

Perturbations to the Active Site of Phosphotriesterase[†]

Jane M. Kuo, Myeong Yun Chae, and Frank M. Raushel*

Department of Chemistry, Texas A&M University, College Station, Texas 77843

Received August 20, 1996; Revised Manuscript Received December 2, 1996[⊗]

ABSTRACT: Phosphotriesterase catalyzes the hydrolysis of organophosphate nerve agents. Four amino acid residues, located within the active site pocket, were mutated in an effort to ascertain the roles that these groups play in the structure and function of this enzyme. Tryptophan-131 is located at the entrance to the binuclear metal center, and the indole ring is positioned to suggest that it could provide a hydrophobic site for interaction of the aromatic leaving group with optimized substrates. The W131F mutant displays catalytic constants for the hydrolysis of paraoxon that are essentially the same as those of the wild type enzyme. However, the K_m value for the W131A mutant is elevated by a factor of 6, consistent with a role for this residue in substrate binding. Aspartate-253 is hydrogen bonded to His-230 which, in turn, is directly ligated to the more solvent-exposed metal ion. The D253N mutant possesses catalytic constants that are virtually the same as those of the wild type enzyme, while the D253A mutant is reduced in activity by 500-fold. These results are consistent with a model where this residue is required to orientate the imidazole side chain of His-230 for proper interaction with the binuclear metal center. Aspartate-301 is a primary ligand to the more buried metal ion. Mutation of this residue to histidine, asparagine, alanine, and cysteine reduces the catalytic activity by factors of 2.6×10^4 , 2.7×10^3 , 5.6×10^2 , and 1.5×10^2 , respectively. These results indicate that alterations to the direct metal ligands, even with residues that can strongly coordinate divalent cations, cause a severe disruption to the proper functioning of the active site. In the wild type enzyme, the side chain of Lys-169 is carbamylated and also acts as a bridge between the two divalent cations. Significant losses in catalytic activity are obtained upon mutation of this residue to either alanine, glutamate, arginine, or methionine. The loss in activity can partially be restored upon inclusion in the assay mixture of short-chain carboxylic acids. A 25-fold enhancement in k_{cat} is observed for the K169A mutant in the presence of 100 mM propionic acid.

Phosphotriesterase (PTE¹) from *Pseudomonas diminuta* is a zinc metalloenzyme which catalyzes the hydrolysis of a variety of phosphotriesters and related phosphonates. The hydrolysis reaction proceeds via an S_N2 -like mechanism, whereby an activated water molecule or hydroxide directly displaces the leaving group, resulting in a net inversion of stereochemistry at the phosphorus center (Lewis et al., 1988). The best substrate found to date is the insecticide diethyl *p*-nitrophenyl phosphate (paraoxon) which is hydrolyzed at nearly the diffusion-limited rate ($k_{cat}/K_m = 5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). The active site of the enzyme consists of a binuclear metal center, which in the native enzyme is comprised of two zinc(II) ions. The precise roles of the two metals are still unclear, although at least one of the metal ions participates in catalysis, since the apoenzyme is essentially inactive. Replacement of the native zinc(II) with cadmium(II), cobalt(II), manganese(II), or nickel(II) results in an enzyme that remains catalytically competent (Omburo et al., 1992). ¹¹³Cd(II) NMR (Omburo et al., 1993) and Mn(II) EPR (Chae et al., 1993) spectroscopy, in conjunction with site-directed

mutagenesis (Kuo & Raushel, 1994; Lai et al., 1994), chemical modification (Banzon et al., 1994), and X-ray crystallographic studies (Benning et al., 1994, 1995; Vanhooke et al., 1996), have demonstrated that six of the seven histidines in phosphotriesterase are clustered around the binuclear metal center, with at least four of these residues, His-55, His-57, His-201, and His-230, participating as direct ligands to the metals.

The solution of the three-dimensional structure of the apo form of phosphotriesterase by X-ray crystallography reveals that the enzyme is a homodimer with each subunit composed of eight strands of parallel β -pleated sheet in an α,β -barrel structure (Benning et al., 1994). The location of the predicted six-histidine cluster at the C terminus of the β -barrel is consistent with the structural motifs of other α,β -barrel enzymes, in which the active sites are invariably found in the C-terminal portion of the β -barrel (Farber & Petsko, 1990). Recently, the three-dimensional structure of the holoenzyme, reconstituted with cadmium(II), has been obtained, and it shows some surprising results. Significant structural rearrangements have occurred in several specific regions between the apo- and holoenzyme structures. Most importantly, and unexpectedly, Lys-169 has been unveiled as one of two bridging ligands between the two metal centers. The ϵ -amino group of this residue in the holoenzyme structure has been modified by reaction with carbon dioxide to form a carbamate group which subsequently coordinates to both metal ions. A water molecule serves as the second bridging ligand. The geometry around the more buried metal ion is trigonal bipyramidal with two nitrogen and three

[†] This research was supported in part by the National Institutes of Health (DK-30343) and the Advance Technology Program from the State of Texas. J.M.K. was supported by a training grant from the NIH to Texas A&M University (T32 GM 08523).

* To whom correspondence may be sent. Fax: 409-845-9452. E-mail: raushel@tamu.edu.

[⊗] Abstract published in *Advance ACS Abstracts*, February 1, 1997.

¹ Abbreviations: PTE, phosphotriesterase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PCR, polymerase chain reaction; RUBISCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

oxygen ligands, and the second metal ion is ligated by two nitrogen and four oxygen ligands in a distorted octahedral arrangement (Benning et al., 1995).

