Identification of the Histidine Ligands to the Binuclear Metal Center of Phosphotriesterase by Site-Directed Mutagenesis[†]

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ABSTRACT: In order to identify which of the seven histidines in phosphotriesterase participate at the active site/binuclear metal center of the enzyme, site-directed mutagenesis has been employed to change, individually, each of the seven histidine residues to asparagine. In addition, the gene for the wild-type enzyme has been subcloned without its leader sequence behind a modified ribosomal binding site, leading to a 5-fold increase in protein expression. The seven mutants, H55N, H57N, H123N, H201N, H230N, H254N, and H257N, exhibit varying degrees of activity compared to the wild-type enzyme. The H123N and H257N mutants are as active as the wild-type enzyme, but all of the other mutant enzymes have 10% or less activity. The metal content of the cobalt-purified mutant enzymes has been determined to be less than that of the wild-type enzyme in all cases. Each of the mutant enzymes has been converted to apoenzyme and reconstituted with 2 equiv of zinc(II), cadmium(II), or cobalt(II). The kinetic parameters, V_{max} and V/K_m, and apparent pK_a's have been determined for each of the reconstituted enzyme derivatives. In almost all cases, the apparent pK_a's have shifted toward higher values. The pH–rate profiles for some of the reconstituted mutant enzymes are significantly different from those for the wild-type enzyme, indicating that other groups may become involved in the reaction mechanism upon mutation of the histidine residue to asparagine. His-123 is the only histidine residue that appears to have no involvement in the catalytic activity of phosphotriesterase. The other six histidine residues appear to be involved in some capacity, either as metal ligands or as the catalytic base or proton-shuttle group. A working model has been proposed that incorporates six of the histidines into the active site/metal centers of phosphotriesterase. The two closely spaced pairs, His-55/His-57 and His-254/His-257, are proposed to act as bidentate ligands, with one pair liganding to the primary catalytic metal and the other pair liganding to the secondary metal, which provides structural and catalytic support. His-230 is proposed to act as the bridging ligand between the two metal ions, and His-201 is proposed to be the catalytic base or proton-shuttle group.

The bacterial phosphotriesterase from *Pseudomonas diminuta* is a zinc metalloenzyme, which catalyzes the hydrolysis of a variety of organophosphate triesters, including the insecticide paraoxon:
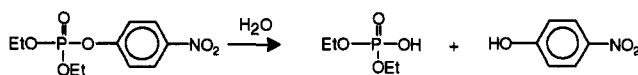
The reaction proceeds via an S_N2-like mechanism and likely involves an amino acid residue acting as a general base to assist in the deprotonation of an activated water molecule, which then directly attacks the substrate (Lewis et al., 1988). pH–rate profiles and inactivation studies with diethyl pyrocarbonate suggest that the most likely candidate for the general base is a histidine residue (Dumas & Raushel, 1990). It has previously been shown that at least one of the two zinc ions coordinated to the phosphotriesterase is essential for enzymatic activity (Dumas et al., 1989; Omburo et al., 1992). Although little is known about the actual roles of the two metal ions, one of the metals may be directly involved in the catalytic mechanism, while the other may be involved in a structural capacity. Apophosphotriesterase prepared with the metal chelator, *o*-phenanthroline, is capable of being fully reactivated

with a variety of different metals. Replacement of the naturally occurring zinc(II) metal with other divalent cations, such as cadmium(II), cobalt(II), manganese(II), or nickel(II), apparently does not alter the conformation of the protein, since the specific activities of the various metal-substituted phosphotriesterase derivatives remain the same or are increased significantly over that of the native zinc enzyme (Omburo et al., 1992).

High-resolution [113]Cd nuclear magnetic resonance (NMR) spectra of the [113]Cd-substituted phosphotriesterase show two cadmium resonances at 212 (M_α site) and 116 ppm (M_β site) downfield from $Cd(ClO_4)_2$, indicating that the chemical environments around the two metal ions are somewhat different from each other (Omburo et al., 1993). The positions of the observed chemical shifts indicate that the ligands to both metal centers are most likely composed of a mixture of oxygen and nitrogen ligands, with the M_α site metal ion possibly ligated to three nitrogen atoms and one oxygen atom and the M_β site metal ion possibly ligated to two nitrogen atoms and two oxygen atoms. Comparison with the [113]Cd nuclear magnetic resonance spectra of other metalloenzyme systems excludes the presence of a sulfur ligand or a ligand set composed exclusively of oxygen atoms. Possible ligands include histidine, aspartate, glutamate, and water.

Electron paramagnetic resonance spectroscopy of the manganese-substituted phosphotriesterase indicates that the two Mn(II) ions are present as an antiferromagnetically exchange-coupled binuclear metal complex (Chae et al., 1993). This requires the two metal sites to be in close proximity and

suggests that the two metal ions are bridged by a common ligand.

The most obvious candidates among the amino acid residues in phosphotriesterase to act as ligands for the metal centers are histidine, aspartate, and glutamate. Histidine is a common metal center ligand in several zinc metalloenzymes (Vallee & Auld, 1990). The activated water molecule proposed in the chemical mechanism of phosphotriesterase is likely to be an oxygen ligand to one of the two metal centers. Since histidine residues have been implicated in both the catalytic mechanism and metal ligation, it seems likely that some, if not all, of the seven histidines in phosphotriesterase are associated with the active site in some capacity. In order to identify which of the histidine residues may be involved in the chemical mechanism and/or metal center ligation, site-directed mutagenesis has been employed to change, individually, each of the seven histidine residues to asparagine. The substitution of a histidine with an asparagine should make that position less effective, both as a ligand and as a general base, but at the same time, the change should be structurally conservative enough not to cause drastic alterations in the conformation of the active site or in the overall structure of the protein. Kinetic studies with these histidine mutants provide further insight into the possible roles of these histidine residues in the structure and function of the phosphotriesterase.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma Chemical Co., Aldrich Chemical Co., Fisher Scientific, or United States Biochemical Co. Bacto-tryptone and Bacto-yeast extract were purchased from Difco Laboratories. Ultrogel AcA54 was purchased from IBF Biotechnics. GeneAmp DNA amplification kits were purchased from Perkin-Elmer Cetus. Sequenase version 2.0 DNA sequencing kits were purchased from United States Biochemical. All enzymes used in the molecular biology experiments were purchased from Promega, United States Biochemical, Stratagene, or New England Biolabs. GeneClean DNA purification kits were purchased from Bio 101. Magic Minipreps DNA purification kits and Magic PCR Preps DNA purification kits were purchased from Promega. All oligonucleotides used in this work were synthesized by the Gene Technology Laboratory of the Biology Department of Texas A&M University.

