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INHIBITORS DIRECTED TOWARDS THE BINUCLEAR METAL CENTER OF PHOSPHOTRIESTERASE

SUK-BONG HONG and FRANK M. RAUSHEL*

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

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The potential roles in binding and catalysis for the binuclear metal center found within bacterial phosphotriesterase were evaluated by characterization of the inhibitory properties of 26 substrate and product mimetics. Phosphonates bearing monofluoro, difluoro, or hydroxyl substituents at the methylene position were found to be noncompetitive inhibitors with K_i values ranging from 0.6–9 mM versus the substrate paraoxon. Phosphoramidates did not significantly inhibit the enzyme. Diethyl phosphate and diethyl dithiophosphate inhibited the Cd-substituted enzyme with K_i values of 10 and 130 μM, respectively. The most effective inhibitor for either the cadmium or zinc substituted enzyme was found to be diethyl thiomethylphosphonate. The competitive inhibition constants for this compound were found to be 60 nM and 2.8 μM for the cadmium- and zinc-substituted enzyme, respectively. The tight binding is attributed to chelation of both metal ions simultaneously.

Keywords: Phosphotriesterase; Binuclear metal center; Phosphonates

INTRODUCTION

The phosphotriesterase from *Pseudomonas diminuta* is a zinc metalloenzyme that catalyzes the hydrolysis of a variety of organophosphorus nerve agents.[1,2] Shown below is the reaction catalyzed for the hydrolysis of the insecticide paraoxon.

* Corresponding author. Fax: 409-845-9452. E-mail: raushel@tamu.edu.

The active site of the native enzyme consists of a binuclear metal center and the two native zinc ions can be substituted with either Co^{2+}, Cd^{2+}, Ni^{2+} or Mn^{2+} to yield catalytically competent enzyme.[3] Site-directed mutagenesis, in conjunction with chemical modification and inactivation studies, has shown that at least four histidine residues are essential for enzyme activity.[4–7] A three-dimensional structure of the Cd^{2+}-substituted holoenzyme by X-ray crystallography has demonstrated that the binuclear metal center at the active site is embedded within a cluster of histidine residues.[8] These results have been confirmed by the solution of the three-dimensional structure of the Zn^{2+}-substituted protein in the presence of a nonhydrolyzable substrate analog.[9]

The precise roles of the binuclear metal center and the rest of the active site residues involved in catalysis and substrate binding are not well understood. However, the pH-rate profiles for the hydrolysis of substrates by the phosphotriesterase indicate that a single group from the enzyme must be unprotonated for full catalytic activity.[3] The variation of the apparent kinetic pK_a value with the specific metal ion bound to the protein suggests that the solvent hydroxide that bridges the two metal ions within the binuclear metal center is the nucleophile that initiates the attack at the phosphorus center. The rather large dependence on the rate of substrate hydrolysis with the pK_a of the leaving group phenol is inconsistent with either Lewis acid or general acid catalysis as an enhancement of the leaving group potential.[10,11] Stereochemical experiments with chiral substrates have demonstrated that the overall reaction proceeds with an inversion of configuration at the phosphorus center.[12] These results are consistent with an S_N2-like reaction mechanism without the participation of a phosphorylated enzyme intermediate. Therefore, one can conclude that the primary catalytic role for this enzyme is directed towards the nucleophilic activation of the hydrolytic water molecule and the electrophilic activation of the phosphorus center. Both of these functions can be accommodated by the binuclear metal center.

In order to more clearly define the critical interactions between the enzyme and substrate it is imperative that the structure of the enzyme–substrate and enzyme–product complexes be determined to a higher level of resolution. These efforts will be significantly enhanced if tightly bound structural analogs of the substrate, transition state, and/or product complexes can be designed, synthesized, and evaluated for binding efficiency. Toward this end we have constructed and tested 26 molecules as potential candidates for nonhydrolyzable probes of enzyme–ligand interactions using X-ray crystallography and/or NMR spectroscopic techniques. These

investigations have revealed the high level of sensitivity of this binding site toward relatively small changes in the structure of the target molecules and have identified a powerful competitive inhibitor with a K_i value of 60 nM toward the enzyme substituted with cadmium.