An examination of the putative active site of phosphotriesterase in the apo- and holoenzyme structures suggests at least four residues, other than the above-mentioned histidines (Trp-131, Lys-169, Asp-253, and Asp-301), which may be of some significance to the catalytic function of this protein. Asp-301 and Lys-169 are in direct contact with the metals (Benning et al., 1995). Asp-253 participates in an electrostatic interaction with the side chain imidazole of His-230, and this interaction may serve to orientate this histidine for optimum ligation to the more solvent-exposed metal ion. The extension and orientation of the side chain of Trp-131 into the pocket of the proposed active site lead to the supposition that this residue could act to position the aromatic ring of the substrate leaving group through π -stacking interactions. On the basis of the three-dimensional structure of the apoenzyme, site-specific mutations have been made at these four residues to help define what roles, if any, they may have in the catalytic mechanism or structural definition of phosphotriesterase.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma Chemical Co., Aldrich Chemical Co., or Fisher Scientific. Molecular biology supplies were purchased from Amersham, Perkin-Elmer Roche, Promega, and Bio 101. Oligonucleotides were synthesized by the Gene Technology Laboratory of the Biology Department of Texas A&M University.

Construction of Mutants. The phosphotriesterase mutants, W131F, W131A, K169M, K169A, K169E, K169R, D253N, D253A, D301N, D301A, D301C, and D301H, were constructed using the overlap extension method of PCR (Ho et al., 1989). The flanking A and D primers were the same ones previously used to create the histidine \rightarrow asparagine mutants (Kuo & Raushel, 1994). The nucleotide sequences of the mutagenic C primers for each new mutant were selected by replacement of the wild type codon with the one for the desired amino acid substitution (sequences not shown). The AB and CD fragments were amplified in a standard PCR reaction mixture using the GeneAmp DNA amplification kit with native Taq polymerase (Perkin-Elmer Roche). Thermocycling consisted of 26 cycles of 1 min each at 95, 50, and 72 °C. The reaction started with an initial melt of 4 min at 95 °C and ended with a final extension of 5 min at 72 °C. The fragments were purified from a 1% agarose gel with the Wizard PCR Preps DNA purification kit (Promega) and used in a second PCR to make the complete AD fragments. A standard reaction mixture was again used. The second thermocycling protocol began with an initial melt of 5 min at 95 °C, followed by 25 cycles of 1 min each at 95, 55, and 72 °C, and ended with a final extension of 7 min at 72 °C. The AD fragments were purified directly with the Wizard PCR Preps DNA purification kit, inserted into the BamHI site of the pBS⁺ phagemid (Stratagene) behind the *lac* promoter, and transformed into XL1-Blue cells. Plasmids with the correct inserts were screened as previously described (Kuo & Raushel, 1994).

Sequencing of Mutants. Double-stranded plasmid DNA was linearized with either *Pst*I or *Eco*RI, digested with T7 Gene 6 exonuclease to make single-stranded DNA, and

sequenced with the Sequenase Version 2.0 DNA sequencing kit (Amersham). The insert for each mutant was completely sequenced to eliminate any possibility of extraneous mutations introduced during the PCR amplifications.

Growth and Purification of Mutants. The plasmids for the phosphotriesterase mutants, pJK10 (W131F), pJK11 (K169M), pJK12 (D253N), pJK13 (W131A), pJK14 (K169A), pJK15 (D253A), pMC02 (K169R), pMC03 (K169E), pMC04 (D301A), pMC05 (D301N), pMC06 (D301C), and pMC07 (D301H), were transformed into BL21 cells, which were grown in cobalt chloride-supplemented medium (Kuo & Raushel, 1994). The expressed mutant enzymes were purified according to published procedures (Omburo et al., 1992).

Preparation and Reconstitution of Mutant Apoenzymes. Enzymes at a concentration of 1–2 mg/mL were incubated at room temperature with 2 mM 1,10-phenanthroline for 70–80 min until there was no detectable activity remaining. The chelator was removed by diafiltration with metal-free 50 mM HEPES (pH 7.5) buffer until there was less than 0.5 μ M 1,10-phenanthroline remaining. Apoenzymes (1–2 mg/mL) were reconstituted with 0, 0.5, 1.0, 1.5, 2.0, 5.0, and 10 equiv of ZnCl₂, CdSO₄, CoCl₂, and MnCl₂ at room temperature and assayed for catalytic activity in 100 mM CHES (pH 9.0) buffer every 2 h up to 12 h and again after 24 h of incubation. The hydrolysis of 1 mM paraoxon at 25 °C was monitored at 400 nm for 1 min. Some of the reconstituted enzymes were subsequently passed through a Pharmacia PD-10 Sephadex G-25 desalting column to remove loosely bound metals. Samples were analyzed for bound metal content with a Perkin-Elmer 2380 atomic absorption spectrophotometer as previously described (Omburo et al., 1992).