Bacterial Strains and Plasmids. The *Escherichia coli* strains used for this study were XL1-Blue (*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F' *proAB, lacI^qZM15,* Tn *10* (tet^r)]) (Bullock et al., 1987) and BL21 (F^- *ompT*r_B-m_B-) (Studier et al., 1990). The pBS^+ phagemid (Stratagene) was used as the vector in all cases. The *opd* gene from *Pseudomonas diminuta,* which was previously subcloned along with its leader sequence into the pBS^+ phagemid at the *Bam*HI site and used under the name of pDD02, was used as the template for further subcloning experiments.

Reconstruction of the opd Gene. The plasmid pDD02 containing the *opd* gene was used as a template for the polymerase chain reaction (PCR). Primer A (shown in Table 1) was a 44-base oligonucleotide composed of a *Bam*HI site, an optimized *E. coli* ribosomal binding site (Skoglund et al., 1990), and the codons for the first six amino acid residues of the mature phosphotriesterase. Primer D (shown in Table 1) was a 33-base oligonucleotide composed of a *Bam*HI site, three successive stop codons, and the codons for the last five amino acid residues of the mature phosphotriesterase. The

Table 1: Flanking Primers Used in PCR

primer A

       *Bam*HI   Met Ser Ile Gly Thr Gly Asp

     ⁵CGCGGATCCGGAGGTTTAAAATATGTCGATCGGCACAGGCGATC³

primer D

        *Bam*HI   Ser Ala Arg Leu Thr

     ⁵CGCGGATCCTCATCATCATGACGCCCGCAAGGT³

PCR reaction mixture consisted of 200 μM each of the dideoxynucleotide triphosphates, 2.5 units of AmpliTaq DNA polymerase, 1 μM each of primers A and D, 1 ng of pDD02 DNA template, and GeneAmp reaction buffer for a final volume of 100 μL. Two drops of mineral oil were added to prevent evaporation during thermocycling. An initial melt at 95 °C for 2 min was followed by 28 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and ended with slow cooling to 4 °C. After phenol/chloroform extraction to remove the mineral oil, the 1051-base-pair (bp) fragment was digested with *Bam*HI to yield a 1039-bp fragment, which was concentrated with the GeneClean system and purified by electrophoresis in a 1% agarose gel. The DNA was extracted from the agarose gel with the GeneClean system. The pBS^+ vector was also cut with *Bam*HI and purified as above. Vector and insert were combined and concentrated with the GeneClean system before ligation with T4 DNA ligase. The recombinant DNA was transformed into XL1-Blue cells, and transformants were selected using the blue/white selection feature of XL1-Blue. Plasmids isolated from the selected colonies were screened for the insertion of the *opd* gene in the correct orientation behind the *lac* promoter in pBS^+ through the following series of restriction digests: *Bam*HI alone (should yield 1039- and 3204-bp fragments), *Eco*RV/*Pst*I (should yield 264- and 3979-bp fragments), and *Eco*RV/*Eco*RI (should yield 811- and 3432-bp fragments). The reconstructed *opd* gene encoding just the mature phosphotriesterase was designated pJK01 and transformed into BL21 cells.

Site-Directed Mutagenesis. The seven histidine residues in phosphotriesterase at positions 55, 57, 123, 201, 230, 254, and 257 were individually changed to asparagine using the method of overlap extension PCR (Ho et al., 1989). The 20-base oligonucleotide primers used to make these mutants are listed in Table 2. Each pair of B and C primers was used to introduce a single 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 and a selected B primer, was used to make an AB fragment. The second set of primers, consisting of the corresponding C primer and primer D, was used to make a CD fragment. The plasmid pJK01 was used as the template in both cases. The AB and CD fragments were purified by agarose gel electrophoresis, followed by either the GeneClean system or the Magic PCR Prep DNA purification system. The two fragments were combined and used in a third PCR, where they acted as both primer and template for each other, since their sequences overlapped in the region around the desired mutation site. Primers A and D were also included to further amplify the resulting AD fragment. The AD fragment was purified from agarose gel, cut with *Bam*HI, concentrated with *Bam*HI-digested pBS^+, ligated with T4 DNA ligase, and transformed into XL1-Blue cells. Colonies were screened as above for those vectors containing inserts in the desired orientation. The plasmids for the seven mutants, H55N, H57N, H123N, H201N, H230N, H254N, and H257N, were designated pJK02–pJK08, respectively, and transformed into BL21 cells.

