Title
Elucidation of function of an E. coli gene

Author
Yool, Brooke E.

Publication Date
2000

Peer reviewed|Thesis/dissertation

Elucidation of Function of an *E. coli* Gene

by

Brooke E. Yool

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Pharmaceutical Chemistry

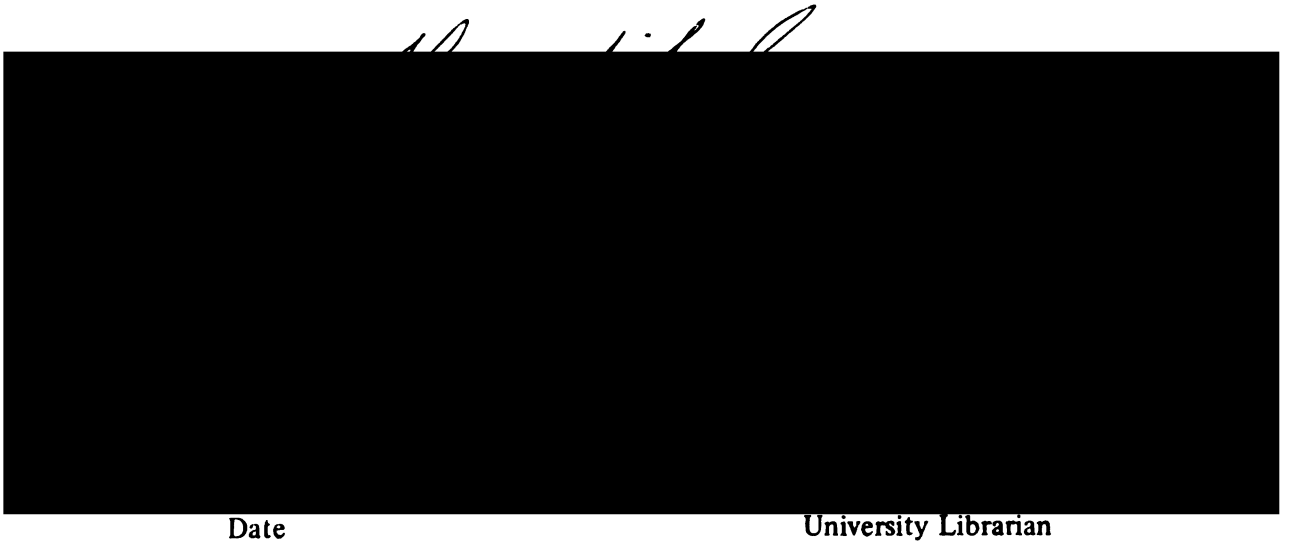
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Date University Librarian

Degree Conferred: . . . . . .

TABLE OF CONTENTS

LIST OF FIGURES/ILLUSTRATIONS

INTRODUCTION

The lineage and evolution of proteins is a topic with which many scientists are fascinated, but mainly on a theoretical level. It is clearly impossible to re-enact millions of evolutionary years in a test tube. Nonetheless, the process by which today's existing proteins obtained their current function is an interesting mystery, and it may let us predict the behavior of certain proteins in environments of the future. Proteins which serve one particular function now may have served a different function in the earth's environment 1000 years ago, and may serve yet another function 1000 years in the future.

One example of apparent evolution to serve differing function is the protein phosphotriesterase (PTE). PTE is a metalloenzyme with a bimetallic zinc center. This protein is encoded on a plasmid in soil bacteria such as *Pseudomonas* and *Flavobacterium*. This protein, as suggested by its name, cleaves phosphotriesters, the bulk of which are synthetic insecticides and nerve gases (fig. 1). At this time, no naturally occurring substrates are known. Interestingly enough, the rate of phosphotriester cleavage is quite fast; the kinetics of cleavage of the best substrate for the enzyme (paraoxon, see fig. 1 for reaction) approach the diffusion-limited rate (k_{cat}/k_m = 5 x 10^7 M^{-1} s^{-1}) (9). This is quite unusual, as these substrates are synthetic and have only been in existence for roughly 50 years. As a comparison, an equally fast enzyme is triose phosphate isomerase, part of the glycolytic pathway, an enzyme which has been around since the dawning of time. Whereas this enzyme has had years to perfect its processes and become more efficient, PTE has had a very short period of time to do so. This, then, is an apparent evolutionary anomaly: how can an enzyme become so catalytically efficient, so quickly? Might it have previously been performing a function in nature very similar to phosphotriester hydrolysis, and just needed a quick evolutionary step, or change, to serve this new purpose?

THE SEARCH FOR RELATIVES

The search for a potential explanation for this phenomenon started with sequence alignment -- do there exist sequence relatives of PTE which could provide insight into this interesting protein? Initial searches yielded a gene sequence coding a 33 kDa protein in *E. coli*, the function of which was unknown. The sequences are 27% identical, namely in the putative active site area of PTE. This was viewed with potential and named PHP, or Phosphotriesterase Homology Protein (11).

Concurrent with the evolution of this project has been the wealth of information about various proteins and their relatives in various databases. This can serve as a very useful tool, particularly when a protein has one or more very close relatives in one of these databases. In such a case, the function of the protein can often be elucidated from the function of its close relatives. In any situation, however, knowledge about proteins related in any degree to the query sequence can generally be helpful, as may be the case in the relationship between PTE and PHP.

STRUCTURE

PTE is native in the alpha-beta barrel configuration, complete with putative active site at the C-terminal end (3). Mutagenesis by Kuo and Raushel have determined putative active site residues in PTE (8). Based upon sequence alignment, 6 out of 7 of these residues are conserved in the PHP sequence. At this time, it is unknown whether the active site pocket of PTE three-dimensionally resembles that of its sequential equivalent in PHP. While the crystal structure of PHP is finished at this time along with

that of PTE, the PHP structure includes two bound zinc molecules, while the PTE structure does not, thus not allowing for accurate comparison or visual overlay. However, one would assume that the active site of PHP is also in the C-terminal end of the barrel, as this is the norm for alpha-beta barrel structured proteins (4).

