

# A Combinatorial Library for the Binuclear Metal Center of Bacterial Phosphotriesterase

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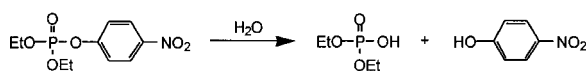
**ABSTRACT** Phosphotriesterase (PTE) is a zinc metalloenzyme that catalyzes the hydrolysis of an extensive array of organophosphate pesticides and mammalian acetylcholinesterase nerve agents. Although the three-dimensional crystal structure of PTE has been solved (M. M. Benning et al., *Biochemistry* 34:7973–7978, 1995), the precise functions of the individual amino acid residues that interact directly with the substrate at the active site are largely unknown. To construct mutants of PTE with altered specificities for particular target substrates, a simple methodology for generating a library of mutants at specific sites was developed. In this investigation, four of the six protein ligands to the binuclear metal site (His-55, His-57, His-201, and His-230) were targeted for further characterization and investigation. Using the polymerase chain reaction (PCR) protocols, a library of modified PTE genes was generated by simultaneously creating random combinations of histidine and cysteine codons at these four positions. The 16 possible DNA sequences were isolated and confirmed by dideoxy-DNA sequencing. The 16 mutant proteins were expressed in *Escherichia coli* and grown with the presence or absence of 1 mM CoCl<sub>2</sub>, ZnSO<sub>4</sub>, or CdSO<sub>4</sub> in the growth medium. When grown in the presence of CoCl<sub>2</sub>, the H57C protein cell lysate showed greater activity for the hydrolysis of paraoxon than the wild type PTE cell lysate. H201C and H230C exhibited up to 15% of the wild-type activity, while H55C, a green protein, was inactive under all assay conditions. All other mutants had <math>10^{-5}</math> of wild-type activity. None of the purified mutants that exhibited catalytic activity had a significantly altered  $K_m$  for paraoxon. *Proteins* 29:553–561, 1997. © 1997 Wiley-Liss, Inc.

**Key words:** metal ligands; mutagenesis

## INTRODUCTION

The phosphotriesterase (PTE) originally isolated from *Pseudomonas diminuta* catalyzes the hydrolysis of a variety of phosphotriesters and correspond-

ing phosphonates, including the insecticide paraoxon as shown in the reaction presented below.



The rate of hydrolysis of the best substrate, paraoxon, approaches the diffusion-controlled limited ( $k_{\text{cat}}/K_m = 5 \times 10^7 \text{ s}^{-1} \text{ M}^{-1}$ ).<sup>1</sup> The enzyme has a broad substrate specificity and will hydrolyze, at variable rates, the insecticides paraoxon, parathion, and coumaphos, as well as the more toxic acetylcholinesterase nerve agents, soman and sarin.<sup>2,3</sup> The broad specificity and high catalytic activity makes phosphotriesterase a potentially useful tool for the hydrolytic detoxification of insecticide-contaminated soils and the destruction of chemical warfare agents. However, to be more effective in these capacities, the specificity for the hydrolytic turnover of individual substrates must be enhanced.

The hydrolytic reaction catalyzed by phosphotriesterase is known to occur with an inversion of configuration at the phosphoryl center.<sup>4</sup> This suggests that the reaction occurs in a single step initiated by attack of the substrate with an activated water molecule. The active site of PTE consists of a binuclear metal center that is catalytically active when two equivalents of Zn<sup>2+</sup>, Cd<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, or Mn<sup>2+</sup> are bound to the protein.<sup>5</sup> The crystal structure of the phosphotriesterase enzyme, reconstituted with Cd<sup>2+</sup>, has been solved to a resolution of 2.0 Å.<sup>6</sup> The determination of the holoenzyme structure has enabled the identification of those amino acids that are required for the binding of each metal ion to the protein. A two-dimensional representation of this binuclear metal center is shown in Figure 1. The more buried metal site is in a trigonal bipyramidal arrangement ligated by the side chains of His-55, His-57, Asp-301, and two bridging ligands: a carbam-

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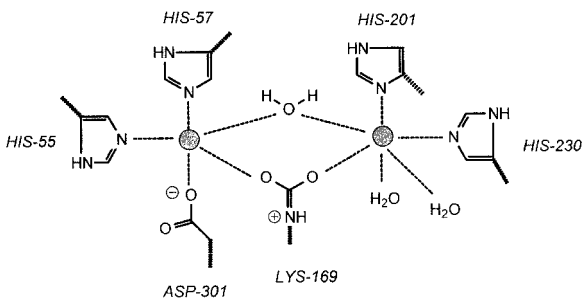


Fig. 1. Two-dimensional representation of the binuclear metal center of the Cd/Cd-phosphotriesterase.<sup>6</sup>

ylated lysine (Lys-169) and a solvent water molecule. The second metal ion is in a distorted octahedral geometry complexed to His-201, His-230, the two bridging ligands, and two additional solvent molecules. The postulated functional roles for the binuclear metal center in this protein are to increase the nucleophilic character of the hydrolytic water molecule and to polarize the phosphoryl oxygen bond of the substrate in order to increase the electrophilic character of the phosphorus reaction center.