MATERIALS AND METHODS

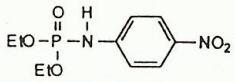
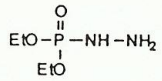
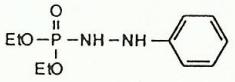
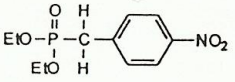
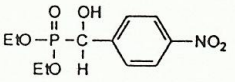
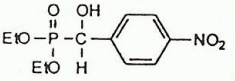
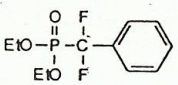
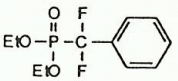
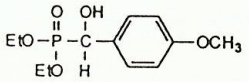
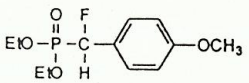
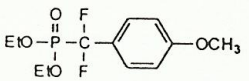
Materials

All chemicals used for the syntheses of the compounds reported in this paper were purchased from either Aldrich or Sigma. The phosphoranilidate (I) and benzyl phosphate (XIV) were obtained as previously described by Caldwell *et al.*[10] Para-substituted benzylphosphonic acid diethyl esters bearing α-hydroxyl, α-fluoro, and α,α-difluoro substituents (V–XI) at the α-methylene group were prepared according to Burke *et al.*[13] The phosphorohydrazidate (II) and phosphorophenylhydrazidate (III) were synthesized as previously described.[14,15] 4-(Dimethylamino)phenol and 2,4-dimethoxyphenol were synthesized according to published procedures,[16,17] and condensed with diethyl chlorophosphate to give the corresponding products (XII and XIII) using the procedure of Caldwell *et al.*[10] Diethyl phosphate (XVI) was prepared by the method of Bielawski and Casida.[18] (S_p)- and (R_p)-*O*-ethyl phenylphosphonothioic acids (XVIII and XIX) were obtained as previously described.[12] Diethyl thiomethylphosphonate (XXVI) was synthesized by the procedure of Linderman *et al.*[19] All of the remaining compounds were commercially available. The structures of all newly synthesized materials were fully verified with ^{1}H NMR, ^{13}C NMR and mass spectral analysis. The phosphotriesterase used in this study was isolated according to the method of Omburo *et al.*[3] The enzyme was subsequently reconstituted with Zn^{2+} and Cd^{2+} as previously described.[5]

Inhibition Studies

The assay solutions containing paraoxon (100 μM–1.5 mM) and various levels of the specific inhibitor in 0.10 M 2-(*N*-cyclohexylamino)ethanesulfonic acid buffer (CHES), pH 9.0, were preincubated at 25°C for 10 min prior to the addition of enzyme. Cd^{2+}-substituted or Zn^{2+}-substituted enzyme was added last to initiate the enzymatic reaction. The rates of reaction were measured spectrophotometrically by following the production of 4-nitrophenolate from the hydrolytic cleavage of paraoxon at 400 nm ($\varepsilon = 1.7 \times 10^4\ M^{-1}cm^{-1}$ at pH 9.0) using a Gilford 260 UV–Vis

TABLE I Inhibition of Phosphotriesterase by Phosphonates and Phosphoramidates

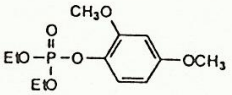
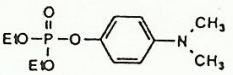
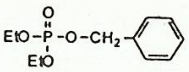
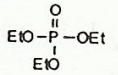
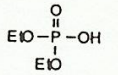
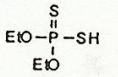
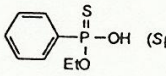
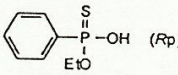
	Compound	Cd/Cd-*enzyme*				Zn/Zn-*enzyme*			
		Type of inhibition[a]	MeOH (%)	$K_{is}(K_i)$ (mM)	K_{ii} (mM)	*Type of inhibition*[a]	MeOH (%)	$K_{is}(K_i)$ (mM)	K_{ii} (mM)
I		No Inhibition[b]				nd	10	(3.4)[c]	
II		nd	0	(15)[c]		nd	0	(30)	
III		NC	4.7	9.2	2.1	nd	2.3	(12)[c]	
IV		nd	10	(5.0)[c]		nd	0	(8.0)[c]	
V		C	1.6	3.7		NC	1.6	4.6	44
VI		NC	5.2	1.6	2.5	NC	5.2	5.5	16
VII		NC	0	0.65	0.50	NC	0	1.5	15
VIII		NC	2.5	1.1	1.3	NC	4.4	2.4	2.6
IX		NC	0	3.2	8.9	NC	0	3.7	5.0
X		NC	0	2.0	1.8	NC	2.3	2.2	25
XI		NC	5.7	2.5	1.4	NC	5.1	4.2	6.4

[a] C = competitive; NC = noncompetitive; UC = uncompetitive; nd = not determined.

