Mechanism-Based Inhibitors for the Inactivation of the Bacterial Phosphotriesterase[†]

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ABSTRACT: 1-Bromovinyl (**I**), *Z*-2-bromovinyl (**II**), 1,2-dibromoethyl (**III**), and a series of 4-(halomethyl)-2-nitrophenyl (**IVa−c**) diethyl phosphate esters were examined as substrates and mechanism-based inhibitors for the bacterial phosphotriesterase. All of these compounds were found to act as substrates for the enzyme. Inhibitor **I** rapidly inactivated the enzyme within 1 min, giving a partition ratio of 230. The newly formed covalent adduct with inhibitor **I** was susceptible to hydrolysis at elevated values of pH and dissociation by NH_2OH. Azide was not able to protect the enzyme from inactivation with inhibitor **I**, implying that the reactive species was not released into solution prior to the inactivation event. The reactive species was proposed to be either an acyl bromide or a ketene intermediate formed by the enzymatic hydrolysis of inhibitor **I**. Compounds **II** and **III** were shown to be relatively poor substrates of phosphotriesterase and they did not induce any significant inactivation of the enzyme. The inhibitor, 4-(bromomethyl)-2-nitrophenyl diethyl phosphate (**IVa),** was found to irreversibly inactivate the enzyme with a K_I = 7.9 mM and k_{inact} = 1.2 min^{-1} at pH 9.0. There was no effect on the rate of inactivation upon the addition of the exogenous nucleophiles, azide, and NH_2OH. The species responsible for the covalent modification of the enzyme by **IVa** was most likely a quinone methide formed by the elimination of bromide from the phenolic intermediate. NMR experiments demonstrated that the quinone methide did not accumulate in solution. The chloro (**IVb**) and fluoro (**IVc**) analogues did not inactivate the enzyme. These results suggest that the elimination of the halide ion from the phenolic intermediate largely determines the partition ratio for inactivation.

Phosphotriesterase (PTE)[1] from *Pseudomonas diminuta* is a zinc containing enzyme that catalyzes the hydrolysis of a wide range of organophosphate triesters. Naturally occurring substrates for this enzyme are unknown, but the enzyme has exhibited a high catalytic efficiency and a potential utility for degrading organophosphorus nerve agents (*1*). The enzyme requires two divalent metal ions for catalytic activity and the natural activator Zn^{2+} can be substituted with either Co^{2+}, Cd^{2+}, Ni^{2+}, or Mn^{2+} with the restoration of full catalytic activity (*2*). X-ray diffraction studies, in conjunction with a number of other mechanistic and structural studies, have provided considerable insight into the structure of the metal-binding sites and identification of important amino acid residues within the active site (*1−13*). The hydrolytic reaction catalyzed by the enzyme has been shown to occur via an S_N2-like mechanism, whereby an activated water molecule attacks the phosphorus center of the substrate resulting in P−O bond cleavage. However, many of the details for the precise functions of the two metal ions and active site residues involved in binding and catalysis remain unknown.

Active site-directed inactivation studies using mechanism-based or suicide substrates are an important tool for the identification of active site residues and elucidation of enzyme reaction mechanisms. Mechanism-based inactivators induce the loss of catalytic activity of an enzyme through the reaction of an active site nucleophile with a highly reactive intermediate generated by enzymatic catalysis (*14*). Recent studies with this phosphotriesterase have demonstrated that alkynyl phosphate esters can act as potent mechanism-based inactivators of the bacterial phosphotriesterase (*15−17*). These studies have demonstrated that His-254 is a crucial amino acid residue for the inactivation of the enzyme by these reagents. Recently, Stowell *et al*. (*18*) have proposed the design and synthesis of other potentially useful enzyme inactivators which have the capacity to promote the formation of other types of chemically reactive intermediates. In order to further develop phosphotriesterase inactivators and incorporate the chemical motifs originally described by Stowell *et al*. (*18, 19*), 1-bromovinyl (**I**), *Z*-2-bromovinyl (**II**), 1,2-dibromoethyl (**III**), and 4-(halomethyl)-2-nitrophenyl phosphate (**IVa−c**) esters were synthesized and evaluated as potential suicide substrates. Chemically reasonable reaction mechanisms for the inactivation process using compounds **I−III** and **IVa−c** are presented in Scheme 1. The enzymatic hydrolysis of compound **I** by the phosphotriesterase would lead to the formation of an acetyl bromide or a ketene intermediate, while hydrolysis of compounds **II** and **III** would potentially yield the common intermediate, α-bromoacetaldehyde. Phosphotriesterase-catalyzed hydrolysis of compounds **IVa−c** would result in the transient formation of a quinone methide. All of these intermediates are known to be highly reactive electrophiles such that the protein would be anticipated to be rapidly inactivated through covalent and irreversible reactions.