Very little is known about how the binuclear metal center accommodates alterations in the specific ligands to either metal ion. Each of the four histidine residues that complex one or the other of the two metal ions has previously been substituted by an asparagine residue.<sup>7,8</sup> These studies have clearly demonstrated that substitution of any one of the native imidazole ligands with a much poorer carboxamide ligand from an asparagine residue results in a significant loss of catalytic activity. However, the tolerance for ligand substitution with other amino acid side chains that are more commonly found as metal binding ligands has not been addressed. This paper elaborates on the utilization of a convenient PCR method for the rapid generation of a library of site-specific mutants within the binuclear metal center. Each one of the original four histidine residues comprising the native ligand set to the binuclear metal site of PTE has been randomized to either histidine or cysteine. The 16 possible permutations of these two amino acid residues at these four sites have been expressed in *Escherichia coli* and the catalytic activities assayed under a variety of assay formats.

## MATERIALS AND METHODS

### Materials

Chemicals were purchased from Sigma Chemical Co., Aldrich Chemical Co., Fisher Scientific, or United States Biochemical Co. Bactotryptone and yeast extract were purchased from Difco Laboratories. UltraGel AcA54 was purchased from IBF Biotechnics. PCR amplification kits were purchased from Gibco BRL or Perkin Elmer Roche (GeneAmp DNA

amplification kit). Sequenase, version 2.0, DNA sequencing kits were purchased from United States Biochemical. T4 DNA ligase and all restriction enzymes were purchased from Promega. GeneClean DNA purification kits were purchased from Bio 101. Wizard Miniprep DNA purification kits and Wizard Magic PCR Prep DNA purification kits were purchased from Promega. All oligonucleotides were synthesized by the Gene Technology Laboratory at Texas A&M University.

### Bacterial Strains and Plasmids

The *E. coli* strains used for this study were XL1-Blue<sup>9</sup> and BL21.<sup>10</sup> The pBS<sup>+</sup> phagemid (Stratagene) was used as the vector for all manipulations. The reconstructed *opd* gene encoding the mature phosphotriesterase (pJK01)<sup>7</sup> was used as the initial template in the mutagenesis experiments.

### Design and Construction of A, B, C, and D Fragments for Combinatorial Library

A standard PCR cocktail was used for the first round of DNA amplification (PCR-1). The template used was the plasmid pJK01, and a list of primer sequences is shown in Table I. Fragment A, generated with primers A<sub>1</sub> and A<sub>2</sub>, extended from the beginning of the *opd* gene past the His-55/His-57 site and was 241 base pairs (bp) in length. Fragment B, generated simultaneously with three mutagenic primers, B<sub>1a</sub>, B<sub>1b</sub>, and B<sub>1c</sub>, and primer B<sub>2</sub>, extended from the His-55/His-57 site past the His-201 site and was 503 bp in length. Primers B<sub>1a</sub>, B<sub>1b</sub>, and B<sub>1c</sub> were the mutagenic primers used to randomize the His-55/His-57 site to His/Cys, Cys/His, and Cys/Cys, respectively. Fragment C, generated with mutagenic primer C<sub>1</sub> and primer C<sub>2</sub>, extended from the His-201 site past the His-230 site and was 140 bp in length. Fragment D, generated with mutagenic primer D<sub>1</sub> and primer D<sub>2</sub>, extended from the His-230 site to the end of the *opd* gene and was 434 bp in length. The thermocycling program used was as follows: 95°C for 5 minutes; 25 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; then, lastly, 72°C for 5 minutes. The DNA fragments were purified from 1% agarose gels with the Wizard PCR Prep DNA purification kit.

### Design and Construction of AB and CD Fragments for Combinatorial Library

Fragments A and B were combined in the second round of PCR, along with primers A<sub>1</sub> and B<sub>2</sub>, to generate a 597-bp fragment, AB, which was now randomized at the His-55/His-57 site for histidine and/or cysteine codons. In a separate tube, fragments C and D were combined along with mutagenic primer C<sub>1</sub> and primer D<sub>2</sub>, to generate a 522-bp fragment, CD, which coded for a cysteine at His-201 and was randomized at the His-230 site. The same reaction conditions used in PCR-1 were used for

**TABLE I. Primers Used in the Generation of the Combinatorial Library of PTE**

Primer designation	Primer sequence (5' → 3')
A <sub>1</sub>	CGCGGATCCGGAGGTTTAAAATATGTC- GATCGGCACAGGCGATC
A <sub>2</sub>	CACATCGACAATCGTTCG
B <sub>1a</sub>	CTGACTCACGAGTGCATCTGCGG
B <sub>1b</sub>	CTGACTTGGGAGCAGCATCTGCGG
B <sub>1c</sub>	CTGACTTGGGAGTGCATCTGCGG
B <sub>2</sub>	CCTTCGGACTCAAAAATGGC
C <sub>1</sub>	GTAACCACTTGCACGGCAGC
C <sub>2</sub>	GCGAGGGCGGTGAGATAGCT
D <sub>1</sub>	GTATGGTTGCAGCGATG
D <sub>2</sub>	CGCGGATCCTCATCATCATGACGCCCGCAAGGT
B <sub>3</sub>	GCTGCCGTGCAAGTGGTTAC
C <sub>3</sub>	GTAACCACTTGCACGGCAGC

PCR-2 and the DNA fragments were purified as above.

### Design and Construction of ABCD Fragment for Combinatorial Library

Fragments AB and CD were combined in the third round of PCR, along with primers A<sub>1</sub> and D<sub>2</sub>, to generate the 1051-bp fragment, ABCD, which was randomized at the His-55/His-57, His-201 and His-230 sites for either histidine or cysteine codons. The same reaction conditions used previously were used for PCR-3, and the DNA fragments were purified as above. A schematic representation for the overall strategy for the construction of the ABCD fragment is shown in Figure 2.