[b] The concentration of I was varied over the range of 0–0.6 mM at a fixed concentration of 0.1 mM paraoxon.

[c] The values were determined from a fit of the data to equation (4) at a concentration of 0.1 mM paraoxon.

TABLE II Inhibition of phosphotriesterase by alternate substrates and reaction products

Compound		Cd/Cd-*enzyme*				Zn/Zn-*enzyme*			
		Type of inhibition[a]	MeOH (%)	$K_{is}(K_i)$ (mM)	K_{ii} (mM)	*Type of inhibition*[a]	MeOH (%)	$K_{is}(K_i)$ (mM)	K_{ii} (mM)
XII		NC	0.6	1.1	1.2	NC	3.0	0.22	9.1
XIII		NC	0	0.65	2.2	NC	0	0.09	2.3
XIV		NC	5.4	6.1	12	NC	2.7	2.2	3.9
XV		NC	0	25	100	NC	0	1.7	62
XVI		NC	0	0.01	0.006	UC	0		0.81
XVII		C	0	0.13		nd	0	(38)[b]	
XVIII		NC	0	0.13	0.04	nd	0	(7.0)[b]	
XIX		NC	0	1.7	0.38	nd	0	(12)[b]	

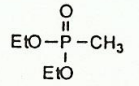
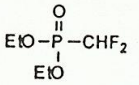
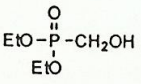
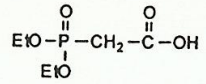
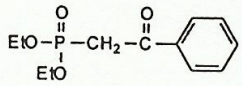
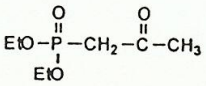
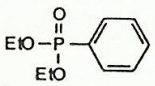
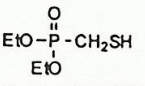
[a] C = competitive; NC = noncompetitive; nd = not determined.
[b] The values were determined from a fit of the data to equation (4) at a concentration of 0.1 mM paraoxon.

spectrophotometer. Because of the limited solubility in water, some of the inhibitors were dissolved in a methanol/water mixture and the final concentration of methanol in each of the assays is shown in Tables I–III.

Data Analysis

All kinetic data were fit to the appropriate rate equations using the computer programs supplied by Savanna Shell Software. Inhibition constants were determined by fitting data from the kinetic patterns to one or more of the following equations for competitive, uncompetitive or noncompetitive

TABLE III Inhibition of phosphotriesterase by substituted phosphates

	Compound	Cd/Cd-*enzyme*			Zn/Zn-*enzyme*			
		Type of inhibition[a]	MeOH (%)	$K_{is}(K_i)$ (mM)	*Type of inhibition*[a]	MeOH (%)	$K_{is}(K_i)$ (mM)	K_{ii} (mM)
XIX		C	0	1.6	nd	0	(34)[b]	
XX		nd	0	(18)[b]	nd	0	(67)[b]	
XXI		nd	0	(15)[b]	nd	0	(140)[b]	
XXII		nd	0	(12)[b]	UC	0		3.9
XXIII		C	0	2.7	UC	0		2.1
XXIV		nd	0	(22)[b]	nd	0	(26)[b]	
XXV		nd	1.6	(24)[b]	nd	1.6	(13)[b]	
XXVI		C	0	6.0×10^{-5}	C	0	2.8×10^{-3}	

[a] C = competitive; UC = uncompetitive; nd = not determined.
[b] The values were determined from a fit of the data to equation (4) at a concentration of 0.1 mM paraoxon.

types of inhibition.

$$v = V_m A/(K_m(1 + I/K_{is}) + A), \quad (1)$$

$$v = V_m A/(K_m + A(1 + I/K_{ii})), \quad (2)$$

$$v = V_m A/(K_m(1 + I/K_{is}) + A(1 + I/K_{ii})). \quad (3)$$

In these equations, v is the initial velocity, V_m is the maximal velocity, K_m is the Michaelis constant, I is the inhibitor concentration, K_{is} is the slope inhibition constant, and K_{ii} is the intercept inhibition constant. Some of the compounds were tested as inhibitors by fixing the concentration of paraoxon near the Michaelis constant (0.1 mM) while varying the

concentration of the inhibitor. These data were fit to equation (4), where V_0 is the initial rate of paraoxon hydrolysis in the absence of inhibitor and $K_{i(app)}$ is the apparent inhibition constant. These inhibition constants are enclosed in parentheses in Tables I–III.

