 1% gelatin. For this step, TBS buffer was prepared by dissolving 2.42 g Tris Base and 29.24 g NaCl in 1 liter distilled deionized water. After incubating the membrane in the 1% gelatin/TBS blocking solution for 2 hours at 37°, the membrane was labeled using rabbit primary antibody (Ab#7000), The membrane was incubated in a solution containing 100 μL of Ab#7000 and 50 mL of 1% gelatin/TBS buffer for 2 hours at 37° C. Following this step, the membrane was rinsed three times with the gelatin/TBS buffer and labeled with the secondary antibody. Goat antirabbit antibody conjugated with horseradish peroxidase (HRP) was used as the secondary antibody. The membrane was incubated for 1.5 hours at 37° C in a solution

containing 50 μL of the secondary antibody and 50 mL of 1% gelatin/TBS buffer. The membrane was then washed three times with gelatin/TBS buffer.

Two solutions were prepared to visualize the protein using the HRP activity. The first is a solution containing 60 mg of 4-chloro-1-naphthol dissolved in 20 mL of cold methanol. The second is 60 μL hydrogen peroxide in 100 mL of TBS buffer.

The membrane was placed in a glass dish with the two visualization buffers. The dish was tipped to mix the solution until purple bands appeared. The bands were allowed to develop until they could be adequately visualized then the membrane was removed, washed with distilled deionized water and allowed to dry. The dry blot was stored in a dark place.

Measuring Paraoxon Activity

Enzyme activity was monitored by visual detection of yellow color as paraoxon was hydrolyzed to diethylphosphate and p-nitropheol. One hundred μL of 1 mM PX in 20 mM CHES buffer at pH 9 were added to each well of a 96 well plate. Ten micro-liters of cell free extract were added to each well, and the time required for yellow color to appear was recorded.

Measuring Demeton-S Activity

The activity of the variant OPH enzymes against demeton-S, an analog of the chemical warfare agent VX, was measured using a modified Ellman assay. The formation of free thiol as a result of the hydrolysis of demeton-S can be monitored using 2-dithiopyridone (2-dTP). The free thiol reacts with 2-dTP to form a product

whose absorption at 343 nm is measured with the spectrophotometer. First 2 x tripart buffer was prepared by mixing 7.808 g MES, 2.58 mL N-ethymorpholine, and 2.15 mL of diethanolamine in 200 mL of distilled deionized water and adjusting the pH to 8 using KOH. A fresh 8 mM demeton-S stock solution was then prepared by first mixing 2.5 ml of 2 x tripart buffer with 6 mL of water and adjusting the pH to 8 with KOH. Then 100 μL methanol, 0.0022 g of 2-DTP and 20.6 μL CHEM SERV demeton-S stock were mixed and added to the solution. The final volume was adjusted to 10 mL using distilled deionized water. The activity of the variants was measured using a final concentration of 2 mM demeton-S. The cuvette contained 100 μL of cell free extract, 640 μL of 0.5 X tripart buffer 1% methanol at pH 8 and 250 μL of the 8 mM demeton-S stock. The absorbance was measured at 343 nm for 5 min, and the slope of the resulting curve was reported.

RESULTS

Generation of Variants

The first phase of the project was the generation of random mutations in the *opd* gene using error prone PCR. A total of 67 *E. coli* colonies expressing putative variant forms of OPH were isolated. Eight of the variants have been sequenced to date (Table 4). Two of the variants, 3 and 30, contained only silent mutations that did not affect the amino acid sequence of the protein. These two variants express wild type protein and were used as positive controls in the activity assays. Based on the mutations found in two of the variants, 13 and 29, no protein expression is expected. The start codon of variant 13 was mutated to a lysine codon, indicating that protein should not be expressed. Variant 29 contains a missense mutation that converted tryptophan 69 to a stop codon. This variant should express a severely shortened nonfunctional form of OPH.