BACTERIAL GENETICS

Many prokaryotic genes are part of operons, clusters of genes transcribed together which function in the regulation of a variety of metabolic processes. This is often done by means of a common promoter for all the genes within the operon, transcribing all linked genes when the promoter is turned on. In prokaryotes, the methods by which intracellular levels of a protein are regulated are much more primitive than in eukaryotes, which possess the additional ability to process and thus change the mRNA between the transcription and translation steps, and the ability to posttranslationally modify their proteins. Prokaryotes often rely on the operon to regulate levels of a particular protein within the cell. The most frequently used example of an operon is the lac operon, by which decreased cellular concentrations of lactose prevent transcription of the lac genes, thus stopping protein production. When lactose is present, it binds to and blocks the repressor site on the DNA, causing transcription of the lac genes. By these means, the cell can control its own levels of lactose (10).

These types of systems are dependent on a compound or item which will alter the transcriptional mechanism by binding to the promoter region and either increasing or suppressing the transcriptional level of the protein itself. There is also a specific site on the DNA to which the regulatory protein will specifically bind, in its presence. Discovering the sequence and location of particular sites can be quite advantageous for purposes of research.

In some cases, this regulatory protein is the protein being produced or a byproduct. In other cases, it is a compound present in the intracellular environment, such as the induction of the SOS response during cellular DNA damage by LexA and RecA. Any range of conditions, then, can affect the level of the production of a protein, whether it be a chemical, a protein, excess heat, or cold. In some cases, knowledge of an inducer of a particular protein can shed some light onto the function of the protein itself.

PHP is part of a cluster of 5 genes located close to each other on the *E. coli* genome. Thus, there is the possibility that these genes are indeed part of an operon. As PHP is related to PTE, PHP may be part of an operon which metabolizes a phosphate-based compound in *E. coli* (11).

MATERIALS AND METHODS

BLAST SEARCH

Basic Local Alignment Search Tool (BLAST) is a fairly recent development which assists in finding potential sequence relatives to a query sequence (1). The PHP sequence was subjected to BLAST.

ACTIVITY OF CRUDE LYSATE

Previously, PHP purified via determined procedures (5) was determined not to show any PTE-like activity. This was tested via incubation of purified PHP with the compound paraoxon. Cleavage of paraoxon produces p-nitrophenol, an aromatic compound which absorbs light at a wavelength of 400 nm, and thus rate and extent of cleavage can be easily measured via UV spectroscopy.

Since no esterase activity could be observed using a purified fraction of PHP, the same experiment was performed using the unpurified crude lysate of cells which had been induced to express PHP. A control batch of cells was also grown to which IPTG had not been added and thus PHP not induced. Tests were performed to assess crude lysate cleavage of various types of ester bonds. Compounds used in this experiment included p-nitrophenyl butyrate, L-alanine p-nitroanilide, p-nitrophenyl acetate, and p-nitrophenyl sulfate.

The lysate was obtained as follows: DH5α cells containing pJWP1 (clone of PHP gene in pTacTac vector, created by Jed Pitera) were grown overnight in 3.5 ml of LB

containing 50 υg/ml ampicillin. Cells were grown at 37° with shaking. The grown culture was split between two flasks, each containing 500 ml LB inoculated with ampicillin at 50 υg/ml. As PHP is naturally a zinc-containing protein, $ZnSO_4$ was added to both flasks to a final concentration of 10 υM. The "induced" flask also contained 2 mM IPTG. Cells were harvested as per the standard procedure for PHP: Cells were first spun at 4000g for 15 minutes at a temperature of 4°. Each pellet was resuspended in 20 ml of 20 mM Bis-Tris-Cl, pH 6.6. Each tube was split into 2 – 10 ml aliquots, and each tube was sonicated 5 times for 1 minute each. Cell debris was spun down at 18,000g for 30 minutes, and supernatant was stored in two separate containers at 4°.

For each experiment, 500 υl of 1 mM substrate (5 compounds listed above) was placed inside a UV cuvette. Immediately prior to measurement in a UV spectrometer, 100 υl of crude lysate was added to the cuvette. The cuvette was quickly mixed by inversion, and the absorption at 400 nm was measured for one minute. Ten total experiments were performed: the first five with uninduced crude lysate, and the second group of five with induced crude lysate.

ISOELECTRIC POINT SHIFT EXPERIMENTS

One potential cause for the overall negative charge of the protein may be the presence of one or more phosphate groups. To assay for the presence of linked phosphate groups on PHP, pure PHP was treated with calf intestinal phosphatase for 1 hour. A sample of CIP-treated PHP was run on a one-dimensional isoelectric focusing gel (Novex) alongside a sample of untreated pure PHP from the same purification batch as a control.

LEVELS OF PHP PRODUCTION

To test the levels of PHP being produced under normal cellular conditions, *E. coli* MG1655 were grown to log phase. This batch of cells was first pelleted by centrifugation, lysed by resuspending cell pellets in 20mM Bis-Tris-Cl and adding 7.5 ml lysozyme, and the flasks were then incubated on ice for one hour. Cellular debris was then pelleted, leaving the intracellular fluid as the supernatant. The intracellular fluid was run on SDS-PAGE to identify whether the protein was present in the intracellular fluid.

The *php* construct was also transformed into a *phoR*- strain (CAG13661, from the Gross lab) to determine if the expression of *php* is under the regulation of the phosphate metabolic regulon of *E. coli*. First, a 10 ml overnight culture of CAG13661 containing the pJWP1 construct was grown in LB at 37° with shaking. This was then used to inoculate 50 ml of LB, which was incubated at 37°. 1.5 ml of culture was taken out at the following timepoints after inoculation (inoculation at t=0): t=0 (A600=0.3055), t=30m (A600=0.4594), t=1h (A600=0.3837), t=1h 30m (A600=0.4460), t=2h (A600=0.4482), t=2h 30m (A600=0.4613). The samples were run on 15% SDS-PAGE using standard technique. Protein was then transfered from the gel onto nitrocellulose using standard Western blot technique, and PHP was detected using rabbit anti-PHP as the primary antibody and goat anti-rabbit alkaline phosphatase conjugate as the secondary antibody. The same experiment was repeated using a 5 ml overnight culture to inoculate the 50 ml of LB, and again, the initial experiment was repeated using a different *phoR*- strain provided by the Gross lab (name not recorded).