Table 2: Primers Used for Site-Directed Mutagenesis

H55N Primer B	5'GATGTGCTCGTTAGTCAGTG3'
H55N Primer C	5'CACTGACTAACGAGCACATC3'
H57N Primer B	5'GCCGCAGATGTTCTCGTGAG3'
H57N Primer C	5'CTCACGAGAACATCTGCGGC3'
H123N Primer B	5'GCCACGATATTAACGTCGGC3'
H123N Primer C	5'GCCGACGTTAATATCGTGGC3'
H201N Primer B	5'GCTGCCGTGTTAGTGGTTAC3'
H201N Primer C	5'GTAACCACTAACACGGCAGC3'
H230N Primer B	5'TCATCGCTGTTACCAATACA3'
H230N Primer C	5'TGTATTGGTAACAGCGATGA3'
H254N Primer B	5'TGCGGGATGTTGTCTAGACC3'
H254N Primer C	5'GGTCTAGACAACATCCCGCA3'
H257N Primer B	5'AATCGCACTGTTCGGGATGT3'
H257N Primer C	5'ACATCCCGAACAGTGCGATT3'

Sequencing of Mutants. The *opd* gene in each of the eight recombinants, pJK01–pJK08, was completely sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977) to ensure that only the desired base changes were present. Double-stranded plasmid DNA (dsDNA) was isolated using the Magic Miniprep DNA purification system and cut 3' to the *opd* gene with *Eco*RI. The linear dsDNA was then incubated with the T7 Gene 6 5'→3' exonuclease to make single-stranded DNA (ssDNA), which was then sequenced using the Sequenase version 2.0 DNA sequencing kit.

Growth Conditions. Overnight cultures of the transformed cells grown in Luria–Bertani (LB) broth (Maniatis et al., 1982) were used to inoculate modified LB medium (Post et al., 1990) containing 1 mM $CoCl_2$ and 50 $\mu g/mL$ ampicillin. The cobalt-supplemented cultures were incubated at 30 °C and induced with isopropyl D-thiogalactopyranoside (IPTG) in the early log phase. Cells were harvested in the stationary phase.

Purification of Wild-Type and Mutant Enzymes. Phosphotriesterase wild-type and mutant enzymes were purified from BL21/pJK01–BL21/pJK08 according to the procedures given by Omburo et al. (1992), with the exception that the purification buffer contained 100 μM $CoCl_2$. SDS–PAGE and Western blots indicated that all of the mutants were of the same size as the wild-type protein.

Preparation of Apoenzyme. Apoenzyme was prepared by incubating enzyme with 2 mM *o*-phenanthroline (OP) at room temperature for 90 min until there was less than 1% residual activity left. The *o*-phenanthroline was removed by diafiltration with metal-free 50 mM HEPES (pH 8.5) buffer over an Amicon PM10 membrane until there was less than 0.5 μM OP remaining.

Reactivation of Apoenzyme with Zn(II), Cd(II), and Co-(II). Apoenzyme at a concentration of between 0.5 and 2 mg/mL was reconstituted with the desired metal by adding 2 equiv of either $ZnCl_2$, $CdSO_4$, or $CoCl_2$ per equivalent of enzyme and incubating the mixture at room temperature for at least 12 h.

Determination of Metal Content. The metal content of the cobalt-purified and metal-reconstituted wild-type and mutant enzymes was determined by atomic absorption spectrophotometry. The cobalt-purified enzymes were dialyzed against metal-free 50 mM HEPES (pH 8.5) buffer, with several buffer changes over a 24-h period at room temperature, to remove the $CoCl_2$ that was included in the purification buffer and any loosely bound metals from the enzymes. The metal-reconstituted enzymes were passed through Pharmacia PD-10 Sephadex G-25 desalting columns to remove any loosely bound metals. All samples were analyzed on a Perkin-Elmer 2380 atomic absorption spectrophotometer. The errors in metal concentration are estimated to be ±15%.

Determination of Enzyme Activity. The activity for each of the enzyme derivatives was measured by monitoring the appearance of *p*-nitrophenol produced by the hydrolysis of paraoxon. The assay mixture consisted of 1 mM paraoxon in 3 mL of 100 mM CHES (pH 9.0) buffer. In the cases of the cobalt-substituted H55N, H57N, H201N, H230N, and H254N mutants, it was necessary to add extra $CoCl_2$ to the assay mixtures in order to obtain maximum activities (50, 80, 200, 10, and 500 μM $CoCl_2$, respectively). All spectrophotometric measurements were made at 25 °C with a Gilford Model 260 spectrophotometer. Rates were determined by monitoring the production of *p*-nitrophenol at 400 nm for 1 min, and the values for V_{max} and V/K_m were calculated as described previously (Caldwell et al., 1991). The extinction coefficient for *p*-nitrophenol at 400 nm was 17 000 M^{-1} cm^{-1}.

pH–Rate Profiles. The apparent pK_a values were determined for the Zn(II), Cd(II), and Co(II) forms of the wild-type and mutant enzymes. Each enzyme derivative was assayed over the pH range 5.0–11.0 at increments of 0.25. The V_{max} values at each pH were approximated by using a saturating amount of substrate in each assay. The assay mixtures consisted of 4 mM paraoxon in 3 mL of 100 mM buffer. The buffers used were sodium acetate, 2-(*N*-morpholino)ethanesulfonic acid (MES), piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), *N*-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), *N*-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid (TAPS), 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS). Other assay conditions were the same as those given above. The apparent extinction coefficient for *p*-nitrophenol at each pH value was calculated using the equation

$$\epsilon_{app} = \epsilon/(10^{pK_a - pH} + 1) \qquad (1)$$

where the extinction coefficient is 17 000 M^{-1} cm^{-1} and the pK_a value for *p*-nitrophenol is 7.14.