### Generation of Combinatorial Library

The purified ABCD fragment was digested with *Bam*HI and cloned into the pBS<sup>+</sup> vector. Plasmids with the correctly sized inserts were screened for mutations through sequencing. Mutants were also screened for the insertion of the *opd* gene in the correct orientation behind the *lac* promoter in pBS<sup>+</sup> through a series of restriction digests. Plasmids containing the *opd* gene in the reverse orientation were digested with *Bam*HI, religated, transformed into XL1-Blue cells and rescreened.

### Site-Directed Mutagenesis of His-201

His-201 was individually mutated to a cysteine residue using the method of overlap extension PCR.<sup>11</sup> The 20-bp oligonucleotide primers used to make this mutant are listed in Table I. The B<sub>3</sub> and C<sub>3</sub> primers were used to introduce a two base change into the wild-type sequence. Overlap extension PCR consisted of performing the reactions with two separate sets of primers. The first set of primers, primer A<sub>1</sub> and primer B<sub>3</sub>, was used to make the AB<sub>3</sub> fragment. The second set of primers, consisting of the mutagenic C<sub>3</sub> primer and primer D<sub>2</sub>, was used to make a

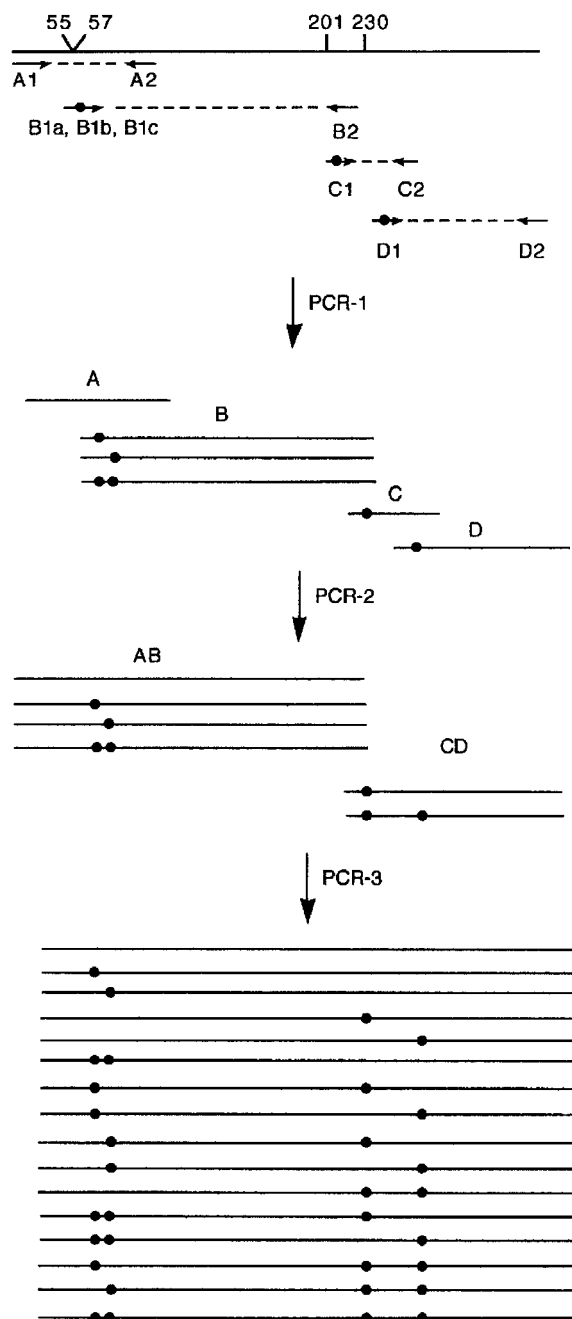


Fig. 2. Schematic diagram for the construction of the combinatorial library of phosphotriesterase mutants at residue positions 55, 57, 201, and 230. These four sites were randomized with histidine and cysteine residues. Additional details are presented in the text.

C<sub>3</sub>D fragment. The plasmid pJK01 was used as the template for both reactions. The AB<sub>3</sub> and C<sub>3</sub>D fragments were purified by agarose gel electrophoresis, combined, and used in a third PCR step, where they acted as both primer and template for each other. Primers A<sub>1</sub> and D<sub>2</sub> were also included to amplify the resulting AD fragment. The AD fragment was puri-

fied from agarose gel, digested with *Bam*HI, and cloned into the pBS<sup>+</sup> vector. Isolated plasmids were screened for the correct orientation of the *opd* gene behind the *lac* promoter as described above.

### Sequencing of Mutants

Each of the mutant *opd* genes generated through PCR was completely sequenced to ensure that only the desired base changes introduced at position 55, 57, 201, and/or 230 were present. Sequencing was performed using the dideoxynucleotide chain termination method.<sup>12</sup>

### Growth Conditions

Overnight cultures of transformed BL21 cells grown in Luria-Bertani (LB) broth<sup>13</sup> were used to inoculate terrific broth (TB) containing 50 µg/ml ampicillin. In some cases, the medium also contained 1 mM CoCl<sub>2</sub>, 1 mM CdSO<sub>4</sub>, or 1 mM ZnSO<sub>4</sub>. The cultures were incubated at 30°C and induced with IPTG in early log phase. Cells were harvested in stationary phase.

### Purification of Mutant Enzymes

Mutant enzymes were purified from BL21 cells according to previously reported procedures.<sup>5,7</sup> The mutants H57C and H57,201C were purified using a purification buffer that contained 1 mM CoCl<sub>2</sub> and 10 mM KHCO<sub>3</sub>. SDS-PAGE indicated that the purified mutants were greater than 99% pure and were the same size as the wild-type enzyme.