Activation of K169A and K169M Mutants by Short-Chain Carboxylic Acids. Cadmium(II)-substituted K169A and K169M mutant enzymes were assayed for enzymatic activity with 1.0 mM paraoxon in 100 mM CHES (pH 9.0) buffer supplemented with 0, 0.1, 1, 10, 50, and 100 mM acetic, propionic, or butyric acid. Cadmium(II)-substituted K169R and K169E enzymes were assayed for catalytic activity with 1.0 mM paraoxon in 100 mM CHES (pH 9.0) buffer supplemented with 0, 1, 5, 10, 50, and 100 mM formic, acetic, or propionic acid or with 0, 1, 5, 10, 40, 70, and 100 mM potassium bicarbonate. Cadmium(II)- and cobalt(II)-substituted K169M, K169A, K169E, K169R, and wild type enzymes were also incubated in 50 mM HEPES (pH 8.5) buffer with 100 mM bicarbonate (K169R and K169E), 100 mM formic acid (K169R and K169E), 100 mM acetic acid (wild type, K169M, K169R, and K169E enzymes), 100 mM propionic acid (wild type, K169A, K169R, and K169E enzymes), or 100 mM ethylamine/1 M potassium bicarbonate (all enzymes). After 25 h of incubation on ice, the enzymes were assayed for catalytic activity with 1.0 mM paraoxon in 100 mM CHES (pH 9.0) buffer with and without the corresponding effectors.

Reactivation of K169R and K169E Mutants by Bicarbonate. The apoenzymes of the K169R and K169E mutants were preincubated at room temperature for 1 h with 0, 10, and 100 mM KHCO₃. Two equivalents of Cd²⁺ was added to initiate the reconstitution. The enzymes were subsequently assayed for catalytic activity with 1.0 mM paraoxon in 100 mM CHES (pH 9.0) buffer as a function of time.

Statistical Analysis of Kinetic Data. The kinetic constants, V_{\max} and V/K_m , for the various mutant forms of PTE were

Table 1: Kinetic Parameters of Purified Mutant Enzymes^a

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
Zn-wild type	68	2300	3.4×10^7
Cd-wild type	460	6000	1.3×10^7
Co-wild type	130	7800	6.0×10^7
Co-W131F	290	4300	1.5×10^7
Co-W131A	710	5600	7.9×10^6
Co-K169M ^b	240	9	3.7×10^4
Co-K169A ^b	4 900	84	1.7×10^4
Zn-K169R	300	0.8	2.7×10^3
Cd-K169R	970	0.7	7.7×10^2
Co-K169R	300	0.5	1.7×10^3
Zn-K169E	58	0.11	1.9×10^4
Cd-K169E	1 000	0.3	3.3×10^2
Co-K169E	170	0.3	2.0×10^3
Co-D253N ^b	68	2300	3.4×10^7
Co-D253A ^b	240	15	6.1×10^4
Zn-D301H	38	0.07	1.7×10^3
Cd-D301H	830	0.4	4.6×10^2
Co-D301H	150	0.3	1.6×10^3
Zn-D301N	29	0.5	1.6×10^4
Cd-D301N	520	2.6	5.0×10^3
Co-D301N	180	2.9	1.6×10^4
Zn-D301A	650	7.8	1.2×10^4
Cd-D301A	320	17	5.4×10^4
Co-D301A	340	14	4.2×10^4
Zn-D301C	24	14	5.8×10^5
Cd-D301C	250	280	1.1×10^6
Co-D301C	17	51	3.0×10^6

^a K_m values are $\pm 10\%$, and k_{cat} values are $\pm 7\%$. The experiments were conducted at pH 9.0 in CHES buffer using variable concentrations of paraoxon as the substrate. ^b CoCl_2 ($40 \mu\text{M}$) included in assay solutions.

determined by fitting the data to eq 1 with the computer program supplied from Savanna Shell Software. In this

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

equation, v is the initial velocity, V is the maximal velocity, K_m is the Michaelis constant, and A is the substrate concentration. The assays were conducted at pH 9.0 in 100 mM CHES buffer using paraoxon as the variable substrate. Initial velocities were calculated from the linear portions of the associated time courses using the data collection program supplied by Savanna Shell Software.

RESULTS

Construction, Expression, and Purification of Mutant Enzymes. The W131F, W131A, K169M, K169A, K169E, K169R, D253N, D253A, D301A, D301N, D301C, and D301H phosphotriesterase mutants were constructed through overlap extension PCR. The genes for these mutants were completely sequenced and shown to encode the correct protein sequences. The mutant proteins were expressed and purified to homogeneity. Yields ranged from 1.3 mg of protein/(g of wet cell paste) to 10.7 mg/gm. Wild type preparations typically yield 2–3 mg/gm. The kinetic parameters, V_{max} and V/K_m , for the purified mutant enzymes revealed W131F, W131A, and D253N to be essentially wild type in activity. The V_{max} and V/K_m values for the rest of the mutations, using paraoxon as a substrate, were all 1% or less when compared to wild type values (Table 1) with the single exception of that of the D301C mutant. An enhancement was observed in the activities of the Lys-169 and Asp-253 mutants upon addition of $40 \mu\text{M}$ cobalt chloride to the assay solution. For the wild type enzyme, it has been