POTENTIAL INDUCERS OF PHP PRODUCTION

1. Low-nutrient Media

Low-phosphate and minimal media were prepared as follows: sterile MOPS media was first prepared (1.05 ml 10mM MOPS buffer, 9.3 ml sterile H_2O, 93 υl 20% glucose, 4 υl 1 υg/υl thiamine). This mixture was then divided into two tubes each containing 5 ml of media. To make low phosphate media, 6 υl of 100 mM $NaHPO_4$ was added to Tube 1 for a final phosphate concentration of 120 υM. To make minimal media, 70 υl of 100 mM $NaHPO_4$ was added to Tube 2 for a final phosphate concentration of 1400 υM.

Eight colonies grown on LB plates were transferred into 8 separate tubes each containing 3.5 ml minimal media. Care was taken not to pick up additional nutrients from the LB media on the plate when suspending the culture. The eight tubes were subject to eight different conditions: media only, heat shock, cold shock, 1 mM p-nitrophenyl butyrate, 1 mM L-alanine-p-nitroanilide, 1 mM p-nitrophenyl acetate, 1 mM p-nitrophenyl sulfate, and 1 mM paraoxon. These compounds were selected in order to sample a range of different types of esters for potential cleavage by the protein. For the latter five cases, the respective compound was added to the media at the same time as the colony, and the tubes (along with the media only tube) were allowed to grow at 37° with shaking overnight.

The heat shock and cold shock tubes were allowed to grow with shaking at 37° until slightly cloudy (approximate mid-log phase). At that point, the heat shock tube was transferred to a 42° water bath and the cold shock tube was transferred to a 10°

water bath. 200 ul samples were removed from each of these two tubes at t=0, 15m, 30m, 45m, 2h 45m, and 4h 45m. These samples were placed at 4° upon removal.

Samples from the overnight cultures were then run on 15% SDS-PAGE using standard procedures and transferred to nitrocellulose for Western blotting using standard procedures with the antibody to PHP. Levels of PHP on the membrane were detected using rabbit anti-PHP as the primary antibody, and an alkaline phosphatase conjugate of goat anti-rabbit as the secondary antibody. The same procedure was followed with a separate gel for the heat shock samples, and a separate gel for the cold shock samples.

2. Chemical induction

Ninety-six well plates in which each well contains a different carbohydrate-based compound were obtained from Biolog (Hayward, CA). To first determine whether the presence of PHP would cause metabolism of any of the substances in the wells, cells grown in the presence and absence of IPTG were tested on two separate Biolog plates. Each well was filled with 150 ul of cell colony which had been suspended in 0.85% saline. To create this solution, LB plates were streaked with BL21 cells containing pJWP1 and allowed to grow 36 hours. Plates containing IPTG were swabbed with 20 ul of 100 mM IPTG prior to streaking cells. Two sterile tubes were each filled with 17 ml of 0.85% sterile saline pre-warmed to 37°. To one tube, 1 mM IPTG was added. To each tube (marked for proper induction status), a few colonies were added by gently rolling a sterile swab (premoistened in sterile 0.85% saline) over the colony on the plate, then suspending the colony in the saline using a vertical motion. Care was taken not to carry over any nutrients from the LB media on the plate. This solution was used to fill the wells in the 2 plates, which had been prewarmed to 37° and marked per induction

status. Plates were then incubated at 37°. Readings of the plates were taken at 6 hours after inoculation and after overnight incubation. Wells in which the substance was metabolized turned purple. Thus presumably, the wells that turn purple contain a compound which is able to be metabolized by the bacteria present in the well. This experiment was performed on both general use plates and also on GN plates, specific to gram-negative bacteria.

DNA AS POTENTIAL SUBSTRATE

The three-dimensional structure of PHP revealed a cylindrical groove in the middle of the protein bearing a positive charge. The shape, size, and charge of this groove suggest that DNA could interact in some way with PHP. To test this theory, 1 υl purified fractions determined to contain PHP via SDS-PAGE analysis were incubated with 0.3 υl of a pJWP1 plasmid miniprep. Added to the mix were 1 υl of NEBuffer #2 (New England Biolabs; 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), found to facilitate this reaction (unpublished data) and water to a total volume of 10 υl. Initial experiments suggested potential time dependence of such a reaction, if it indeed exists, so time dependent reactions were set up. Seven reactions were set up, each at a different time interval prior to running the gel (t=2h, 1h 30m, 1h, 45m, 30m, 15m, 0m). The tubes were incubated in a 37° water bath for the respective amount of time prior to removal and run on agarose gel.

To test if this response was limited to PHP DNA, a similar experiment was run using a different plasmid as DNA substrate. This experiment also tests the effect of removing PHP following the 30 minute reaction. Sample 1 included 1 υg pJWP1, 1 υl purified PHP protein, 1 υl 10X buffer (NEBuffer 2), and water to 10 υl. Sample 2 contained the same ingredients as sample 1 and was treated in the same fashion,

except it was phenol extracted and ethanol precipitated following the reaction to remove the PHP prior to running the sample on an agarose gel. The third tube contained regular PHP and buffer along with 0.85 υg HEGO (estrogen receptor plasmid). Three controls were run containing pJWP1 alone, HEGO alone, and purified PHP alone. All reactions were incubated at 37° for 30 minutes.

The temperature dependence of this interaction was then tested. Four tubes were mixed with the following contents: 1 υg DNA, 1 υl purified PHP, 1 υl NEBuffer 2, and 7 υl water to a total of 10 υl. The four tubes were placed, one each, at the following four temperatures: 16°, room temp (left on bench), 37°, and 45°. The tubes were incubated one hour, and the results were run on 1% agarose.