### Preparation and Reconstitution of Apoenzyme

Apoenzyme was prepared by incubating enzyme with 2 mM *o*-phenanthroline at 4°C overnight or until there was less than 1% residual activity. The *o*-phenanthroline was removed by diafiltration with metal-free 50 mM HEPES (pH 8.5) buffer using an Amicon PM10 membrane or by passing the mixture through a Pharmacia PD-10 Sephadex G-25 desalting column. Apoenzyme was reconstituted with the desired metal by adding 5 equivalents of either ZnSO<sub>4</sub>, CdSO<sub>4</sub>, or CoCl<sub>2</sub> per mole equivalent of enzyme in the presence of 10 mM KHCO<sub>3</sub> and incubating at 4°C for at least 2 hours.

### Determination of Metal Content

The metal content of the cobalt-purified H55C mutant was determined by atomic absorption spectrometry. The purified enzyme was passed through a Pharmacia PD-10 Sephadex G-25 desalting column to remove any loosely bound metals. The sample was analyzed with a Perkin-Elmer 2380 atomic absorption spectrometer.

### Determination of Enzyme Activity

The catalytic activity for each of the mutants was measured by monitoring the appearance of *p*-nitrophenol at 400 nm during the hydrolysis of 1.0

mM paraoxon at 25°C, pH 9.0. The assays were conducted as previously reported<sup>7</sup> except for the addition of 100 µM of either ZnSO<sub>4</sub>, CdSO<sub>4</sub>, or CoCl<sub>2</sub> to some of the reaction mixtures. The kinetic constants were obtained by a fit of the data to Equation 1 by using the software supplied by Savanna Shell Software:

$$v = VA/(K_a + A) \quad (1)$$

where  $v$  = velocity,  $V$  = maximum velocity,  $A$  = substrate concentration, and  $K_a$  is the Michaelis constant.

## RESULTS

### Generation of Combinatorial Library

The randomization of PTE residues 55, 57, 201, and 230 with either histidine or cysteine yields 16 possible permutations at the four original imidazole ligand sites located within the binuclear metal center of phosphotriesterase. This modest combinatorial library of PTE containing the randomized mutations was swiftly and economically generated through three rounds of PCR amplification with a limited set of mutagenic primers. The mutant genes were subsequently cloned into the pBS<sup>+</sup> vector behind the *lac* promoter and then transformed into XL1-Blue cells. Plasmids isolated from 17 individual colonies were screened for the desired mutations by partial sequencing of the corresponding DNA. Of the 16 possible combinations of histidine and cysteine codons at positions 55, 57, 201, and 230, 7 were identified in this manner: wild-type (all histidines); H57C; H230C; H55,230C; H57,230C; H201,230C; and H55,57,230C. Rather than screen for additional colonies, we elected to create the H201C mutant by a more conventional site-directed mutagenesis approach, employing the method of overlap extension PCR.<sup>11</sup> The remaining 8 mutants were then constructed by utilizing the unique *Pst*I and *Not*I restriction sites within the plasmids containing the variant phosphotriesterase genes (Fig. 3). The *Pst*I-*Not*I restriction fragments from the wild-type, H201C, and H201,230C genes were replaced with the corresponding restriction fragments from the H55,230C, H57,230C and H55,57,230C mutants to create the other half of the library (H55C; H55,57C; H55,201C; H57,201C; H55,57,201C; H55,201,230C; H57,201,230C; and H55,57,201,230C).

### Exhibition of Catalytic Activity

All 16 of the phosphotriesterase histidine/cysteine variants were expressed in BL21 cells grown in 50-ml cultures in the presence and absence of 1 mM CoCl<sub>2</sub>, CdSO<sub>4</sub>, or ZnSO<sub>4</sub>. The cell lysates were tested for their ability to hydrolyze paraoxon. The results, expressed as µmoles of paraoxon hydrolyzed per minute per gram of wet cell paste are summarized in

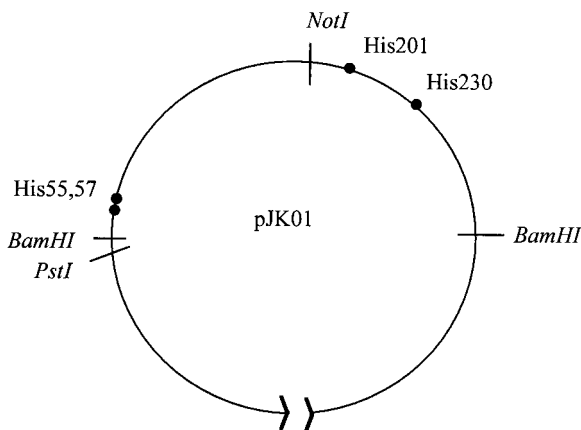


Fig. 3. Map of the pJK01 plasmid. The gene encoding the mature phosphotriesterase was ligated in to the multiple cloning site of the PBS<sup>+</sup> plasmid at the *Bam*HI restriction site.<sup>7</sup> The locations of the *Pst*I, the *Not*I and the two *Bam*HI restriction sites relative to the four mutagenic sites are illustrated.

Table II. The net expression of the wild-type enzyme was very dependent on the exact growth conditions. In the presence of 1 mM CoCl<sub>2</sub> in the growth medium there was a 40-fold increase in the observed rate of paraoxon hydrolysis. This is consistent with previous observations.<sup>5</sup> The catalytic activity of the single mutants H55C, H57C, H201C, and H230C varied greatly. H55C had essentially no activity under all growth conditions, while H57C had between 50 to 200% of wild-type activity. H201C had approximately 10% of the wild type level of activity but H230C had less than 1% of this value except in the presence of 1 mM Zn<sup>2+</sup>. The relative activities of the double and triple mutants were less than 0.1% of the wild-type values.