Variant	Position of Mutations
Variant 3	One Silent Mutation: A to T change in Thr 279
Variant 5	Three Mutations: Ala 68 to Val, Asp 233 to Glu, and Leu 283 to Ser
Variant 12	Two Mutations: Thr 33 to Ser, and Trp 277 to Arg
Variant 13	Four Mutations: Met 29 to Lys, Silent A to G Mutation in Gly 107, Thr 147 to Ala, and Ile 313 to Val
Variant 15	One Mutation: His 254 to Leu
Variant 27	Three Mutations: Thr 52 to Ala, Lys 82 to Met, Phe 104 to Leu
Variant 29	Two Mutations: Trp 69 to stop codon, and Tyr 248 Asn
Variant 30	One Silent Mutation: A to C in Arg 139

Table 4: Sequence Data

The reaction conditions used for the mutagenesis were designed to produce an average of 3 mutations per 1,000 bp. Since the *opd* gene is 1,014 bp long, these conditions should produce an average of slightly more that 3 mutations per gene copy. Based on the 8 variants sequenced, the average number of changes per gene was 2.1. Although the average number of mutations per gene is less than the expected 3, this is most likely not an indication that the mutagenesis reaction is not working as designed. The sample size of 8 variants is to small to be a good indicator of the total pool of variants, and the 8 variants sequenced were not selected randomly.

Characterization of Variant Activity

To determine the effect of the mutations on the activity of the enzyme, qualitative measurements of paraoxonase activity were performed. These measurements are summarized in Table 5. As expected variants 3 and 30, which contained only silent mutations, retained maximum activity against paraoxon. Surprisingly, variant 13, also showed paraoxonase activity in spite of the mutation in the start codon. This may be due to the use of the AGG codon as a start codon or alternatively it might be due to sample contamination.

Variant	Time To Color Development
Variant 3	< 1 minute
Variant 5	> 12 hours
Variant 12	> 5 minutes
Variant 13	1-5 minutes
Variant 15	> 12 hours
Variant 27	> 12 hours
Variant 29	> 12 hours
Variant 30	< 1 minute

Table 5: Paraoxon Activity

The activity of the variants against demeton-S, a VX analog, was also determined. The results of this assay are given in Table 6. Although the same amount of cell free extract was used in all of the reactions the amount of enzyme in each reaction was not standardized. Differences in growth rate and the level of OPH expression may have led to large variations in the concentration in OPH in the cell free extract. These factors help to explain the difference in activity seen between variant 3 and 30, which are expressing the same wild type enzyme. The measured value for variant 29 is most likely due to experimental error, since further work with variant 29 showed that protein was not being expressed (data not shown).

Variant	Demeton-S Activity (OD/min)
Variant 3	50×10^{-4}
Variant 5	2×10^{-4}
Variant 12	26×10^{-4}
Variant 13	2×10^{-4}
Variant 15	3×10^{-4}
Variant 27	3×10^{-4}
Variant 29	18×10^{-4}
Variant 30	13×10^{-4}

Table 6: Demeton-S Activity

DISCUSSION AND CONCLUSIONS

The first step in understanding how mutations affect variant enzyme activities is to review what is currently known about OPH structure. The three important regions, the dimer interface, the metal binding site, and the active site, have been identified. OPH is a dimeric enzyme, and the residues of the dimer interface have been identified[8] (Figure 3). Changing these residues might be expected to the affect the stability of the enzyme. The residues involved in metal binding have also been identified; these residues are necessary to correctly position the metal ions in the active site[9]. Mutations to this region will affect the activity of the enzyme. The active site has also been characterized in some detail[6]. The residues of the active site interact directly with the substrate, and mutations of this region will most likely affect the activity of the enzyme and influence the enzyme's specificity.

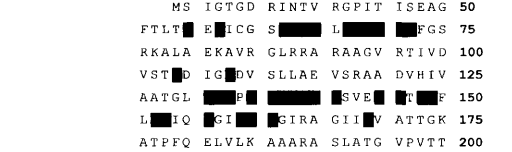


Figure 3: Important OPH Residues

The effect of the mutations found in variants 13 and 29 are obvious. These variants contain mutations that prevent the protein from being expressed. Although these variants also contain other mutations, since no protein is available the effects of these mutation can not be determined. No new information can be determined from variants 3 or 30, since these variants contain silent mutations and so express wild type enzyme.