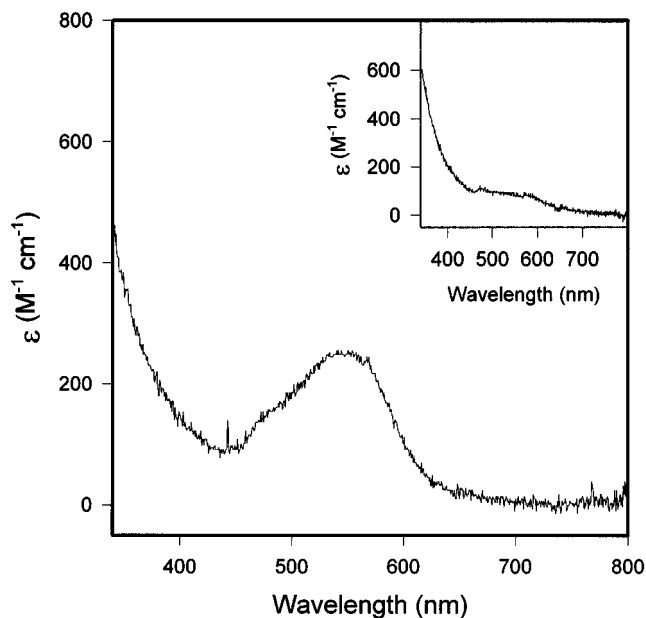


FIGURE 1: Visible absorbance spectrum for the K169M mutant of phosphotriesterase at 1.0 mg/mL at pH 8.5. The visible spectrum for the wild type enzyme substituted with cobalt is shown as a reference in the upper right-hand corner. The spectra were recorded on a Cary 2200 UV-Vis spectrophotometer after subtraction of a blank containing only the buffer solution.

demonstrated that for the hydrolysis of paraoxon the rate-limiting step for V_{max} is the release of products while the rate-limiting step for V/K_m is the rate of association of free enzyme with the substrate (Caldwell et al., 1991). With the mutant enzymes reported here, it is unknown which step in the reaction mechanism now limits these two kinetic parameters, but it is likely that the cleavage of the P–O bond in paraoxon limits these kinetic constants.

An unusual feature of the purified Lys-169 mutants was that they appeared pink in color. An absorbance scan from 800 to 300 nm on a CARY 2200 spectrophotometer indicated a small absorbance peak around 536 nm for the K169A and K169M mutants when compared to the spectrum for the purified wild type enzyme (Figure 1). The calculated extinction coefficients were determined to be $250 \text{ M}^{-1} \text{ cm}^{-1}$ for K169M and $60 \text{ M}^{-1} \text{ cm}^{-1}$ for K169A (data not shown). The K169R and K169E mutants were not visibly colored. The addition of 100 mM acetate to a solution of the K169M mutant did not perturb the visible spectrum.

Reactivation of K169A and K169M Apoenzymes. Apoenzymes were prepared from the K169A and K169M mutants with the metal chelator, 1,10-phenanthroline. Reactivation experiments with varying amounts of ZnCl_2 , CdSO_4 , CoCl_2 , and MnCl_2 were conducted to determine how many equivalents of metal were required to reactivate each enzyme fully. Both mutants appeared to be reactivated within 4 h with zinc(II) and cadmium(II). Between 1 and 2 equiv of Cd(II), Co(II), and Zn(II) required to maximally activate the K169A mutant. With the K169M mutant, between 1 and 2 equivalents of Cd(II) and Zn(II) were required to maximally activate the enzyme. Excess Mn(II) was required to fully activate the K169A and K169M mutants, and excess Co(II) was needed to fully activate the K169M mutant. The reactivation curves for the K169M mutant with ZnCl_2 , CdSO_4 , CoCl_2 , and MnCl_2 at 24 h are shown in Figure 2.

Activation of K169A and K169M Mutants by Short-Chain Carboxylic Acids. The K169A and K169M mutants were

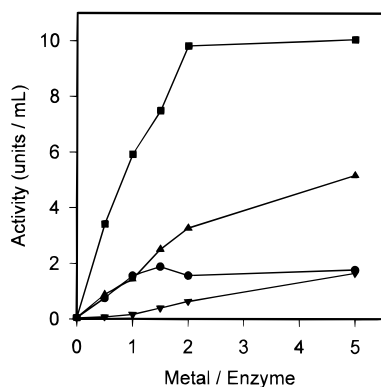


FIGURE 2: Reactivation profiles for the addition of divalent cations to the K169M mutant of phosphotriesterase. The divalent cations [ZnCl₂ (●), CdSO₄ (■), CoCl₂ (▲), and MnCl₂ (▼)] were added to the apoenzyme, and the relative catalytic activity was assayed after 24 h of incubation at room temperature.

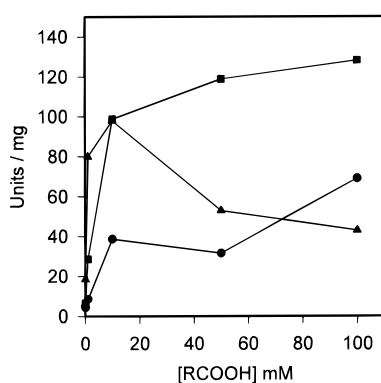


FIGURE 3: Effects of varying concentrations of acetic (●), propionic (■), and butyric (▲) acid on the catalytic activity of the cadmium(II)-K169A mutant. Additional details are given in the text.

assayed for paraoxonase activity in the presence of varying amounts of the short-chain carboxylic acids, acetic acid, propionic acid, and butyric acid. The K169R and K169E mutants were also assayed in the presence of formic acid. An enhancement in activity was observed with all three carboxylic acids for the K169A mutant, with propionic acid being the best activator. Enhancements in activity ranged up to 25-fold, depending on the concentration and type of carboxylic acid added. Acetic acid was the best activator for the K169M mutant, which showed increases in activity up to 5-fold. The K169R mutants showed no enhancement in activity with any of the carboxylic acids. Formic acid was the only carboxylic acid to enhance the rate of activity of the K169E mutant by about 4-fold. The wild type enzyme showed no enhancement of activity by any of the carboxylic

acids and, in fact, was increasingly inhibited by the increased length and concentration of the added acids. The effects of varying concentrations of acetic, propionic, and butyric acid on the activities of the K169A mutant are shown in Figure 3.