#### Purification of Mutants

Seven of the mutants (H55C; H57C; H201C; H230C; H57,201C; H57,230C; and H201,230C) were purified to homogeneity from cells grown in the presence of CoCl<sub>2</sub> using the standard purification protocol.<sup>5</sup> The specific activities of these preparations are presented in Table III. Purification of the H55,57C mutant was not possible due to the apparent physical instability of the protein. The relative activity of the purified H57C mutant was below expectations based upon the high initial activity observed in the unfractionated cell lysate. Upon further dilution of the protein concentration, the specific activity of the H57C mutant rapidly dropped another 10-fold. Attempts to reactivate the mutant in the presence of 10 mM KHCO<sub>3</sub> and 1 mM CoCl<sub>2</sub> yielded enzyme were partially successful (Fig. 4). However, purification of this mutant in the presence of 10 mM KHCO<sub>3</sub> and 1 mM CoCl<sub>2</sub> resulted in a preparation of protein that had a specific activity of approximately 10,000 U/mg.

#### Kinetic Parameters of Reconstituted Enzyme Preparations

*o*-Phenanthroline was used to remove metals from the active sites of those mutants of PTE that exhibited activity using paraoxon as a substrate. The enzymes were then reconstituted with 5 equivalents of CoCl<sub>2</sub>, CdSO<sub>4</sub>, or ZnSO<sub>4</sub> in the presence of 10 mM KHCO<sub>3</sub>. All of the enzymes obtained maximum activation in a period of 2–4 hours. The kinetic parameters for the reconstituted enzymes are shown in Table IV. The *K<sub>m</sub>* values for paraoxon with the Co<sup>2+</sup>, Cd<sup>2+</sup>, or Zn<sup>2+</sup> reconstituted enzymes did not vary significantly from that observed for the wild-type phosphotriesterase.

#### Characterization of H55C

The H55C mutant of phosphotriesterase did not hydrolyze paraoxon under any assay conditions. The addition of excess metal and KHCO<sub>3</sub> to the assay mixture and preincubation of the enzyme with low levels of metal and/or KHCO<sub>3</sub> was unsuccessful in the elicitation of catalytic activity. All attempts to make apo-enzyme resulted in the precipitation of the protein. The purified H55C mutant is a bright green color and the visible absorption spectrum is shown in Figure 5. The green color is the result of significant absorption at 440 and 630 nm. Analysis of the metal content of the desalted enzyme after purification in buffer containing 100 μM CoCl<sub>2</sub> indicated that only one Co<sup>2+</sup> was bound to the protein.

#### DISCUSSION

Phosphotriesterase has a diverse substrate specificity and is capable of hydrolyzing a wide variety of organophosphate compounds. The rate of catalysis for the substrate paraoxon approaches the diffusion controlled limit; however, the enzyme is significantly slower with other substrates.<sup>2,5</sup> In order for phosphotriesterase to be more effective in the detoxification of chemical warfare agents it must be modified to be more efficient with these other substrates. There are two general approaches that can be used for the manipulation of substrate specificity. The first approach is a "rational" design that uses information gathered from biophysical studies of the protein to select targets for site-specific mutation. X-ray crystal structures and sequence homology studies are often used in the rational redesign of native enzymes. The second approach is the "random" generation of many mutant proteins simultaneously. These latter protocols involve spiked oligonucleotides<sup>14</sup>, cassette mutagenesis<sup>15</sup>, and/or DNA shuffling techniques.<sup>16</sup> Random approaches can be applied to whole sequences as well as to particular regions of the protein or specific amino acids. This methodology has the potential to generate enormous numbers of mutant proteins but requires an efficient screening method to select mutants with the properties of interest.