The p*K* values for each metal-substituted mutant were calculated using one of the following equations:

$$\log V = \log(C/(1 + 10^{-pH}/K_1)) \qquad (2)$$

or

$$\log V = \log(C/(1 + K_2/10^{-pH})) \qquad (3)$$

or

$$\log V = \log(C(1 + 10^{-pH}/K_1)/(1 + (10^{-pH}/K_2)(1 + 10^{-pH}/K_3))) \qquad (4)$$

where *V* is the pH-dependent value of V_{max} and *C* is the pH-independent value of V_{max}, or

$$\log V = \log((Y_l + Y_h(K_1/10^{-pH}))/(1 + K_1/10^{-pH})) \qquad (5)$$

or

Table 3: Activities and Metal Content of Cobalt-Purified Wild-Type and Mutant Enzymes

enzyme	(mg of enzyme)/ (g of cell paste)[a]	K_m (μM)[b]	V_{max} (units/mg)	k_{cat}/K_m (M^{-1} s^{-1})	metal content[c]		
					Co(II)/enzyme	Zn(II)/enzyme	Fe(II)/enzyme
WT	3.0	110	9600	5.0×10^7	2.1	0.4	0.1
H55N	0.5	110	34	1.8×10^5	0	0.02	
H57N	2.3	430	350	4.9×10^5	0	0.08	
H123N	2.3	99	8000	4.9×10^7	1.0	0.2	0.03
H201N	1.4	9	16	1.1×10^6	1.0	0.2	0.01
H230N	1.2	320	240	4.5×10^5	1.1	0.1	0.04
H254N	1.7	41	670	9.7×10^6	0.5	0.1	0.06
H257N	1.2	430	4700	6.6×10^6	1.0	0.6	0.06

[a] Cells were grown in CoCl$_2$-supplemented medium. [b] Assays were done at pH 9.0 and 25 °C with paraoxon as the substrate. [c] The cobalt-purified proteins were dialyzed against metal-free buffer before atomic absorption was performed.

$$\log V = \log((Y_h + Y_l(10^{-pH}/K_1))/((1 + 10^{-pH}/K_1)(1 + K_2/10^{-pH}))) \quad (6)$$

where V is the pH-dependent value of V_{max}, Y_l is the minimum pH-independent value of V_{max}, and Y_h is the maximum pH-independent value of V_{max}.

Circular Dichroism Studies. Wild-type and mutant enzymes were exchanged into 50 mM potassium phosphate (pH 8.0) buffer using Pharmacia PD-10 Sephadex G-25 desalting columns and scanned over the far-UV range (250–190 nm) in a 0.2-mm cell using a Jasco J-600 spectropolarimeter.

RESULTS

Construction, Expression, and Purification of Recombinants. The *opd* gene from *Pseudomonas diminuta* was subcloned without its leader sequence into an *E. coli* expression system. In the reconstructed *opd* gene, the codon for Ser-30, which is the first amino acid residue in the mature phosphotriesterase, was placed immediately after the Met start codon. Substitution of the *P. diminuta* ribosomal binding site with an optimized *E. coli* ribosomal binding site increased the expression of phosphotriesterase by 5-fold. The seven histidine residues of phosphotriesterase were individually mutated to asparagines by site-directed mutagenesis, employing the method of overlap extension PCR. The DNA sequences for the wild-type, H55N, H57N, H201N, and H230N recombinants contained no extraneous base changes. The sequences for the H123N, H254N, and H257N recombinants, however, in addition to the desired mutations, contained a few silent base changes. In the H123N gene, the codons for Arg-91 and Ala-126 were altered (AGA→AGG and GCG→GCA). In the H254N gene, the codons for Ala-281 and Ser-307 were altered (GCT→GCC and TCG→TCA). Finally, the codon for Ile-260 in the H257N gene was altered (ATT→ATA). The mutant proteins were expressed and purified to homogeneity by the same methods used for the wild-type protein; however, none of the mutants was expressed as well as the wild-type protein (Table 3). The expression of the H55N mutant was especially low, with a yield of less than 20% compared to the wild-type enzyme. The metal content, as well as the kinetic parameters, V_{max} and V/K_m, of the cobalt-purified mutant enzymes ranged in value from less than 1% to 100% of the wild-type values (Table 3). The H55N and H201N mutants had less than 1% activity, and the H57N, H230N, and H254N mutants had less than 10% activity, while the H123N and H257N mutants had at least 50% activity compared to wild-type phosphotriesterase. All of the cobalt-purified mutants bound fewer metals than the wild-type enzyme, with the H55N and H57N mutants showing no bound metals at all after dialysis with metal-free buffer.
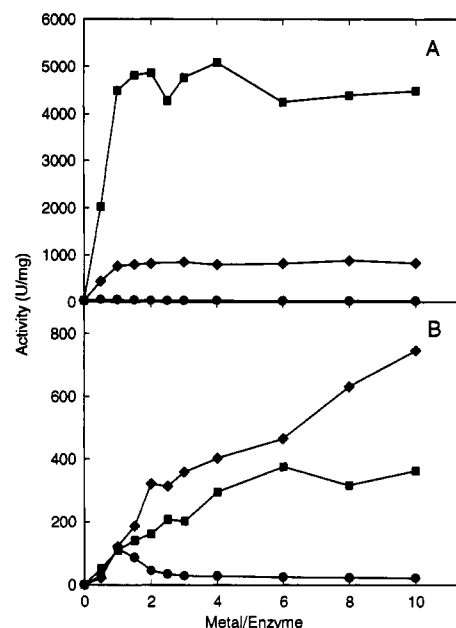

FIGURE 1: Reactivation of mutants H254N and H230N with varying amounts of metal. The apoenzymes were incubated with 0–10 equiv of ZnCl$_2$ (●), CdSO$_4$ (■), or CoCl$_2$ (◆), and activities (units/mg) were measured after 12 h of incubation at room temperature. (A) The H254N mutant required only 1 equiv of metal per enzyme to be fully reactivated. (B) The H230N mutant required an excess of metals to be fully reactivated.