The K169A and K169M mutant enzymes were also preincubated with either a short-chain carboxylic acid or ethylamine/bicarbonate before being assayed for paraoxonase activity in the presence of the respective activation agents. Both mutants showed a substantial reduction in activity after 25 h of incubation in buffer alone, with the K169A mutant showing the greater loss; however, the wild type enzyme showed no loss of activity after incubation under these conditions. The cobalt(II) form of the K169A mutant was activated by propionic acid to 20 times its activity prior to the preincubation and by ethylamine/bicarbonate to 150% of its initial activity. The cobalt(II) form of the K169M mutant, on the other hand, was activated to 7 times its initial activity by acetic acid and showed no activation by ethylamine/bicarbonate. The cobalt(II) form of the wild type enzyme lost 20–30% of its activity when incubated with acetic acid, propionic acid, or ethylamine/bicarbonate. For the cadmium(II) forms of these enzymes, the results were somewhat different. Wild type enzyme lost 60–90% of its activity in the presence of the carboxylic acids or ethylamine/bicarbonate, and neither mutant was activated by ethylamine/bicarbonate. Preincubation of the K169M mutant with acetic acid achieved only 50% of the activity obtained without incubation. For the K169A mutant, preincubation in propionic acid resulted in activity 3 times higher than that with no incubation. The effects of the short-chain carboxylic acids upon enzyme activity appeared to be immediate, since activation was achieved without preincubation, and assaying the preincubated enzymes without their respective activators resulted in a reversal of their effects. The results of these experiments are summarized in Table 2.

Activation of K169R and K169E Mutants by Bicarbonate. Incubation of the inactive apo forms of the K169R and K169E mutant enzymes with 10 mM bicarbonate and 2 equiv of Cd²⁺ enhanced the rate of reactivation by a factor of about 3 for the K169R mutant and about 5 for the K169E mutant relative to the rate observed in the absence of added bicarbonate. The endogenous bicarbonate concentration was estimated to be approximately 0.5 mM. In the absence and presence of added bicarbonate, the first-order rate constant for the appearance of catalytic activity was 0.23 and 1.1 h⁻¹, respectively, for the K169E mutant. For the K169R mutant, the first-order rate constant for reactivation increased from a value of 0.44 h⁻¹ in the absence of added bicarbonate to a value of 1.5 h⁻¹ in the presence of 10 mM added

Table 2: Effects of Short-Chain Activators on Lys-169 Mutants^{a,b}

activator	cobalt(II) form ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)			cadmium(II) form ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)		
	K169A	K169M	wild type	K169A	K169M	wild type
none	0.38	2.0	10100	0.09	7.7	9200
100 mM		109	8500		30	3800
acetic acid		(2.8) ^c	(9200) ^c		(10) ^c	(9200) ^c
100 mM	2600		7200	370		780
propionic acid	(290) ^c		(10200) ^c	(38) ^c		(6700) ^c
100 mM ethylamine/1 M bicarbonate	210	1.0	6 800	0.26	0.18	2100
	(35) ^c	(1.7) ^c	(11800) ^c	(0) ^c	(2.2) ^c	(7500) ^c

^a Enzymes were incubated with activator for 25 h at 0 °C before being assayed with same activator in assay buffer. ^b Values are $\pm 10\%$. ^c Assayed without activator in assay buffer.

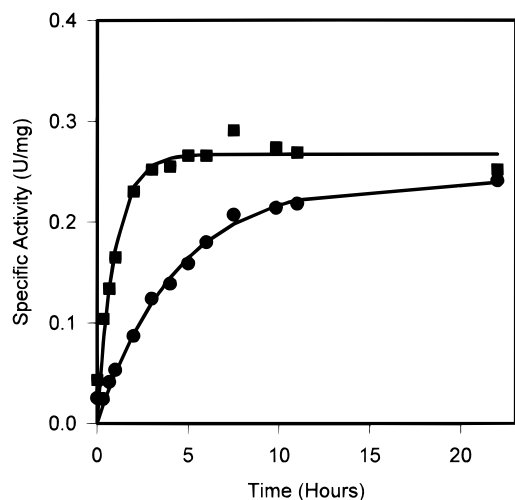


FIGURE 4: Reactivation of the apo form of the K169E mutant of phosphotriesterase. The enzyme was incubated with 2 equiv of Cd^{2+} and either no added bicarbonate (●) or 10 mM bicarbonate (■). Additional details are given in the text.

bicarbonate. The time courses for the K169E enzyme are shown in Figure 4.

DISCUSSION

Structure of the Binuclear Metal Center and Active Site. Of all the enzymes whose structures are known, approximately one out of ten has an eight-stranded α,β -barrel domain. It has been proposed that these enzymes may have diverged from a common ancestor, because, although in most cases they share no sequence similarity and catalyze a diverse range of reactions with varied cofactor requirements, they all possess certain similar structural features. Their active sites are all located in the C-terminal portion of the β -barrel, and without exception, all proteins with the eight-stranded α,β -barrel motif are enzymes (Farber & Petsko, 1990). The solution of the three-dimensional structure of the apo form of phosphotriesterase by X-ray crystallography to 2.1 Å resolution has revealed this enzyme to be a member of the α,β -barrel enzyme family (Benning et al., 1994). The overall fold of the molecule consists of an α,β -barrel with eight strands of parallel β -pleated sheet, in addition to two antiparallel β -strands at the N terminus. Furthermore, the six histidines that were predicted to be at or near the binuclear metal center/active site are indeed clustered at the C terminus of the β -barrel.