The next experiment was similar to the first, but PHP had first been incubated 5 minutes in boiling water to kill off any nucleases which may produce confounding results.

An additional experiment was performed to assure that activity was due to PHP, involving immunoprecipitation of PHP out of the protein solution being used in the experiment. MagnaBind Goat Anti-Rabbit beads from Pierce were used to remove protein from solution, in addition to using rabbit anti-PHP antibody as the primary antibody to which the MagnaBind beads would bind. Four tubes were created containing both PHP and primary antibody; the first tube contained no antibody, the second contained antibody at a 1:10,000 dilution, then 1:15,000 dilution, and 1:20,000 dilution, adding water to 40 υl total volume. All tubes were incubated 1 hour on ice to insure optimal binding. To each tube was added 200 υl secondary antibody bound to the magnetic beads, and the tubes were incubated on ice with shaking for 30 minutes. The protein was then precipitated out of solution using the magnet provided by Pierce.

The tubes were allowed to rest on the magnet until the solution visibly cleared. The solution from each tube was aspirated and placed in a clean tube. These solutions were also run on 15% SDS-PAGE to assure the removal of PHP protein. This protein solution was then used in an experiment with DNA, similar to the first experiment listed in this section, to test the effects. Four samples were prepared using the supernatants from the immunoprecipitation reactions, and 15 υl of each reaction was placed in the appropriate tube along with 1.6 υg DNA, 2 υl NEBuffer 2, and water to a total volume of 20 υl. Control reactions were set up using PHP which had not been subject to immunoprecipitation, DNA alone, and protein alone at an identical ratio to the other fractions. All were mixed and incubated for 30 minutes at 37°. This entire experiment was also performed after having blocked both primary and secondary antibody with 5% milk for 1 hour with shaking on ice.

RESULTS

BLAST SEARCH

A current BLAST search using PHP as the query sequence resulted in 5 relatives of the protein. Results are shown in Figure 2. Though no significant conclusions can be made from the BLAST search, as not much is known about the found sequences, it is interesting that all the proteins other than PTE seem to be more closely related based on the percent similarity. In fact, the mouse and rat proteins are 90.8% identical (7). The majority of identity among all of the proteins is within the active site residues of PTE, suggesting that all proteins share a similar mechanism.

It is also interesting to note that expression of the PHP relative found in rat (rPHP) increases upon renal damage. Additionally, rPHP appears to bind to resiniferatoxin, which is similar in structure to phosphotriesters but bears a carbon in place of the phosphorus. This suggests similar shape in the binding pockets of rPHP and PTE.

ACTIVITY OF CRUDE LYSATE

Results of this experiment are posted in figures 3a-e. There is no apparent difference between crude lysate containing PHP and not containing PHP in each case.

ISOELECTRIC POINT SHIFT EXPERIMENTS

The isoelectric point did not significantly shift when PHP was treated with phosphatase. It would appear that there are no phosphate groups covalently linked to the PHP molecule. This does not rule out the possibility of another type of association with a phosphate group.

LEVELS OF PHP PRODUCTION

Transformation of pJWP1 into the *phoR*- strain did not seem to induce PHP expression in the first attempt of this experiment. This result was replicable in the other two variants of this experiment, including using the other *phoR*- strain.

POTENTIAL INDUCERS OF PHP PRODUCTION

1. Low Nutrient Media

E. coli DH5α cells containing the pJWP1 plasmid will not grow on phosphate-free media. One can conclude from this that exogenous phosphate sources are necessary for cell growth even in the presence of *php*. If any cells had grown, it would be interesting to see if the levels of PHP production would be any higher than they were when grown on regular LB plates.

2. Chemical Induction

Experiments using the Biolog plate were performed several times. Although one or more compounds had been metabolized in each set of experiments, the results could never be replicated, neither did there seem to be an overall theme in the chemistry of

the substances metabolized. One or more of the compounds on the plate may still be an inducer of PHP expression, which will need further experimental clarification.

DNA AS POTENTIAL SUBSTRATE

Earlier experiments performed by Stephenson suggested that PHP interacts with DNA; incubating the two together caused an upward band shift, in addition to a split into two separate bands, with samples run on 1% agarose (unpublished results). This result was repeated and further explored.

First, the time dependence of the reaction was studied. All cases (t=15m through t=2h) showed the two band migration. However, the larger of the two bands is slightly more populated in the fractions incubated between 15m and 1h.

Next, dependence on the type of DNA was tested by performing the experiment with two different samples using both the PHP clone and a random clone. No difference between the two samples was detected, so this result would appear to be independent of the actual sequence of the DNA. Removing PHP by phenol extraction after the 30 minute incubation also did not seem to affect the band shift.

The temperature dependence of the reaction was tested. Indeed, in all four cases, band shifting did occur. However, in both the 16° and room temperature cases, the smaller of the two bands was shifted farther downward (smaller) than its counterpart in the heated reactions.

Immunoprecipitation of PHP out of the solution caused little change. The band shift in the IP samples was not as dramatic as the standard reaction, but there is still a bit of an upward band shift. The same result occurs after blocking both primary and

secondary antibody with milk. It could be the case that not 100% of PHP is being precipitated out of solution, and the small amount which exists could be performing the bandshift reaction.

Boiling PHP seemed to cause a slight difference in the bandshift; however, it did not return to the baseline value.

The cause of the bandshift is still unknown at this time. It could also be due to contamination of the protein sample. Prior to making any conclusions, this series of experiments would have to be repeated using a new purified PHP pool. This experiment was also attempted using linearized DNA, but the linearization process used still needs to be optimized, as the yield of linearized DNA was quite low.

DISCUSSION AND FUTURE DIRECTIONS

Sequence analysis is often a useful tool to gain insight into protein function. However, sometimes the puzzle is missing key pieces and cannot be solved without these missing links. This seems to be the case for PTE and PHP at this time; unfortunately not enough information is known about the proteins in the aligned family, nor does PHP have any apparent phosphotriesterase function. Nonetheless, we have identified a new family of proteins related by sequence and similar in PTE's active site region.