Reactivation of the Apoenzymes. The native Zn(II) metal in the mutant enzymes could be replaced with other divalent metals, such as Cd(II) and Co(II). Apoenzyme was prepared through incubation with the metal chelator, *o*-phenanthroline. Reactivation experiments were conducted with varying amounts of ZnCl$_2$, CdSO$_4$, and CoCl$_2$ to determine how many equivalents of metal were required to reactivate each enzyme fully. Apoenzyme was incubated at room temperature with 0–10 equiv of metal and assayed for activity every 2 h up to 12 h and again after 24 h of incubation. All of the enzymes were fully reactivated by 12 h. Typical reactivation curves of the H230N and H254N mutant enzymes with ZnCl$_2$, CdSO$_4$, and CoCl$_2$ at 12 h are shown in Figure 1. The H254N and H257N mutants required only 1 equiv of metal per equivalent of enzyme to achieve maximal activity under the conditions of the reconstitution experiment. The H123N and H201N mutants required between 1 and 2 equiv of metal, while the H55N, H57N, and H230N mutants required an excess of metal to be fully reactivated.

Metal Content of Zinc-, Cadmium-, and Cobalt-Reconstituted Mutants. In order to determine whether either of the metal binding sites in phosphotriesterase was affected by any of the histidine replacements, atomic absorption spectrophotometry was conducted with each metal-substituted

Table 4: Metal Content of Reconstituted Enzymes[a]

enzyme	Zn(II)/enzyme	Cd(II)/enzyme	Co(II)/enzyme
wild-type	1.5	1.6	1.3
H55N	0.6	0	0
H57N	1.4	0	0
H123N	1.5	1.6	1.3
H201N	1.5	1.0	0.8
H230N	1.6	1.2	1.1
H254N	1.6	1.6	1.2
H257N	1.6	1.7	1.3

[a] Apoenzymes were reconstituted with 2 equiv of the indicated metal and desalted through Sephadex G-25 before atomic absorption spectrophotometry was performed.

mutant. Apoenzyme was incubated at room temperature with 2 equiv of $ZnCl_2$, $CdSO_4$, or $CoCl_2$ for 12 h. The metal-reconstituted enzymes were then passed through G-25 Sephadex desalting columns to remove any loosely bound metals before atomic absorption spectrophotometry was performed. The zinc-reconstituted wild-type and histidine mutant enzymes, except for H55N, all appeared to have approximately 1.5 equiv of zinc bound per enzyme. The H55N mutant bound less than 1 equiv of zinc per enzyme. In the case of the cadmium-reconstituted enzymes, the wild-type, H123N, H254N, and H257N mutants indicated at least 1.6 equiv of cadmium bound per enzyme. The H201N and H230N mutants, on the other hand, indicated only 1 equiv of cadmium per enzyme, while the H55N and H57N mutants showed no cadmium bound at all. For the cobalt-reconstituted enzymes, all except the H55N and H57N mutants indicated between 0.8 and 1.3 equiv of cobalt bound per enzyme. The H55N and H57N mutants showed no cobalt bound. Results are summarized in Table 4.

Kinetic Properties of Zinc-Substituted Mutants. The zinc-substituted enzymes showed the lowest activities of the three metal derivatives, even though zinc seemed to be the most tightly bound of the three metals. The kinetic parameters, V_{max} and V/K_m, for the hydrolysis of paraoxon with the H123N mutant were essentially identical to those of the wild-type enzyme. The H257N mutant had higher activity compared to the wild-type enzyme, but a lower V/K_m value. The H201N, H230N, and H254N mutants all had V_{max} values around 1% that of the wild-type enzyme, although the V/K_m values varied widely. The V_{max} and V/K_m values for the H57N mutant were 10% of those for the wild-type enzyme. The H55N mutant was the least active of the seven histidine mutants, showing V_{max} and V/K_m values of less than 1.0%.

Kinetic Properties of Cadmium-Substituted Mutants. The cadmium-substituted wild-type enzyme was more than twice as active as the zinc enzyme. The cadmium mutants behaved somewhat differently from the zinc mutants, although the H123N mutant was again essentially the same as the wild-type enzyme. The V_{max} and V/K_m values for the H57N, H254N, and H257N mutants were within 20–60% of the wild-type values, while the kinetic values for the H201N and H230N mutants were 10% or less. The H55N mutant had the lowest activity, with V_{max} and V/K_m values of less than 1% relative to the wild-type enzyme.

Kinetic Properties of Cobalt-Substituted Mutants. The cobalt-substituted enzymes were the most active of the three forms. The H123N and H257N mutants were as active as the wild-type enzyme, although the V/K_m value for the H257N mutant was only 20% of the wild-type value. The H254N mutant was only 10% as active, but had a V/K_m value one-half that of the wild-type value. The H57N and H230N mutants were 5% as active, with equally low V/K_m values.

The H55N and H201N mutants were less than 1% as active as the wild-type enzyme. The H55N mutant was again the least active of the seven histidine mutants.

pH-Rate Profiles of Zinc-Substituted Mutants. The pH-rate profiles for the zinc wild-type enzyme and most of the zinc-substituted mutants were fit to eq 2. The H123N mutant had the same pK_a value as the wild-type enzyme, but the other mutants all showed a shift to higher pK_a values. The pH-rate profile for the H55N mutant was fit to eq 5. The pH-rate profile for the H257N mutant was fit to eq 6 and gave both a pK_a and a pK_b value. The H57N mutant was the most unusual in that it appeared to have only a pK_b value; its pH-rate profile was fit to eq 3.

pH-Rate Profiles of Cadmium-Substituted Mutants. The pK_a value for the cadmium-substituted wild-type enzyme was shifted to over 2 units higher compared to that for the zinc-substituted wild-type enzyme. The cadmium-substituted H123N, H254N, and H257N mutants had similar pK_a values compared to the wild-type enzyme, while the H55N, H57N, H201N, and H230N mutants had higher values. The pH-rate profiles for all but the H55N and H254N mutants were fit to eq 2. The pH-rate profile for the H55N mutant was fit to eq 5, and the pH-rate profile for the H254N mutant was fit to eq 4.