**TABLE II. Activity of Phosphotriesterase Mutants in Unfractionated Cell Lysate**

Enzyme	CoCl <sub>2</sub> * U/g cell <sup>  </sup>	No added metal <sup>†</sup> U/g cell	CoCl <sub>2</sub> ‡ U/g cell	CdSO <sub>4</sub> § U/g cell	ZnSO <sub>4</sub> ¶ U/g cell
Wild type	$8.2 \times 10^3$	$1.6 \times 10^2$	$2.4 \times 10^2$	$1.3 \times 10^3$	$1.4 \times 10^2$
H55C	$2.7 \times 10^{-2}$	$\leq 10^{-4}$	$5.0 \times 10^{-3}$	$\leq 10^{-4}$	$\leq 10^{-4}$
H57C	$1.2 \times 10^4$	$3.7 \times 10^2$	$5.2 \times 10^2$	$6.2 \times 10^2$	$8.7 \times 10^1$
H201C	$1.3 \times 10^3$	7.1	$2.0 \times 10^1$	$1.6 \times 10^2$	4.5
H230C	$2.7 \times 10^1$	$8.0 \times 10^{-3}$	$1.4 \times 10^{-1}$	1.8	6.4
H55,57C	$3.2 \times 10^{-2}$	$\leq 10^{-4}$	$5.0 \times 10^{-3}$	$4.0 \times 10^{-3}$	$\leq 10^{-4}$
H55,201C	$4.3 \times 10^{-2}$	$\leq 10^{-4}$	$5.7 \times 10^{-3}$	$9.8 \times 10^{-3}$	$\leq 10^{-4}$
H55,230C	$3.6 \times 10^{-2}$	$1.7 \times 10^{-2}$	$6.5 \times 10^{-3}$	$2.8 \times 10^{-3}$	$1.6 \times 10^{-2}$
H57,201C	$1.4 \times 10^{-2}$	$\leq 10^{-4}$	$6.1 \times 10^{-3}$	$1.2 \times 10^{-2}$	$5.5 \times 10^{-2}$
H57,230C	$2.7 \times 10^{-2}$	$\leq 10^{-4}$	$5.8 \times 10^{-3}$	$\leq 10^{-4}$	$\leq 10^{-4}$
H201,230C	$3.4 \times 10^{-2}$	$\leq 10^{-4}$	$6.7 \times 10^{-3}$	$3.5 \times 10^{-2}$	$\leq 10^{-4}$
H55,57,201C	$8.8 \times 10^{-2}$	$\leq 10^{-4}$	$1.1 \times 10^{-2}$	$\leq 10^{-4}$	$3.2 \times 10^{-3}$
H55,57,230C	1.6	$\leq 10^{-4}$	$\leq 10^{-4}$	$\leq 10^{-4}$	$\leq 10^{-4}$
H55,201,230C	$6.2 \times 10^{-2}$	$2.6 \times 10^{-2}$	$\leq 10^{-4}$	$2.4 \times 10^{-2}$	$\leq 10^{-4}$
H57,201,230C	$8.2 \times 10^{-2}$	$6.0 \times 10^{-3}$	$6.2 \times 10^{-2}$	2.2	$\leq 10^{-4}$
H55,57,201,230C	$1.7 \times 10^{-1}$	$\leq 10^{-4}$	$2.0 \times 10^{-3}$	$\leq 10^{-4}$	$2.4 \times 10^{-3}$
pbs <sup>+</sup>	$6.0 \times 10^{-3}$	$8.1 \times 10^{-3}$	$9.9 \times 10^{-3}$	$7.5 \times 10^{-3}$	$1.0 \times 10^{-3}$

\*Cells were grown in the presence of 1 mM CoCl<sub>2</sub>, and the lysate was assayed in the presence of 100 μM CoCl<sub>2</sub>.

†Cells were grown in the absence of additional metal, and the lysate was assayed in the absence of additional metal.

‡Cells were grown in the absence of additional metal, and the lysate was assayed in the presence of 100 μM CoCl<sub>2</sub>.

§Cells were grown in the presence of 1 mM CdSO<sub>4</sub>, and the lysate was assayed in the presence of 100 μM CdSO<sub>4</sub>.

¶Cells were grown in the presence of 1 mM ZnSO<sub>4</sub>, and the lysate was assayed in the presence of 100 μM ZnSO<sub>4</sub>.

<sup>||</sup>A unit (U) is defined as a micromole of paraoxon hydrolyzed per minute.

**TABLE III. Purification of Phosphotriesterase Mutants**

Mutant	Yield (mg)	Specific activity (U/mg)*
H55C	44	$<10^{-5}$
H57C	71	250
H201C	62	234
H230C	33	5
H57,201C	15	0.6
H57,230C	20	0.08
H201,230C	66	0.02

\*pH 9.0, 25°C.

Since the first reports describing site-directed mutagenesis,<sup>17,18</sup> many studies have been undertaken with the goal of creating mutant enzymes with new activities. These investigations have served to highlight the many factors that influence the activity and specificity of an enzyme catalyzed reaction.<sup>19-23</sup> The inability to accurately predict the many overlapping effects of a mutation on the structure and function of an enzyme has lead investigators to use a combinatorial approach to protein modification. Combinatorial cassette mutagenesis can be utilized when fully randomized sequences are placed into the wild type gene.<sup>24</sup> These mutated cassettes can then be combined or shuffled to create proteins with greater variability.<sup>25,26</sup> Combinatorial mutagenesis is utilized in both rational and random strategies. In a more

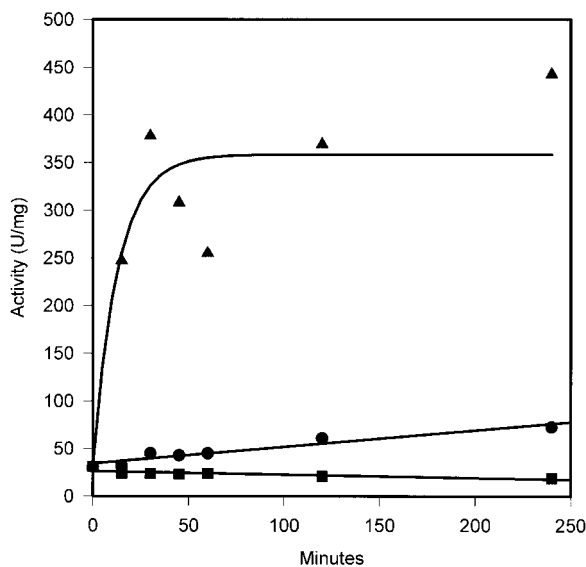


Fig. 4. Time course for recovery of catalytic activity of the purified H57C mutant. Activity was measured as a function of time after addition of 1 mM CoCl<sub>2</sub> (●) and 1 mM CoCl<sub>2</sub>/10 mM KHCO<sub>3</sub> (▲). The control experiment (■) shows no increase in activity over the same time period.

rational based protocol, amino acids that have been determined beforehand to play an important structural or catalytic role in an enzyme are individually or simultaneously mutated and the overall