Variant 5 contains three mutations: one in the dimer interface and two in regions whose functions have not been determined. The first mutation, an alanine to valine mutation at residue 68, occurs at a critical residue in the dimer interface. This reside fits tightly into a pocket in the other subunit (Figure 4). The amount of buried surface area for this alanine residue increases from 25% in the monomer form to 98% in the dimer[8]. The increased size of the valine residue compared to the alanine may have destabilized the enzyme's structure leading to the low activity seen for this variant. The second mutation, an aspartic acid to glutamic acid mutation at 233, is on the surface of the enzyme and involves very similar amino acids. For these reasons, this mutation would not be expected to significantly affect the enzyme's activity. The third mutation, a leucine to serine substitution at 283, also occurs at the enzyme's surface and therefore is also not likely to affect the enzyme's activity.

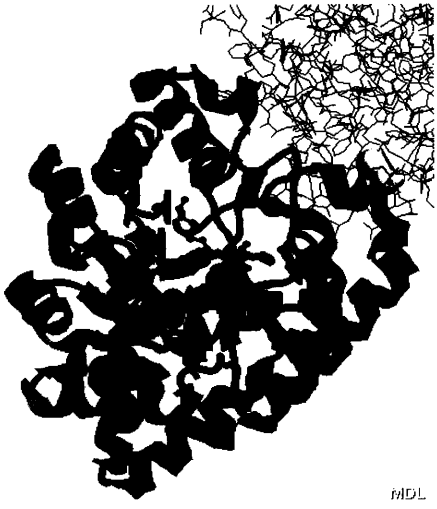


Figure 4: Variant 5 The position of the Ala 68 to Val mutation is shown in light blue. The positions of the Asp 233 to Glu, and Leu 283 to Ser mutations are shown in dark red. The substrate is shown in black and the two zinc ions in orange. (Created using Protein Explorer, http://proteinexplorer.org)

Variant 12 contains two mutations that decreased the enzyme's activity. The first, a threonine to serine mutation, occurred at amino acid 33. This residue is part of an unstructured leader region, located only 4 residues form the N-terminus. Mutations in this region probably do not effect the protein's activity. The second mutation is a tryptophan to arginine mutation at position 277. Although this mutation

is on the surface of the protein it may have some affect on the protein's stability. The mutation changed position 277 from a polar tyrosine to a positively charged arginine. This positions two positively charged residues very close together and may destabilize the protein (Figure 5).

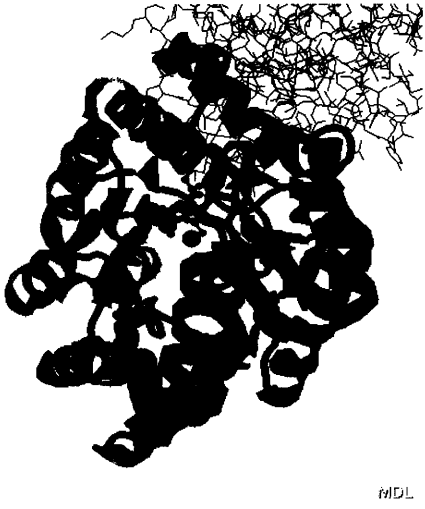


Figure 5: Variant 12 Trp 277 is shown in dark red and the neighboring Arg 331 is shown in blue. Thr 33 is not shown. The substrate is shown in black and the two zinc ions in orange. (Created using Protein Explorer, http://proteinexplorer.org)

Only one mutation is present in variant 15, but this mutation severely disrupted enzyme activity. The sole mutation is a histidine to leucine mutation at position 254 (Figure 6). The histidine residue at position 254 is important in

providing stability to the active site. There are three histidine residues in the active site, His 230, His 254 and His 257, which are closely spaced and stabilized by Van der Waals interactions[6]. Substituting a leucine at position 254 may disrupt this interaction, then destroying the enzyme's activity.