The solution of the three-dimensional X-ray crystallographic structure of the cadmium(II)-substituted holoenzyme to 2.0 Å resolution provides a definitive picture of the binuclear metal center/active site of phosphotriesterase that was not available with the apoenzyme structure (Benning et al., 1995). Significant structural rearrangements have occurred upon binding of the cadmium ions to the enzyme. The phosphotriesterase binuclear metal center contains several unexpected surprises. The more buried metal adopts a trigonal bipyramidal geometry with four of the ligands contributed by the protein (His-55, His-57, Lys-169, and Asp-301) and the fifth by a water molecule. Lys-169 and the water molecule act as bridges to the second more solvent-exposed metal, which is also ligated to His-201, His-230, and two additional water molecules in a distorted octahedral geometry.

The biggest surprise in the holoenzyme structure is that the bridging lysine side chain has been carbamylated by reaction with carbon dioxide. The enzyme may use a carbamylated lysine instead of a glutamate residue because the lone pair of electrons on the nitrogen can be delocalized to allow both oxygen ligands to carry negative charges. Moreover, a shorter glutamate side chain may be incapable of reaching both metal ions in the present active site geometry. Hong et al. (1995) have shown that high concentrations of bicarbonate can accelerate the reconstitution of the native apoenzyme with metal and that degassing of the buffer solution or the addition of formaldehyde can inhibit the reactivation process. They have further shown with ^{13}C NMR experiments that the bicarbonate used to reconstitute the enzyme is found as the carbamate bridge. These experiments, coupled with the fact that Lys-169 is not carbamylated in the apoenzyme structure, demonstrate that the carbamylated lysine is an easily formed but unstable species. However, the carbamate is stabilized by the metal ions, which in turn need the carbamate to bind and hold them within the active site.

Carbamylated lysine ligands have also been found in two other α,β -barrel metalloenzymes, namely ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), which binds magnesium(II) (Hartman & Harpel, 1993), and, most recently, urease from *Klebsiella aerogenes*, which binds two nickel(II) ions (Jabri et al., 1995). Urease is especially interesting because its protein-derived ligands to the binuclear nickel center are identical to the protein-derived ligands to the binuclear metal center of phosphotriesterase, and both enzymes are activated *in vitro* by the addition of carbon dioxide. The two nickel ions in urease are coordinated to four and five ligands, respectively, in a pseudotetrahedral and a distorted trigonal bipyramidal or distorted square pyramidal geometry, which is fewer ligands than what is observed in phosphotriesterase; however, it must be mentioned that the first metal-bound phosphotriesterase structure has been obtained with cadmium(II) and not the native zinc(II), so a change in the local environment around the binuclear metal center may occur depending on the state of the enzyme. In fact, the subsequent solution of the structure of the Zn/Zn-phosphotriesterase indicates that the more solvent-exposed metal ion does not coordinate to the two nonbridging solvent molecules in the Cd/Cd-PTE structure (Vanhook et al., 1996). Phosphotriesterase, like RUBISCO, is readily deactivated by metal chelators, but it is not known if the activation process *in vivo* is aided by specific proteins, as has been observed for urease. Jabri et al. (1995) note that the α,β -barrel of urease is structurally similar to that of adenosine deaminase (Wilson et al., 1991), a member of the α,β -barrel enzyme family that contains one zinc per active site. The two enzymes also share a high degree of active site similarity. The residues which coordinate to the urease binuclear nickel center are conserved in adenosine deaminase, with the exception of the bridging lysine, which has been replaced by an aspartate residue. In the adenosine deaminase active site, the side chain of the histidine equivalent to one of the histidine ligands of the second urease nickel changes its orientation to take the place of the missing lysine residue in metal coordination.

Structural Role for the Participation of Aspartate-253. In the apoenzyme structure (Benning et al., 1994), the carboxylate side chain of Asp-253 acts as an electrostatic bridge

between the imidazole side chains of His-55 and His-254. However, in the holoenzyme structure (Vanhook et al., 1996), the carboxylate of Asp-253 instead appears to be hydrogen bonded to only the imidazole side chain of His-230. This interaction could serve to orientate His-230 for optimal ligation with the more solvent-exposed metal ion. This role in the structural stabilization of the binuclear metal center is supported by the catalytic properties of the two mutants of Asp-253. When this residue is mutated to one that cannot participate in hydrogen bonding (D253A), there is a 500-fold drop in k_{cat} and a 1000-fold drop in $k_{\text{cat}}/K_{\text{m}}$. However, when the carboxylate group is substituted with a carboxamide from an asparagine, then the observed reductions in k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ are only 2-fold. This would serve to indicate that the hydrogen bond stabilization of His-230 is maintained by the presence of the asparagine at position 253.

Hydrophobic Contribution from Tryptophan-131. The potential roles of Trp-131 in binding and catalysis are less clear. In the apoenzyme structure, the indole side chain of Trp-131 is adjacent to the histidine cluster which suggests that it may facilitate the binding of the aromatic leaving group in substrates such as paraoxon. This relative positioning is maintained in the recently determined structure of the Zn/Zn-PTE complexed with a bound substrate analog (Vanhook et al., 1996). In this complex, the nitrogen of the indole ring is 3.3 Å from the phosphoryl oxygen of the substrate analog and the rest of the aromatic ring is positioned against the putative leaving group. The k_{cat} values for the W131F and W131A mutants are only slightly perturbed relative to the values obtained for the wild type enzyme. However, with the substitution of a methyl group for the indole ring of tryptophan, there is an elevation in the Michaelis constant for paraoxon of approximately 6-fold. This result is consistent with a hydrophobic contribution from the tryptophan to the binding pocket of the active site of PTE. This contribution can be effectively maintained with substitution of a phenyl substituent for the indole ring.