pH-Rate Profiles of Cobalt-Substituted Mutants. The pK_a value for the cobalt-substituted wild-type enzyme was intermediate to those of the zinc and cadmium forms. The pK_a value for the H123N mutant was again consistent with that of the wild-type enzyme. The H254N mutant had an unexpectedly lower pK_a value compared to the wild-type enzyme, but the other mutants behaved as expected by having higher pK_a values, with the exception of the H55N and H230N mutants. It was not possible to obtain a fit of the pH-rate profiles of the H55N and H230N mutants to any of the given eq 2–6. The profile for the H230N mutant showed a significant dip in its V_{max} values between pH 7.5 and 9.0 that did not fit to eq 4, while the profile for the H55N mutant showed a linear increase in V_{max} up through pH 11.0, with no signs of leveling off. With the exception of the H257N mutant, all of the pH-rate profiles were fit to eq 2. The pH-rate profile for the H257N mutant was fit to eq 5. A representative example of each type of pH-rate profile is given in Figure 2. The kinetic parameters and pK_a values for the various histidine mutant enzymes are summarized in Table 5.

Circular Dichroism Studies. Circular dichroism studies were carried out to determine whether there were any gross changes in the overall structures of the seven histidine mutants compared to the wild-type enzyme. A scan of the far-UV region for each mutant indicated that all of the mutants were very similar in structure to the wild-type protein (data not shown) and, furthermore, indicated that the phosphotriesterase contains a significant amount of α-helical secondary structure. The spectra for the H201N and H55N mutants indicated that those samples may have contained some denatured protein, but an expansion of their experimental curves gave the same spectra as that of the wild-type enzyme.

DISCUSSION

Histidine residues are known to be essential in many zinc metalloenzymes. In the zinc enzymes whose structures are known, histidine residues appear to be the most common ligand to catalytic zinc centers. In a comparison of the structures of 12 zinc metalloenzymes, Vallee and Auld (1990a,b) show that most of the zinc ions are coordinated to four ligands, regardless of whether they are catalytic or structural centers.
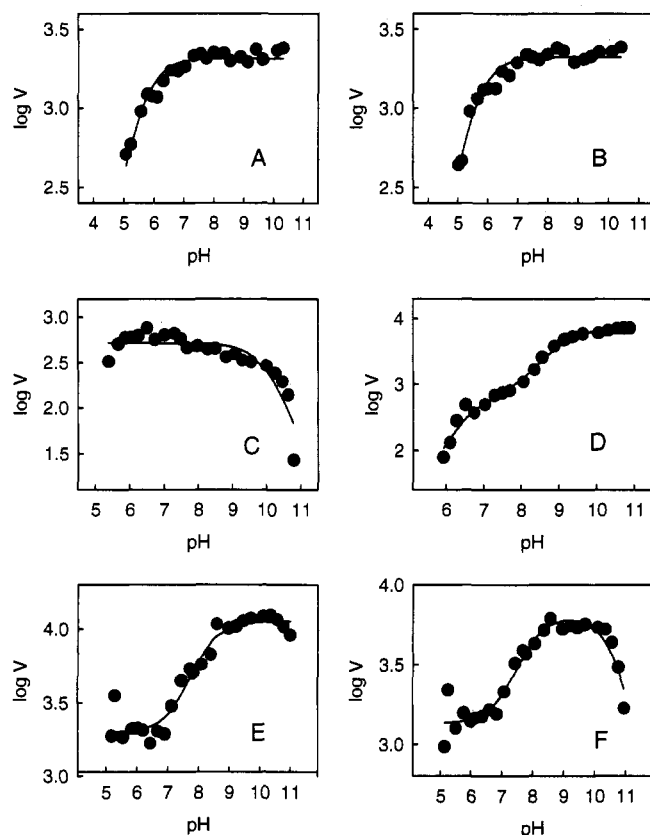
FIGURE 2: pH–rate profiles for selected metal-activated mutants: (A) Zn(II)–wild-type fit to eq 2; (B) Zn(II)–H123N fit to eq 2; (C) Zn(II)–H57N fit to eq 3; (D) Cd(II)–H254N fit to eq 4; (E) Co(II)–H257N fit to eq 5; and (F) Zn(II)–H257N fit to eq 6.

For catalytically active zinc ions, three of the ligands are derived from amino acid residues, with histidine residues being the most common, followed by glutamate, aspartate, and cysteine. An activated H_2O is the universal fourth ligand at all catalytic zinc sites. In the case of structural zinc centers, all four ligands are invariably composed of amino acid residues, with cysteine being the most common ligand. The spacing of the liganding amino acid residues in the primary sequences of the enzymes has also been noted. The three amino acid ligands to catalytic metal centers appear to have a pattern to their spacing within the protein sequence. Two of the residues are usually separated by a short spacer of 1–3 amino acid residues, while the third residue is usually separated by a long spacer of 20–120 residues. It is proposed that the two closely spaced amino acid residues act to form a bidentate zinc complex and that the long spacer of the third ligand confers flexibility to the three-dimensional alignment of the active site, allowing for the approach of substrate or for the required positioning of the zinc atom for catalysis. The close spacing of the four ligands to structural zinc atoms, on the other hand, imparts rigidity to the metal centers, consistent with their role in stabilizing both overall protein structure and local conformation.