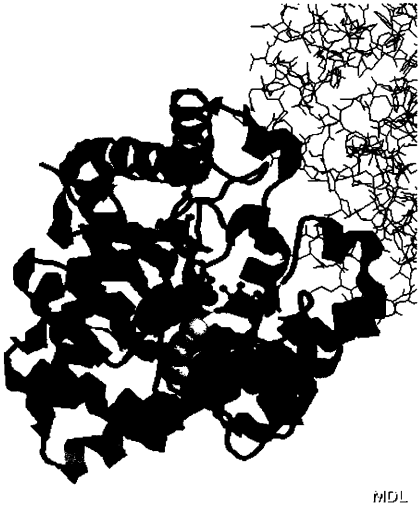


Figure 6: Variant 15 The position of the His 254 to Leu mutation is shown in light pink. His 230 is shown in green and His 257 is shown in magenta. The substrate is shown in black and the two zinc ions in orange.
(Created using Protein Explorer, http://proteinexplorer.org)

Variant 27 contains three mutations: one in the dimer interface, phenylalanine 104 to leucine, and two in regions of unknown function, threonine 52 to alanine and lysine 82 to methionine (Figure 7). These mutations obliterate the enzyme's activity.

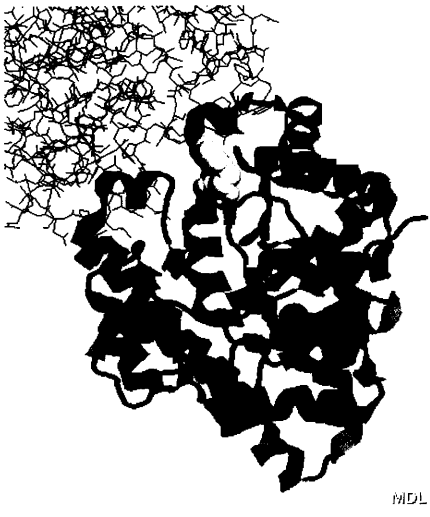


Figure 7: Variant 27 The position of the Phe 104 to Leu mutation is shown in light blue. The Thr 52 to Ala, and Lys 82 to Met mutations are shown in dark red. (Created using Protein Explorer, http://proteinexplorer.org)

The aim of this project was to identify new regions of the OPH structure that are important to enzyme function. A pool of 67 variant enzymes was created using error prone PCR. Eight of these variants were sequenced, and their activities were examined. From these eight, four of the variants were further analyzed. Of these four, three contained mutations in one of the three previously identified regions, reaffirming the importance of these regions. Variant 12 contained two mutations and may have identified a new region important for enzyme activity.

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VITA

Claire E. Rowe was born in Fort Worth, Texas on September 12, 1980 the only child of Farrell and Sally Rowe. Less than a year after her birth the family moved to Austin, TX. Claire graduated from Austin's Science Academy magnet program at Lyndon Baines Johnson High School in May of 1999. She began her undergraduate career at Texas A&M University in August of 1999. During the spring of 2002, Claire joined Dr. James R. Wild's laboratory where this work was conducted. During the summer of 2000, Claire worked at UT M.D. Anderson Cancer Center's Science Park Research Division in the laboratory of Dr. Rodney Nairn studying the role of the cyclin D genes in *Xiphophorus* skin cancers. During the summer of 2001, Claire worked at UT Southwestern Medical Center in the lab of Dr. Philip Perlman studying DEAD box RNA helicases. She graduated from Texas A&M University with honors in May of 2003 with a double major in Biochemistry and Genetics. Claire was a 2003 Senior Merit Winner and the recipient of President's Endowed Scholarship. In addition to participating in the Research Fellows program, Claire also graduated with University Honors. After graduating, Claire went on to pursue a Ph.D. in Biochemistry at the University of California San Francisco.

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