Metal Ligation by Aspartate-301. In the holoenzyme structure, the more buried metal ion within the binuclear metal cluster is ligated to His-55 and His-57 in addition to the carboxylate side chain originating from Asp-301 (Benning et al., 1995). The structural sensitivity to changes in ligand identity for the residues at positions 55 and 57 has previously been addressed by an extensive characterization of those mutants prepared by substitution of the imidazole side chain with the carboxamide from asparagine (H55N and H57N) (Kuo & Raushel, 1994; Lai et al., 1994). These studies have demonstrated that substitution of an asparagine for the histidine at position 55 is far more deleterious than an identical substitution at position 57. The H55N mutant is reduced in activity by a factor of between 10^3 and 10^4 relative to the wild type enzyme, while the same substitution with His-57 results in enzyme preparations that are 10–50% as active as the wild type enzyme, depending on the specific activating metal ion. The carboxylate side chain of Asp-301 is nearly as sensitive to changes as the His-55 residue. Replacement of the aspartic acid group with either histidine, asparagine, or alanine results in a dramatic loss in catalytic activity. These mutants (D301H, D301N, and D301A) are less active by a factor of approximately 1000 than the corresponding wild type enzyme. However, it should be noted that significantly more activity is retained upon substitution with a cysteine residue. Approximately 5% of

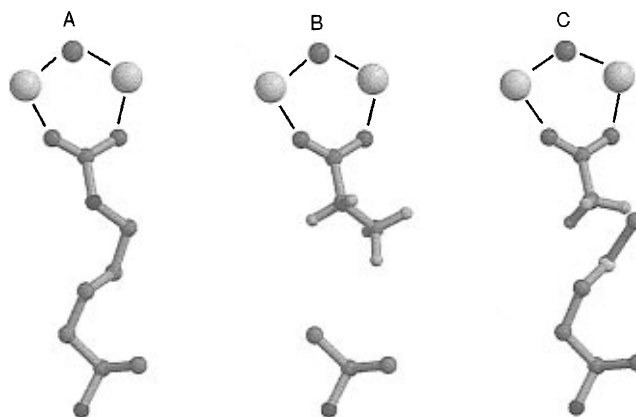


FIGURE 5: Orientation of the carbamylated lysine coordinated to the two divalent cations within the active site of the phosphotriesterase (A), modeled structure showing the K169A substitution with propionate ligated to the binuclear metal center (B), and modeled structure showing the K169M substitution with acetate ligated to the binuclear metal center (C). Coordinates for the wild type structure were taken from Benning et al. (1995).

the original activity is retained with the D301C mutant when the activating metal ion is cadmium.

Modification to Lysine-169 and the Carbamate Bridge. In order for the side chain of Lys-169 to serve as the bridging ligand to the two metals within the binuclear metal center, it must first be carbamylated through a reaction with carbon dioxide. It is not intuitively obvious why the bridging ligand must originate from a carbamate functional group rather than from a more simple carboxylate from either aspartic or glutamic acid. Perhaps this post-translational modification also functions as a regulatory feature similar to the control of the functioning of RUBISCO. Alternatively, the carbamate group might have some underappreciated chemical properties that endow this group to properly function for this purpose. Alanine, methionine, glutamate, and arginine mutants have been constructed at position 169 in an effort to determine the functional role of this group within the structure and function of phosphotriesterase.

Significant changes in the structure and function of the binuclear metal center are obtained upon mutation of the active site Lys-169 to alternative amino acids. For example, the visible color changes in the K169A and K169M mutants clearly indicate that the geometry for metal coordination has been altered upon removal of the lysine side chain. The substantial increase in the extinction coefficient for the absorption maximum centered at 536 nm would strongly suggest that the coordination number for one or both of the metal ions has been reduced upon replacement of the lysine with a methionine (Bertini & Luchinat, 1985). In addition, it appears that the carboxylate of glutamic acid cannot effectively substitute for the carbamate functional group since this mutant (K169E) is reduced in activity by 4 orders of magnitude when either Zn(II), Cd(II), or Co(II) is used as the activating divalent cations. Perhaps the side chain of glutamic acid is too short to reach the two divalent cations for effective ligation. To overcome this potential problem, we attempted to construct a protein with an alanine insertion immediately before the new glutamate (K169AE). Unfortunately, we did not obtain any protein expression of this construction when cells containing the mutant plasmid were grown. It would thus appear that this additional alanine insertion is quite destabilizing to the overall tertiary fold.

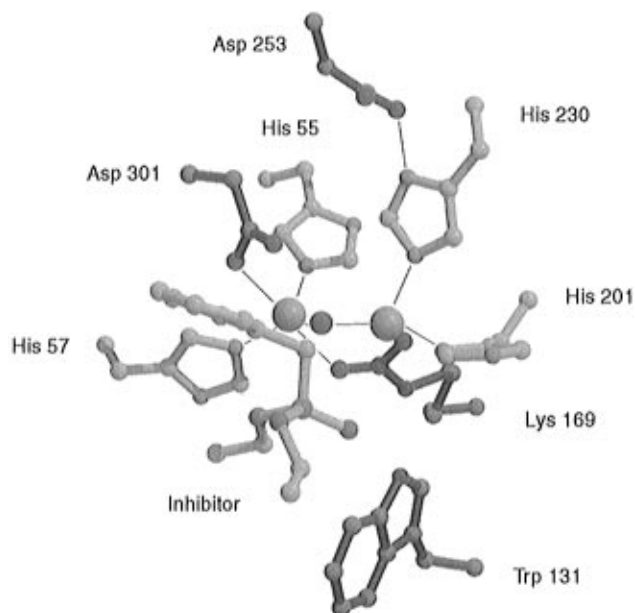


FIGURE 6: Structure of the binuclear metal center and a bound substrate analog (diethyl 4-methylbenzylphosphonate) in the active site of the bacterial phosphotriesterase. The residues mutated in this investigation are highlighted (Trp-131, Lys-169, Asp-253, and Asp-301). The coordinates were obtained from the structure of Zn/PTE (Vanhook et al., 1996).