An examination of the structures of four multi-zinc enzymes reveals similar motifs (Vallee & Auld, 1993). Alkaline phosphatase, phospholipase C, P1 nuclease, and leucine aminopeptidase all possess cocatalytic sites consisting of two or more zinc atoms closely spaced together. In these systems it is thought that the metals act in concert to bring about catalysis, with one of the zinc ions acting as the catalytic metal and the other(s) assisting in coordinating to substrate and/or product. The amino acid sequences of these enzymes reveal that the ligands to the zinc ions also have the pattern

Table 5: Kinetic Parameters for the Reconstituted Wild-Type and Mutant Enzymes[a,b]

enzyme	K_m (μM)	V_{max} (units/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)	pK_1	pK_2	pK_3
wild-type							
Zn	68	3800	2300	3.4×10^7	5.7		
Cd	460	10000	6000	1.3×10^7	8.1		
Co	130	13000	7800	6.0×10^7	6.5		
H55N							
Zn	16	1.4	0.83	5.2×10^4	9.7		
Cd	720	4.4	2.6	3.6×10^3	9.1		
Co	660	0.26	0.16	2.4×10^2			
H57N							
Zn	78	420	250	3.2×10^6		10.0	
Cd	1100	5100	3100	2.8×10^6	8.8		
Co	310	650	390	1.2×10^6	8.1		
H123N							
Zn	64	3500	2100	3.3×10^7	5.6		
Cd	440	8300	5000	1.1×10^7	8.2		
Co	130	9400	5600	4.3×10^7	6.6		
H201N							
Zn	11	31	19	1.7×10^6	8.2		
Cd	90	230	140	1.6×10^6	9.2		
Co	16	42	25	1.6×10^6	8.4		
H230N							
Zn	67	44	26	3.9×10^5	6.8		
Cd	530	260	150	2.8×10^5	9.5		
Co	290	830	500	1.7×10^6			
H254N							
Zn	1.3	56	34	2.6×10^7	7.0		
Cd	540	6000	3600	6.7×10^6	8.0	9.0	6.6
Co	27	1400	900	3.3×10^7	5.3		
H257N							
Zn	550	7300	4400	8.0×10^6	7.8	10.7	
Cd	780	5500	3300	4.2×10^6	8.2		
Co	640	12000	7000	1.1×10^7	8.1		

[a] Assays were done with paraoxon at pH 9.0, 25 °C. [b] V_{max} values are ±10%, K_m values are ±20%, and pK values are ±0.1 pH unit.

of two of the amino acid residues separated by a short spacer and a third amino acid residue separated by a long spacer. The multi-zinc sites have an additional feature of two of the metal atoms being bridged by a common ligand. In the enzymes under consideration, this shared ligand is an aspartic or glutamic acid residue, but the number of enzymes in this comparison is quite small. On the other hand, the X-ray structure of bovine Cu,Zn superoxide dismutase (Tainer et al., 1982) reveals that the metal-bridging ligand can also be a histidine residue.

Comparison of the amino acid sequences of several zinc enzymes whose structures are known reveal that, within the same families, there is a high degree of conservation in the residues around the vicinities of the ligands to catalytically active zinc atoms. Given what is known so far about the coordination to the metal centers in zinc metalloenzymes, the fact that phosphotriesterase has at least one catalytically active zinc center and only seven histidine residues increases the probability that some or all of the histidines are coordinated to the two zinc atoms. A comparison of the sequences around each histidine residue in phosphotriesterase to the GenBank and EMBL database files using software from the Genetics Computer Group, Inc. (Madison, WI) reveals no homology to any known sequence, indicating that phosphotriesterase does not belong to any of the families of zinc metalloenzymes whose structures are known to date. Since phosphotriesterase does not appear to belong to any of the known families of zinc metalloenzymes, elucidation of the identity of the ligands to its two zinc centers will provide further insight into the conformations of catalytic zinc sites and ultimately lead to a better understanding of the specificity of these enzymes and their mechanisms of action.

In order to determine the importance of each of the histidines in phosphotriesterase to its catalytic activity, each of the histidine residues was converted to an asparagine residue through site-directed mutagenesis, and the metal content, reactivation parameters, kinetic parameters, and pH–rate profiles were determined for each of the mutants with a variety of metals incorporated into their metal sites. His-123 is the only one of the seven histidines in phosphotriesterase that is unequivocally uninvolved in any way with the catalytic activity or structural integrity of this enzyme. The reconstituted metal content, the kinetic parameters V_{max} and V/K_m, and the pH–rate profiles determined for the Zn(II)-, Cd(II)-, and Co(II)-substituted H123N mutant are identical to the values for the wild-type enzyme. In addition, a ^{113}Cd NMR spectrum of the ^{113}Cd-substituted H123N mutant gives resonance peaks in the NMR spectrum that are identical to those observed for the wild-type enzyme (L. Mullins and J. Kuo, unpublished observations). The data for the other six histidine mutants are somewhat more difficult to interpret. On the basis of the observed activities and pH–rate profiles, it is possible to say that these histidines are all involved in some capacity at the metal centers and/or active site. On the other hand, the substitution of an asparagine for a histidine may cause enough of a disruption to the catalytic or binding site conformation in a particular mutant to affect its activity without that residue being directly involved with the metal centers. The H257N mutant derivatives are as active as the wild-type enzyme, but the V/K_m values are lower by over 60% relative to wild-type values, suggesting that this mutant may not bind substrate as tightly as the wild-type enzyme. The pH–rate profiles for the Zn(II) and Co(II) derivatives of this mutant further suggest that in this mutant a hitherto uninvolved group may now be affecting the catalytic activity. The mutant H254N is also nearly as active as the wild-type enzyme in its Cd(II) form, but its specific activity is reduced 10–100-fold in its Zn(II) and Co(II) forms. However, the V/K_m values for all three metal derivatives of this mutant are close to the wild-type values, suggesting that substrate binding in this mutant remains relatively unaffected. The pK_a values for the three enzyme derivatives differ from the wild-type values, but the general shapes of the pH–rate profiles are similar, indicating that some group on the H254N mutant must be deprotonated for full enzymatic activity, as is the case with the wild-type enzyme. The metal contents of the reconstituted forms of mutants H257N and H254N are the same as for the wild-type enzyme. Aside from His-123, the mutation of residues His-257 and His-254 has the least overall effect on the properties of phosphotriesterase.