TABLE IV. Kinetic Parameters of Purified Enzymes\*

Enzyme	Co <sup>2+</sup> -reconstituted enzyme		Cd <sup>2+</sup> -reconstituted enzyme		Zn <sup>2+</sup> -reconstituted enzyme	
	V <sub>max</sub> (sec <sup>-1</sup> )	K <sub>m</sub> (μM)	V <sub>max</sub> (sec <sup>-1</sup> )	K <sub>m</sub> (μM)	V <sub>max</sub> (sec <sup>-1</sup> )	K <sub>m</sub> (μM)
WT	5.1 × 10 <sup>3</sup>	290	2.5 × 10 <sup>3</sup>	670	7.8 × 10 <sup>2</sup>	51
H55C	≤10 <sup>-5</sup>	—	—	—	—	—
H57C <sup>a</sup>	2.3 × 10 <sup>2</sup>	150	2.8	470	1.1	50
H57C <sup>b</sup>	2.8 × 10 <sup>3</sup>	90	1.0 × 10 <sup>3</sup>	310	1.2 × 10 <sup>3</sup>	124
H201C	5.1 × 10 <sup>1</sup>	110	7.6 × 10 <sup>1</sup>	350	4.4	250
H230C	7.8	130	4.8	320	1.3 × 10 <sup>1</sup>	190
H57,201C	2.4 × 10 <sup>1</sup>	82	1.5 × 10 <sup>-1</sup>	130	1.4 × 10 <sup>-1</sup>	47
H57,230C	6.0 × 10 <sup>-2</sup>	170	1.4 × 10 <sup>-1</sup>	998	4.0 × 10 <sup>-3</sup>	21
H201,230C	1.6 × 10 <sup>-2</sup>	170	2.4 × 10 <sup>-3</sup>	490	1.0 × 10 <sup>-3</sup>	48

\*Enzymes were assayed at pH 9, 25°C, and the kinetic data were fit to Equation (1).

<sup>a</sup>This enzyme was purified using the standard protocol without added bicarbonate.

<sup>b</sup>Enzyme was purified using purification buffer containing 10 mM KHCO<sub>3</sub> and 1 mM CoCl<sub>2</sub>.

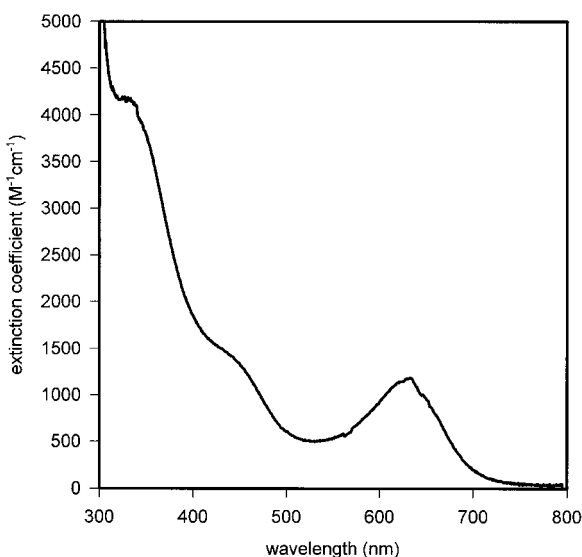


Fig. 5. Absorption spectrum of the Co<sup>2+</sup>-substituted H55C protein. Spectrum was measured at 25°C in 50 mM Hepes, pH 8.5.

mutagenic effects are measured. Combinatorial mutagenesis can alter all of the target residues in the protein to a single amino acid such as alanine,<sup>27</sup> or a single site can be substituted with the other 19 amino acids.<sup>28</sup> Mutations made at multiple sites have been compared to individual mutations and have been observed to be additive, synergistic, and independent.<sup>29,30</sup>

The crystal structure of phosphotriesterase shows that it folds in an  $\alpha/\beta$  barrel motif. The active site of phosphotriesterase is defined by the 8 loops between the  $\beta$  strands and  $\alpha$  helices. At the onset of this series of experiments, the interactions between the enzyme and the substrate bound at the active site were not clearly defined. A study was designed to create a limited number of mutants to more fully explore the role of the four histidines that are known to be ligands to the binuclear metal site. The binuclear metal site is proposed to play a role in the polarization of the phosphorus–oxygen bond and in the

activation of the hydrolytic water molecule. It was anticipated that changing the ligands would alter the kinetic properties and/or specificity of the enzyme.

A three-step PCR method of generating a combinatorial library of site-specific mutants in a single reaction tube was developed for the enzyme phosphotriesterase. The method was used to generate the 16 mutants possible upon changing His-55, His-57, His-201, and/or His-230 to cysteine. The initial sequencing of 17 colonies identified 7 of the desired mutant proteins. The number of colonies screened was nearly the same as the total number of possible mutants. Further screening of additional colonies most likely would have identified more mutants. It is likely that controlling the ratio of the different fragments may have resulted in a more equal distribution of mutants. The technique is quite effective at rapidly generating a specific library of mutants. This is especially true if the number of mutants created in a round of PCR were increased beyond 16.

The role of the binuclear metal site from phosphotriesterase in substrate binding and activity was examined using the library of His/Cys mutants created. It has previously been demonstrated using a Zn<sup>2+</sup>/Cd<sup>2+</sup> hybrid of phosphotriesterase that one of the sites (designated M <sub>$\alpha$</sub> ) determines the kinetic properties of the enzyme while the second site (designated M <sub>$\beta$</sub> ) has a more secondary role in binding and catalysis.<sup>31</sup> The recently determined crystal structure of PTE has identified the ligands to the two metal sites and led to the suggestion that the bridging water molecule may be the nucleophile in the displacement reaction.<sup>6</sup> In order to gain further information about the role of each metal ion at the active site, the activity of all of the mutants in the cell lysate were measured, several of the mutants were purified and their kinetic parameters were determined.