No significant activity is observed when the lysine is mutated to an arginine residue.

The loss in catalytic activity with some of the K169 mutants can be recovered with the addition of low-molecular weight carboxylic acids as first noted by Hong et al. (1995). This procedure for chemical rescue was first developed by Toney and Kirsch (1989) with aspartate aminotransferase. Moreover, the magnitude of the enhancement is dependent on the size of the cavity that is presumably created after mutation of the active site lysine residue. For example, the initial activity of the K169A mutant can be enhanced over 25-fold when 100 mM propionic acid is added to the assay mixture. Significantly, lower levels of activity are observed when either acetic or butyric acid is added to the assay mixture. In contrast, the optimum carboxylic acid for the activation of the K169M mutant, which has a bulkier methionine residue, is acetic acid. These results are consistent with a model whereby the carboxylate group from these acids is now bridging the binuclear metal center and contributing to the thermodynamic and kinetic stabilization of this complex. A model for the bound complexes with the K169M and K169A mutants is illustrated in Figure 5. The observed enhancement in the activity of the K169 mutants with these short-chain carboxylates suggests that there is nothing particularly special about the carbamate bridge since the catalytic activity of the K169A mutant in the presence of 100 mM propionic acid reaches 1/3 of that of the cobalt-substituted wild type enzyme.

It is also curious to note that the reconstitution of the apo form of the K169E mutant is enhanced measurably in the presence of added bicarbonate. These results would suggest either that the bridge itself can be satisfied with carbonate or bicarbonate or, alternatively, that the carboxylate side chain of glutamate might form a mixed anhydride via a direct reaction with CO_2 . The structure of the K169E mutant is currently under intensive investigation. A representation of the active site of the phosphotriesterase holoenzyme with a bound substrate analog and the four residues investigated in this report is depicted in Figure 6.

ACKNOWLEDGMENT

We thank Hazel M. Holden, Matthew M. Benning, and Janeen Vanhook of the University of Wisconsin for their continued support of this project. We are indebted to Dr. Leisha S. Mullins for the preparation of Figures 5 and 6 and to Dr. Linette M. Watkins for the measurement of the visible spectra of the K169M mutant in the presence and absence of acetate.

REFERENCES

- Banzon, J. A., Kuo, J. M., Fischer, D. R., Stang, P. J., & Raushel, F. M. (1995) *Biochemistry* 34, 750–754.
- Benning, M. M., Kuo, J. M., Raushel, F. M., & Holden, H. M. (1994) *Biochemistry* 33, 15001–15007.
- Benning, M. M., Kuo, J. M., Raushel, F. M., & Holden, H. M. (1995) *Biochemistry* 34, 7973–7978.
- Caldwell, S. R., Newcomb, J. R., Schlecht, K. A., & Raushel, F. M. (1991) *Biochemistry* 30, 7438.
- Chae, M. Y., Omburo, G. A., Lindahl, P. A., & Raushel, F. M. (1993) *J. Am. Chem. Soc.* 115, 12173–12174.
- Chae, M. Y., Omburo, G. A., Lindahl, P. A., & Raushel, F. M. (1995) *Arch. Biochem. Biophys.* 316, 765–772.
- Farber, G. K., & Petsko, G. A. (1990) *Trends Biochem. Sci.* 15, 228–234.
- Hartman, F. C., & Harpel, M. R. (1994) *Annu. Rev. Biochem.* 63, 197–234.
- Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene* 77, 51–59.
- Hong, S., Kuo, J. M., Mullins, L. S., & Raushel, F. M. (1995) *J. Am. Chem. Soc.* 117, 7580–7581.
- Jabri, E., Carr, M. B., Hausinger, R. P., & Karplus, P. A. (1995) *Science* 268, 998–1004.
- Kuo, J. M., & Raushel, F. M. (1994) *Biochemistry* 33, 4265–4272.
- Lai, K., Dave, K. I., & Wild, J. R. (1994) *J. Biol. Chem.* 269, 16579–16584.
- Lewis, V. E., Donarski, W. J., Wild, J. R., & Raushel, F. M. (1988) *Biochemistry* 27, 1597–1597.
- Omburo, G. A., Kuo, J. M., Mullins, L. M., & Raushel, F. M. (1992) *J. Biol. Chem.* 267, 13278–13283.
- Omburo, G. A., Mullins, L. M., & Raushel, F. M. (1993) *Biochemistry* 32, 9148–9155.
- Toney, M. D., & Kirsch, J. F. (1989) *Science* 243, 1485–1488.
- Vanhook, J. L., Benning, M. M., Raushel, F. M., & Holden, H. M. (1996) *Biochemistry* 35, 6020–6025.
- Wilson, D. K., Rudolf, F. B., & Quioco, F. A. (1991) *Science* 252, 1278–1282.

BI962099L