Experimental results for the remaining four mutants, H55N, H57N, H201N, and H230N, suggest that these four histidine residues are likely candidates for catalytic metal center ligands in phosphotriesterase, provided the amino acid residue substitutions have not disrupted the active-site conformations of the mutants significantly. The H55N and H57N mutants show the least number of metals bound in the various metal-reconstituted forms. In fact, the amounts of Cd(II)- and Co(II) in the Cd(II) and Co(II)-substituted forms of these two mutants were below detection levels, although in each case the Cd(II) form of the enzyme was the most active of the three derivatives. These data suggest that the metal binding affinity in both of these mutants has been weakened, possibly by the removal of one of the metal-coordinating ligands. Both the V_{max} and V/K_m values for these two mutants have been significantly reduced compared to those of the wild-type enzyme, lending further credence to the possibility that
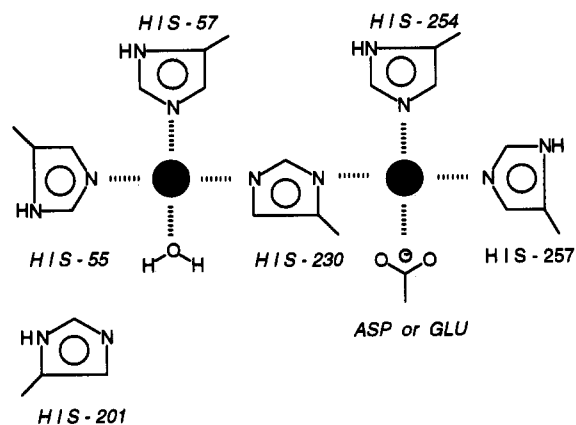

FIGURE 3: Working model of the active site/binuclear metal center of phosphotriesterase.

His-55 and His-57 are metal-coordinating ligands. The pH–rate profiles for the H55N mutant exhibit a wave pattern, indicating that the enzyme retains residual activity at low pH values where the proposed catalytic base is protonated and inactive. Since the overall pK_a values of this mutant have been shifted upward considerably, there is the possibility that this pattern might have appeared for the wild-type enzyme if a wider pH range had been assayed. If this were the case, it would provide a new possibility for the chemical mechanism of phosphotriester hydrolysis. Instead of directly attacking the activated water molecule, the putative catalytic base group could instead be acting as a proton-shuttle group to remove a proton from the active site to the surrounding outer environment, similar to the mechanism proposed for carbonic anhydrase II (Tu et al., 1989). The pH–rate profiles for the H57N mutant, on the other hand, exhibit the same shapes as those of the wild-type enzyme except for the Zn(II)-substituted mutant, which behaves totally unexpectedly by having the opposite pattern, suggesting that a group needs to be protonated for enzymatic activity. Further investigation is required to explain this anomaly.

The H201N and H230N mutants, in their Zn(II)-reconstituted forms, possess the same number of metals as does the wild-type enzyme, but their Cd(II) and Co(II) forms contain less, indicating that these two metals do not bind well to these mutants. The V_{max} and V/K_m values for both mutants are also quite low compared to the wild-type values, so that it is quite likely that these two histidine residues are involved in the catalytic process. The pH–rate profiles of the H201N and H230N mutants do not show any unusual features, as has been noted in some of the other mutants. However, it should be noted for H201N that the pK_a of the group that must be unprotonated for activity has been increased by 1.1–2.5 pK units, depending on the metal ion activator. A preliminary ^{113}Cd NMR spectrum of the ^{113}Cd-substituted H230N mutant indicates two resonance peaks upfield of the two resonances that appear in spectra for the wild-type enzyme (unpublished observations). One possibility suggested by these data is that His-230 acts as the bridging ligand to both metal sites in phosphotriesterase, and thus the two resonance peaks would shift upfield together in the H230N mutant upon the replacement of a nitrogen ligand with an oxygen ligand.

On the basis of the data obtained thus far for the seven histidine to asparagine mutants, a working model for the active site/metal centers of phosphotriesterase that incorporates six of the histidines (Figure 3) can be proposed. In this model, the two metal ions are bridged by the His-230 residue. One of the metal ions acts as the primary catalytic metal with the

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coordinated water ligand, while the other provides secondary structural and catalytic support. Omburo et al. (1993) discovered that the Zn/Cd hybrid derivative of the wild-type enzyme exhibited kinetic properties that were identical to those of the Zn/Zn-substituted enzyme. ^{113}Cd NMR analysis of the Zn/Cd hybrid clearly demonstrated that the Zn was occupying the M_α site, while the Cd was occupying the M_β site. Therefore, the particular metal occupying the M_α site appears to dictate the kinetic properties of the phosphotriesterase, and the metal occupying the M_β site appears to play a more secondary role in binding and catalysis. Two critical histidines, His-55 and His-57, are proposed in this model to form a bidentate zinc complex to the metal occupying the M_α site, while two other histidines, His-254 and His-257, are proposed to form a bidentate zinc complex with the other metal at the less critical M_β site. A water molecule is the fourth ligand to the catalytic metal, and a carboxyl group, possibly a glutamate or aspartate, acts as the fourth ligand to the secondary metal. The sixth histidine, possibly His-201, is proposed to be poised near the catalytic H_2O to act as either a catalytic base or a proton-shuttle group. His-123 is not involved in catalysis or the binding of substrates or metal. In this working model, the metal occupying the M_α site would function as an activator of the hydrolytic water molecule and possibly polarize the phosphoryl oxygen bond. The other metal might also polarize this same bond in addition to providing charge neutralization to the leaving group. The model proposed in Figure 3 is very reminiscent of that observed for the active site of bovine Cu,Zn superoxide dismutase (Tainer et al., 1982).

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