Previous results in Stephenson's experiments suggested that PHP does not have esterase activity. The same experiment was performed in this case using crude lysate from cells expressing PHP, in the case that the purification process had washed away a cofactor necessary for reactivity. There was no notable difference in activity between lysate containing PHP and the control (which did not contain PHP) when tested against 5 potential ester substrates. This indicates that either none of these are suitable substrates for PHP, or that another cofactor – either exogenous or inducible – is needed to perform this reaction. In the first case, it may not necessarily be the case that the scissile bonds need to be changed; the side groups on the substrate may not be the proper size and shape to fit into the active site region, despite the fact that the compound may chemically be a candidate for hydrolysis by PHP.

It would be interesting to be able to compare the overall structure of each of these proteins. In the case of the enolase family of proteins, the family bears more structural than sequential similarity; and while the proteins don't catalyze the same overall reaction, all enzymes in the enolase family share the same first step in their

overall reaction scheme, which is abstraction of an alpha proton. All of the enolase family enzymes bear active sites which are capable of neutralizing the strong negative charge once the proton is removed (2). Perhaps this is the case with the PHP superfamily – perhaps one step is commonly shared among the enzymes even though the overall reactions, and the substrates modified, are different.

Mutagenesis is a commonly-used "what if" tool which allows molecular biologists to alter their system, seeing what effects certain changes might make. While mutagenizing one amino acid residue may result in confounding results due to overall structural changes, mutagenesis is a commonly used, well-respected tool to determine characteristics of a protein. It may be interesting to mutagenize PHP so that all the putative active site residues are identical to those of PTE, and then test the PHP variant for esterase capabilities. In this case, one protein's function may be able to be "pasted" onto another scaffold, which may lead to some interesting conclusions regarding the role of the overall scaffold in the function of the protein itself.

Recently, Stemmer has developed a method known as gene shuffling, or sexual PCR (6). This method allows one to shuffle the sequence of one or more genes in a random fashion, resulting in many mutants of the original sequence. Gene shuffling of *php* would then result in many variants of *php* with varying degrees of similarity to the initial sequence. These mutants could then be tested for their ability to hydrolyze paraoxon. Those capable of hydrolysis could then be crystallized and the structure determined. Then, the sequence of the paraoxon-hydrolyzing PHP mutant could be compared with that of PTE, to see what might cause the difference in substrate specificity, whether it be structural, specific ligands in the active site, or a combination thereof.

Mutagenesis can also be done "in silico" with the wide range of software products available for analysis. A first step would be to determine the near-precise positioning of the interaction between PTE and paraoxon: where are individual residues of the active site relative to the scissile bond, and any side chains on the phosphotriester substrate? The crystal structure of PTE with diethyl 4-methylbenzylphosphonate may be a good enough resource for this information (12). The program DOCK can then be used to try to fit paraoxon into the putative active site of PHP in a similar position, three-dimensionally, to the fit into PTE. This would give some information on potential areas in the PHP active site which prevent sufficient binding of paraoxon, and thus hydrolysis. At this point, parts of PHP could be mutagenized based on the structural differences, to see if modifying the dimensions of the active site can provide catalytic capability. This could first be attempted using a computer, by creating a three-dimensional model of the mutant and simulating paraoxon's approach to the active site by using a modeling program such as DOCK (13). If this appears successful, the actual mutant could be created and crystallized, to study how paraoxon binds into this mutant. In addition, observing how paraoxon does or does not bind into unmodified PHP may lend some insight into potential candidates for PHP substrates. Observing the differences in size and shape of the putative active sites can give clues as to other phosphotriesters which may be cleaved, perhaps with different size and shape to the side chains.

The universe's genomes are being sequenced at an expanding rate, and new gene sequences are always being added to genomic databases. In addition to the 6 known relatives to PHP, there are likely more that exist which have not yet been sequenced. Regular searching of genomic databases, then, is important, in the chance that a well-characterized relative may show up. Perhaps knowledge gleaned about PHP and PTE as we know them now may shed some light on newly discovered proteins in the future.