The single-site mutations (H55C, H57C, H201C, and H230C) all had distinct effects on the activity of the enzyme. In the cell lysate and after purification, H55C had no activity under all assay conditions.

Attempts to make apoenzyme resulted in precipitation of the protein. The mutant H57C had wild-type or greater levels of activity in the cell lysate, but initial attempts at purification resulted in enzyme with less than 5% of wild-type activity. When the enzyme was purified in buffer containing  $\text{KHCO}_3$  and a 10-fold higher concentration of  $\text{CoCl}_2$ , activity was retained. This suggests that the H57C mutation results in a reduced ability of the binuclear metal site to retain the metal ion at the active site and that loss of the metal results in an irreversibly damaged protein. Mutation of the histidines coordinated to the second metal site resulted in enzyme with greatly reduced catalytic activity. H201C had between 3% and 15% of wild-type activity in the cell lysate and H230C had between 0.1% and 4% wild-type activity, depending on the metal present at the active site. When purified, these mutants retained the same levels of activity, but were unstable in solution. The affinity for paraoxon was not significantly changed in H57C, H201C, or H230C.

These results from the single-site mutants correspond well with earlier mutagenesis studies that were directed at identifying the histidine residues acting as ligands to the bound metals of phosphotriesterase.<sup>7</sup> Each of the histidines was mutated to asparagine and the resulting proteins were purified and characterized. H55N had the lowest level of activity under all conditions while H57N had higher levels of activity. H201N and H230N retained intermediate levels of activity. In these studies, changing either one of the histidines coordinating to the more buried metal (H55 or H57) resulted in a weakly bound metal.

In similar studies with the  $\text{Zn}^{2+}$  binding protein human carbonic anhydrase II, each of the histidine ligands in the  $\text{His}_3$  metal site was individually mutated to a cysteine.<sup>32,33</sup> The resulting proteins were kinetically characterized and crystal structures were obtained. In all cases the result was a protein with a decreased affinity for zinc. The negatively charged cysteine residue also resulted in an increase in the  $\text{pK}_a$  of the metal bound hydroxide, a decreased affinity for the transition state analogue and a decrease in activity.<sup>33</sup> The more solvent accessible cysteine ligand was prone to oxidation resulting in irreversible loss of the metal.<sup>32</sup> Crystal structures of the mutants showed that the replacement of any of the histidine residues with cysteine resulted in a conformational change in the  $\beta$  sheet backbone as well as in the placement of the  $\text{Zn}^{2+}$  in the metal site.<sup>34</sup> In the H96C mutant of carbonic anhydrase, the cysteine was not a direct ligand to the metal but was replaced by a solvent water molecule.

The double, triple, and quadruple His/Cys phosphotriesterase mutants all had significantly decreased activity in the cell lysate. Only four of the double mutants were purified. The H55,57C mutant was unstable and could not be purified to homogene-

ity. The H57,201C, H57,230C and H201,230C mutants all had less than  $10^{-4}$  of wild-type activity, but the affinity for paraoxon was not significantly changed. Multiple changes resulted in decreases in metal affinity and decreased stability.

The H55C mutant had no activity in the cell lysate nor after purification. The purified  $\text{Co}^{2+}$ -protein was a bright green color and spectral characterization of the colored protein showed strong absorption bands at 350, 440, and 630 nm. Atomic absorption spectroscopy indicated that there is a single metal tightly bound to the protein. Optical absorption spectra can be used to determine information about the ligands in the first coordination sphere of a metal.<sup>35</sup> The observed visible spectrum of this mutant is characteristic of tetrahedral coordination around the metal ion and the  $\lambda_{\text{max}}$  and extinction coefficient of the band at 630 nm are consistent with a  $\text{Co}^{2+}$  complex that has two cysteine ligands in a tetrahedral environment. The compounds  $[\text{Co}(\text{S}-2,4,6\text{-}i\text{-Pr}_3\text{C}_6\text{H}_2)_2(1\text{-Me-imid})_2]_2$  and  $[\text{Co}(\text{S}-2,4,6\text{-}i\text{-Pr}_3\text{C}_6\text{H}_3)_2(2,9\text{-Me}_2\text{phen})]$  were examined as model compounds for spectral comparison.<sup>36,37</sup> These compounds contain two sulfur and two nitrogen ligands tetrahedrally coordinated to a  $\text{Co}^{2+}$  and both exhibit electronic spectra similar to that of the H55C mutant. Since there is not another cysteine present in the binuclear metal center of the native enzyme, amino acids surrounding the metal site were examined as possible alternative ligands. When a single metal was modeled into the active site of the H55C mutant, Cys-227 was found to be 7 Å from Cys-55. It can be proposed that the H55C mutation causes a conformational change around the binuclear metal site that precludes catalysis. The exact rearrangement of the metal site awaits x-ray crystallography data.

The information gathered from the library of His/Cys mutants of phosphotriesterase supports the general model for the active site reaction and the identification of the  $M_\alpha$  and  $M_\beta$  metal sites proposed from crystallography data.<sup>6,38</sup> These data suggest that the substrate interacts with the more solvent accessible metal site (ligated to His-201 and His-230) displacing the bridging water molecule toward the more buried metal (ligated to His-55 and His-57). The activated water molecule is then in alignment for attack of the phosphoryl group of the substrate and displacement of the leaving group.

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