$$v = V_0 I/(K_{i(app)} + I) \quad (4)$$

RESULTS

Inhibition by Benzylphosphoric Acid Diethyl Esters and Phosphoramidates

A series of nonhydrolyzable mimetics of phosphotriesters were constructed and subsequently tested as inhibitors of the Zn^{2+}- and Cd^{2+}-substituted forms of the bacterial phosphotriesterase. The kinetics of inhibition by these compounds were measured spectrophotometrically using paraoxon as a control substrate and the results are summarized in Table I. Replacement of the P–O bond within the paraoxon framework by either a P–N or a P–C bond resulted in an array of phosphotriester analogs that exhibited modest affinity for the enzyme as demonstrated by the magnitude of the K_i values found for these compounds. The inhibition of enzyme activity by phosphonates bearing monofluoro, difluoro, and hydroxyl substituents attached to the central methylene carbon was of the noncompetitive type, except for compound II which was competitive for the Cd^{2+}-substituted enzyme. The observed inhibition constants for this group of phosphotriester analogs are measurably larger than the K_m values of paraoxon for either the Cd^{2+}- or Zn^{2+}-substituted enzymes (0.4 mM and 0.09 mM, respectively).

Inhibition by Phosphotriesters and Reaction Products

The inhibition constants exhibited by phosphotriesters and various reaction products versus paraoxon are presented in Table II. Phosphotriesters XII, XIII and XIV act as noncompetitive inhibitors of the enzyme versus paraoxon. The K_i values for the aryl esters (XII and XIII) are smaller than those for the alkyl esters (XIV and XV). The K_i values for the reaction products (XVI–XIX) using the Cd^{2+}-enzyme are significantly lower than those found with the Zn^{2+}-enzyme. In contrast, the phosphotriesters (XII–XV) are more effective inhibitors of the Zn^{2+}-enzyme than the Cd^{2+}-enzyme. Introduction of chirality at the phosphorus center significantly alters the observed K_i for the Cd^{2+}-enzyme. The K_i for the

(S_p)-enantiomer (XVIII) of the reaction product from *O*-ethyl *O*-(4-nitrophenyl)benzenephosphonothioate (EPN) for the Cd^{2+}-enzyme is 10-fold lower than for the (R_p)-enantiomer (XIX).

Inhibition by Substituted Phosphonates

The inhibitory properties of a series of substituted phosphonates were also measured for the bacterial phosphotriesterase. The incorporation of fluorine, hydroxyl, thiol, carbonyl, and carboxyl groups adjacent to the phosphoryl group was designed to assist in the direct coordination with one or both of the metal ions contained within the structure of the binuclear metal center. Of the compounds tested the diethyl thiomethylphosphonate (XXVI) was by far the most potent inhibitor identified. The inhibitory properties of these compounds are presented in Table III.

DISCUSSION

The bacterial phosphotriesterase has been shown to hydrolyze a wide variety of organophosphate triesters through the cleavage of P–O, P–F, and P–S bonds.[2,10,11,20] However, a naturally occurring substrate has not, as yet, been identified. Nevertheless, the rapid substrate turnover, and the prospects for exploitation of the demonstrated catalytic activity for the detoxification of organophosphate nerve agents have made this enzyme an attractive target for mechanistic and structural investigations. The heart of the active site machinery has been shown to be a coupled binuclear metal cluster.[8,9,21] It has been previously proposed that these two metal ions function in catalysis by lowering the pK_a of the hydrolytic water molecule and through polarization of the phosphoryl oxygen bond of the bound substrate.[11] Thus far it has not been possible to unambiguously determine the structural factors from the enzyme and substrates that control the orientation of the bound complexes. In order to probe these factors in more detail, a series of nonhydrolyzable substrate analogs, slow substrates, and reaction products were constructed and their binding properties evaluated through the inhibitory effects on the hydrolysis of good substrates.