REFERENCES

1. Altschul, S.F., et al. (1997) Nucleic Acids Res. 25:3389-3402.

2. Babbitt, P.C., et al. (1996) Biochemistry 35:16489-501.

3. Benning, M.M., et al. (1994) Biochemistry 34:7973-78.

4. Branden, C., and Tooze, J. Introduction to Protein Structure. New York: Garland Publishing, Inc., 1991.

5. Buchbinder, J.L., et al. (1998) Biochemistry 37:5096-5106.

6. Crameri, A., et al. (1998) Nature 391(6664):288-91.

7. Davies, J.A., et al. (1997) FEBS Letters 410:378-82.

8. Kuo, J.M., et al. (1994) Biochemistry 33, 4265-70.

9. Kuo, J.M., et al. (1996) Biochemistry 36:1982-88.

10. Lehninger et al. Biochemistry, 2nd Edition. New York: Worth Publishers, 1993.

11. Scanlan, T.S., and Reid, R.C. (1995) Chemistry and Biology 2(2):71-5.

12. Vanhooke, J.L., et al. (1998) Biochemistry 35:6020-25.

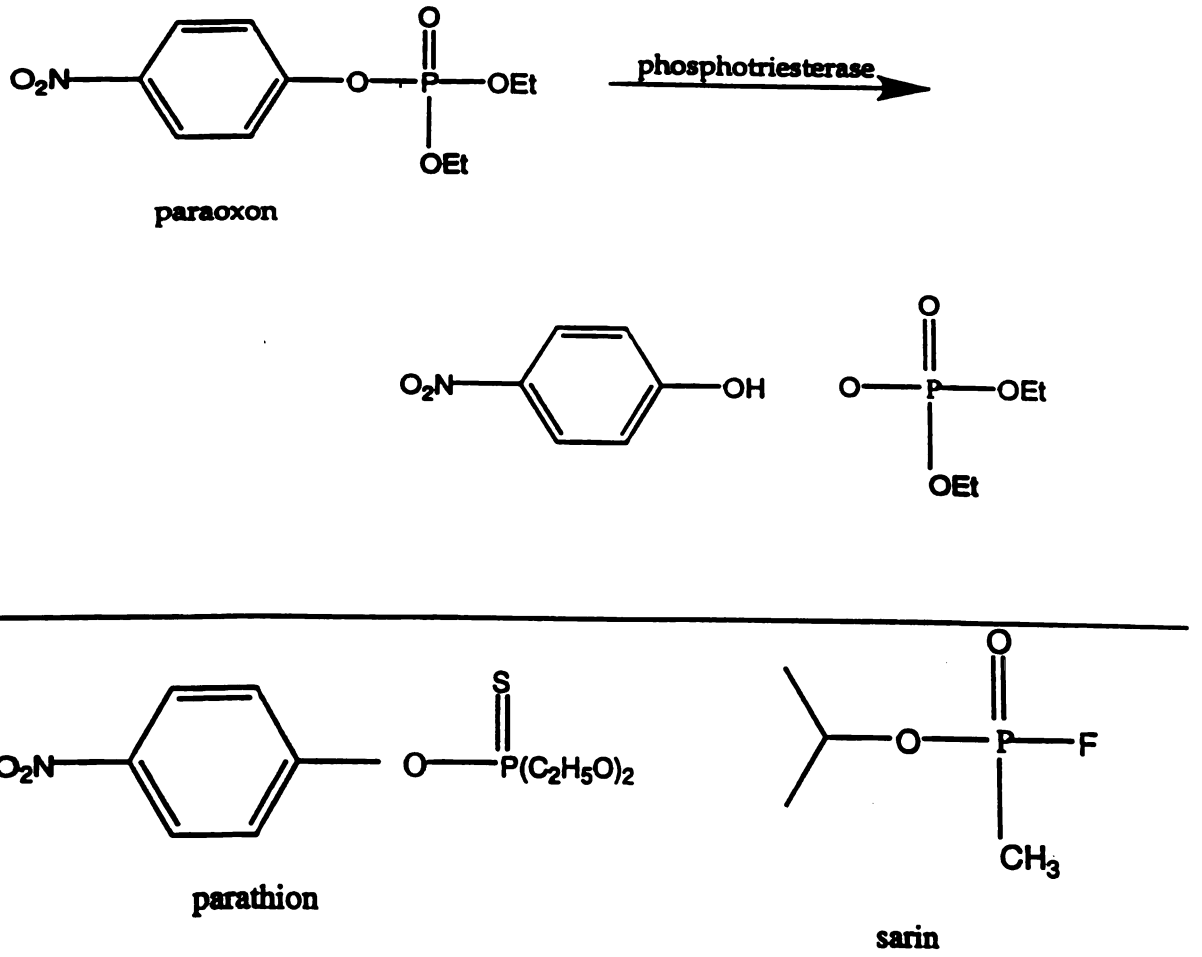


Figure 1. Reaction pathway of phosphotriesterase, and other examples of known substrates.

Organism	Function known?
E. coli (PHP)	No
Pseudomonas, Flavobacterium spp. (PTE)	Yes
Mus musculus	No
Rattus norvegicus	No
Mycobacterium tuberculosis	No
Mycoplasma	No
Deinococcus radiodurans	No

Figure 2. Relatives of PHP found using the program BLAST. All sequences shown bear 25% or greater identity to the query sequence.

Figure 3. Incubation of PHP crude lysate with various ester compounds to check for potential activity. In each figure, 1 = crude lysate from uninduced cells, and 2 = crude lysate from induced cells.

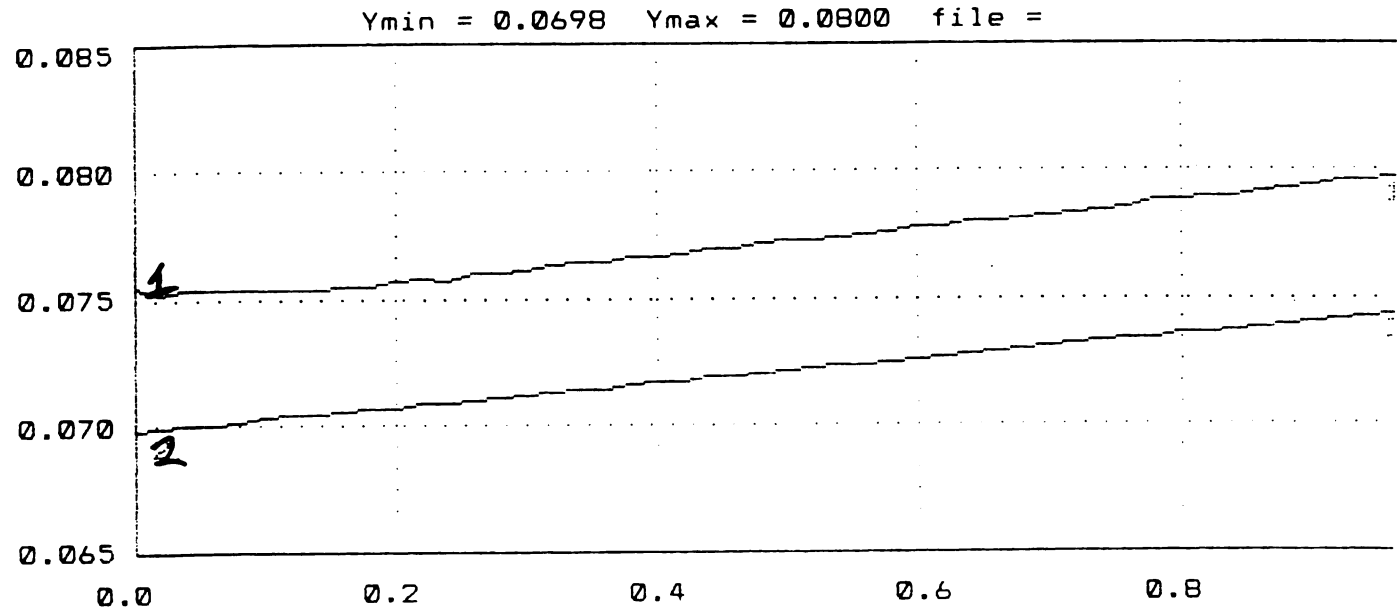


Figure 3a. p-nitrophenyl butyrate.