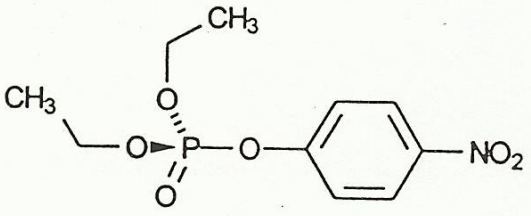
Our initial strategy for the construction of nonhydrolyzable inhibitors of phosphotriesterase was to replace the P–O bond at the site of cleavage in paraoxon with either a P–NH (I) or a P–CH_2 (IV) substitution. Neither of these compounds were particularly effective as inhibitors of

phosphotriesterase. The phosphoramide (I) did not inhibit the cadmium-substituted enzyme and was only weakly inhibitory towards the Zn-enzyme. The phosphonate analog (IV) was not significantly better. The apparent K_i values of 5 and 8 mM for the phosphonate (IV) are significantly higher than the K_m values of 0.40 and 0.09 mM for the Cd- and Zn-substituted forms of phosphotriesterase with paraoxon, respectively. Improvements were sought in binding efficiency by elaboration of the bridging methylene group of phosphonate IV by replacement of one or both of the hydrogens with either an OH or F. These substituents should more closely mimic the hydrophilic oxygen present in the phenolic leaving group of the parent substrate, paraoxon. This approach has successfully been used by Burke *et al.* in the design of inhibitors for tyrosine-specific protein kinases.[13] Modest improvements in the K_i values were found when either a hydroxyl (V) or a fluorine (Vl) was added to the methylene group of phosphonate (IV). Further alterations in the compounds were pursued by the subsequent removal of the nitro-substituent from the phenyl ring and replacement with either a hydrogen (VII and VIII) or methoxy substituent (IX–XI). The phosphonate compound in this series with the lowest K_{is} value for either the Cd- or Zn-substituted enzyme was VII where the K_{is} values were found to be 650 μM and 1.5 mM, respectively.

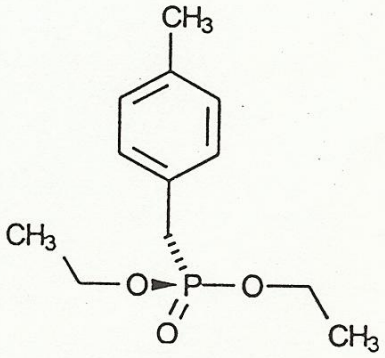
Since the alteration of the P–O bond in paraoxon with either a P–NH or P–CH_2 group proved relatively ineffective for the design of a good inhibitor we next elected to construct a genuine phosphotriester with a much poorer leaving group than the *p*-nitrophenolate of paraoxon. This approach was relatively easy to carry out since the measured values for k_{cat} and k_{cat}/K_m with this phosphotriesterase are very much dependent on the pK_a of the leaving group phenol ($\beta_{lg} = \sim -2$). Therefore, compounds with leaving groups having pK_a values >10 are hydrolyzed approximately a million fold more slowly than is paraoxon (p$K_a \sim 7$). This strategy proved reasonably effective since the K_i values for either XII or XIII are lower than nearly all of the previously constructed compounds. In fact, the K_i values observed for XIII are very close to the reported values for the K_m of paraoxon.

These studies serve to illustrate two points. First, this enzyme is extraordinarily sensitive to small changes in the substituent bonded directly to the central phosphorus atom. The compounds containing either the P–NH or the P–CH_2 substitutents do not serve as substrates and they also do not bind particularly tightly to the active site of the phosphotriesterase. This observation is also supported by the relatively poor turnover exhibited by a series of compounds containing substituted thiophenols as leaving groups.[11] Further structural support for this proposal comes from the X-ray crystal

structure of a bound complex of diethyl 4-methylbenzylphosphonate with the phosphotriesterase.[9] In the crystal structure of the Zn-phosphotriesterase with this compound, the inhibitor did not orient itself within the active site in a manner predicted for optimal binding of paraoxon. It appears that the *pro-S* ethoxy group of diethyl 4-methylbenzylphosphonate is occupying the position expected for the *p*-nitrophenolate of paraoxon as illustrated in the scheme below. Therefore, it is not the phenyl group which is directing the relative orientation of this compound in the active site but it is the bridging oxygen from one of the two ethoxy substitutents.



Paraoxon



Diethyl 4-methylbenzylphosphonate

The other aspect of these studies worth noting is the almost universal appearance of noncompetitive inhibition rather than competitive inhibition by these compounds. The physical reason for this situation is not quite clear but there are at least two possibilities. First, as stated above, these phosphonates are prone to bind in an alternative orientation relative to the rotation about the P=O bond. These orientation effects within the active site may accommodate the binding of an inhibitor in the presence of another substrate. Second, the crystal structures of the cadmium- and zinc-substituted enzymes, in the presence of diethyl 4-methylbenzylphosphonate, have revealed that a molecule of the phosphonate analog is found bound to the interface between the two subunits of the dimeric protein. It is not known whether typical substrates such as paraoxon can also bind at this site. However, it would appear that this secondary inhibitor binding site is the most likely cause for the noncompetitive inhibition.