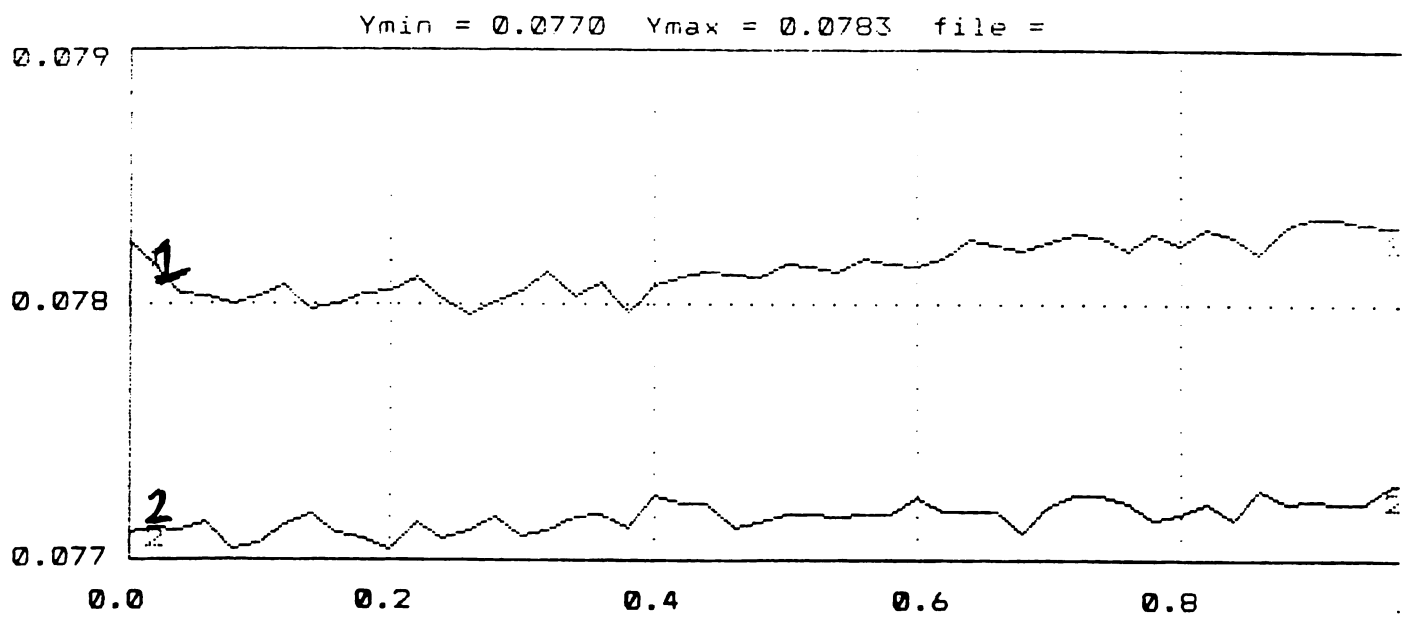


Figure 3b. L-alanine-p-nitroanilide.

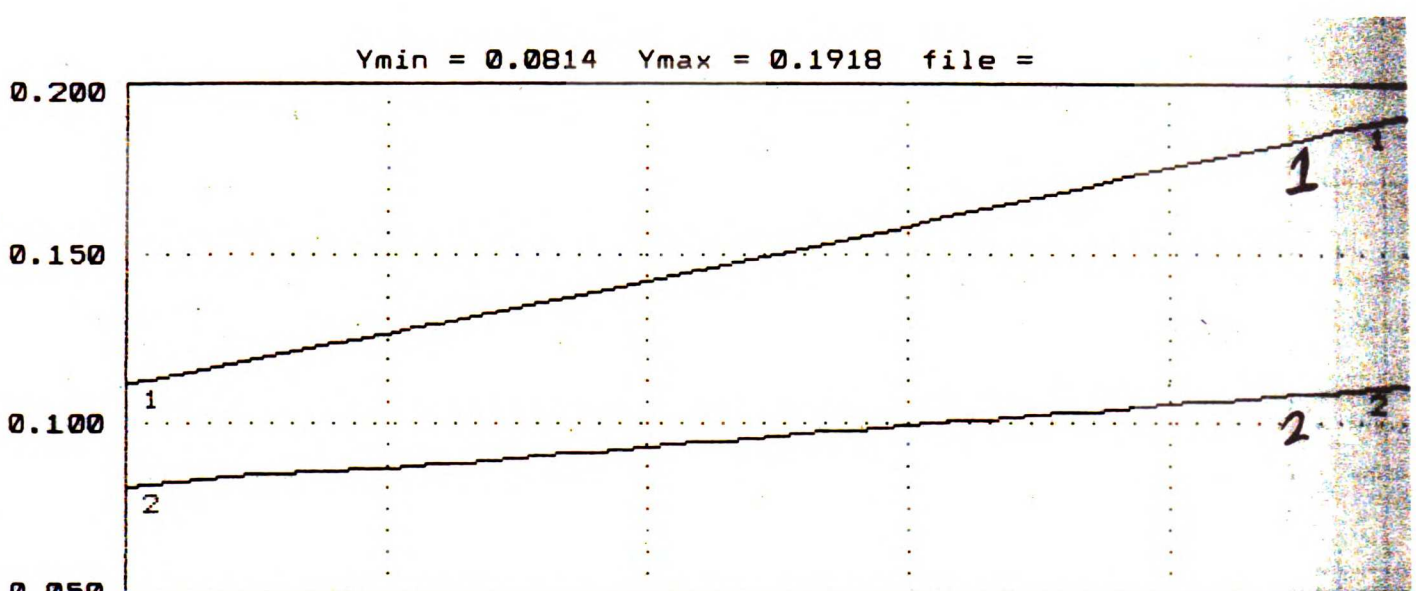


Figure 3c. p-nitrophenyl acetate.