A total of four products of the catalytic reaction (XVI–XIX) were tested as inhibitors of the phosphotriesterase. Diethyl phosphate (XVI) proved to be the most potent inhibitor of any of the reaction products tested for either the Cd- or Zn-substituted enzyme. Surprisingly, the Cd-substituted enzyme was very much more sensitive to inhibition by diethyl dithiophosphate than is the Zn-substituted enzyme. A plausible explanation for the relatively

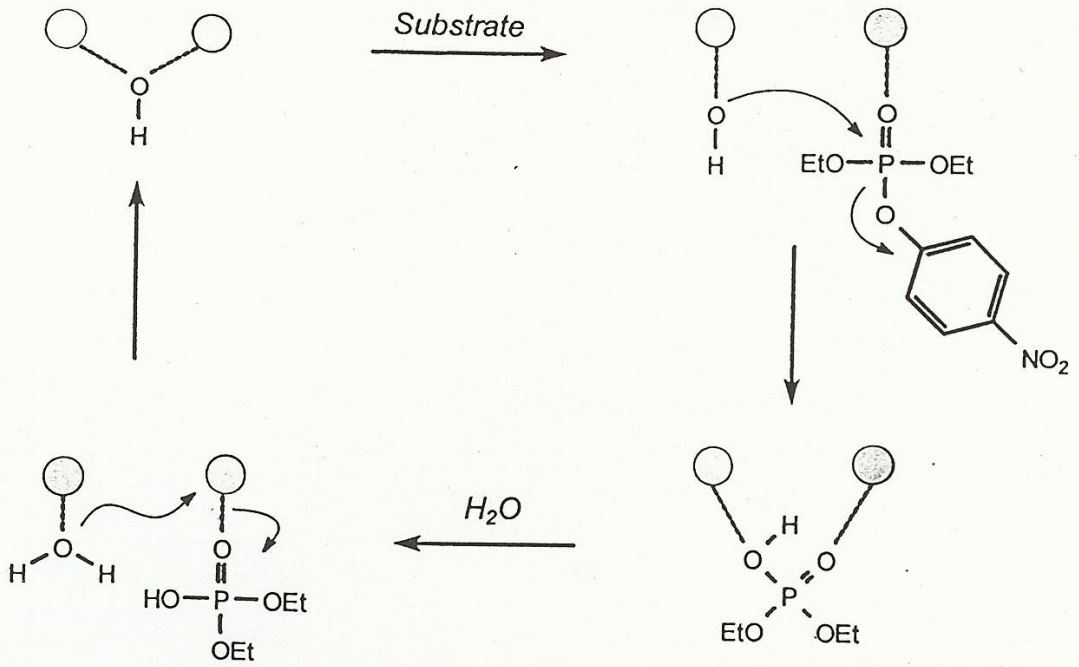
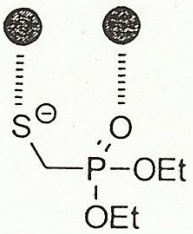


FIGURE 1 Working model for the hydrolysis of substrates by the bacterial phosphotriesterase.

tight binding of these two compounds to the active site is a chelation of both metal ions by the phosphate or thiophosphate as illustrated below:

The final group of inhibitors was designed to provide an additional substituent that would permit the complexation of both metal ions simultaneously (XXI–XXIV, XXVI). Most of these compounds were mediocre inhibitors of the phosphotriesterase. However, diethyl thiomethylphosphonate turned out to be the best inhibitor constructed for this enzyme thus far. The very tight binding is interpreted to result from the chelation of both metal ions simultaneously as indicated in the scheme below:



Structural confirmation for this proposal is not yet available at this time but we predict that the thiol group would be complexed to the more solvent shielded metal ion while the phosphonyl oxygen would be coordinated to the more solvent exposed metal ion within the binuclear metal center. It should be noted that the oxygen analog of this inhibitor binds five orders of magnitude weaker to the enzyme active site.

Overall these results are consistent with our working model for the binding of substrates and products to this enzyme (Figure 1). In this model the phosphoryl oxygen of the bound substrate will be initially coordinated to the more solvent exposed metal ion. Nucleophilic attack would then be initiated from the hydroxide complexed to the more solvent shielded metal ion. A transition structure would include coordination of each oxygen to separate metal ions as illustrated in the mechanism below.

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