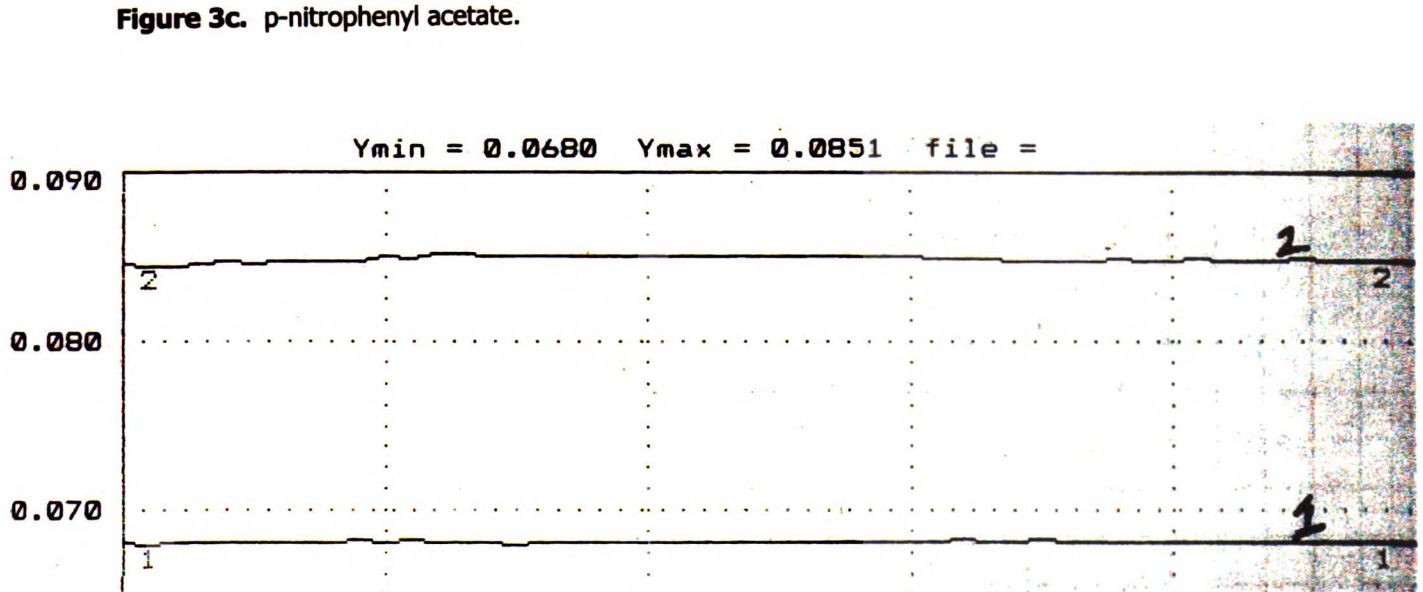


Figure 3d. p-nitrophenyl sulfate.

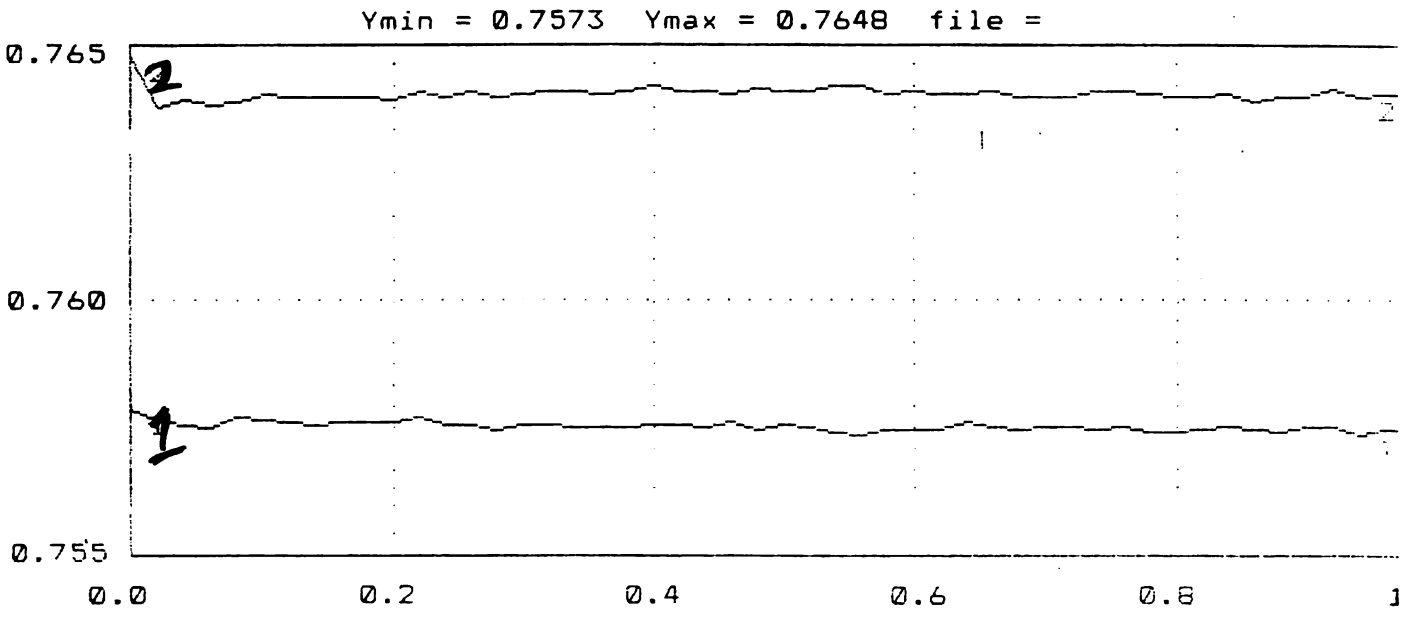


Figure 3e. Paraoxon.