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[1] Abbreviations: PTE, phosphotriesterase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid); TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; BSA, bovine serum albumin.

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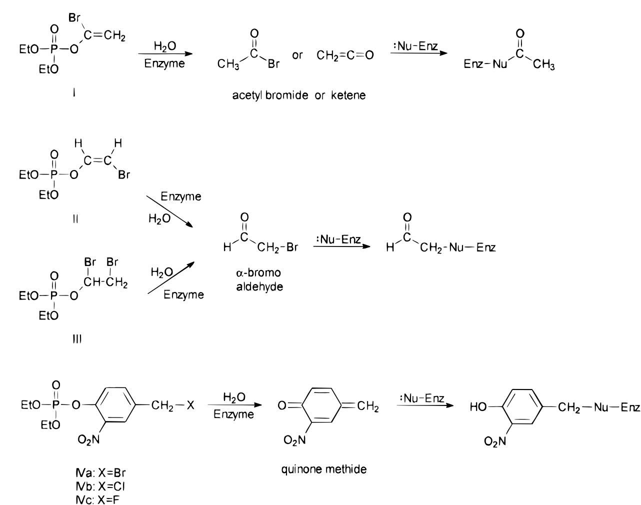
Scheme 1



MATERIALS AND METHODS

Materials. Paraoxon was purchased from Sigma and purified according to the procedure of Omburo *et al*. (*2*). Unless otherwise noted, all of the other chemicals were purchased from either Aldrich or Sigma and used without further purification. Compounds **I−III, IVa−c, V,** and **VI** were synthesized as previously reported (*18−20*). The wild-type phosphotriesterase was purified as described previously, and reconstitution of the apo-enzyme with Cd^{2+} was performed according to the method of Omburo *et al*. (*2*).

Enzymatic Hydrolysis. Rates of enzymatic hydrolysis of compounds **I−III** were determined in a final volume of 3 mL containing 35 mM phosphate buffer, pH 7.0, 5 μM $CdSO_4$, 15% D_2O (v/v), and 2.6 mM of 1-bromide (**I**), 2.7 mM of *Z*-2-bromide (**II**) or 2.2 mM of dibromide (**III**), respectively. Dibromide **III** was dissolved in deuterated methyl alcohol (CD_3OD) such that the final concentration of CD_3OD was 15% (v/v). The reactions were followed by ^{1}H NMR spectroscopy at 500 MHz with a Varian Unity 500 NMR spectrometer. The hydrolysis rates were determined from the normalized integration of signals for reactants and products as a function of time. Kinetic constants for the enzyme-catalyzed hydrolysis of 4-(halomethyl)-2-nitrophenyl phosphates (**IVa−c**) as substrates were determined spectrophotometrically at 25 °C with a Gilford Model 260 spectrophotometer. Due to their limited solubility in water, stock solutions of **IVa−c** were prepared with acetonitrile as a cosolvent. The final concentration of acetonitrile in the assays was 20%. This concentration of acetonitrile had no effect on enzyme activity. The reaction of the benzylic halides **IVa−c** was initiated by addition of phosphotriesterase and was monitored by measuring the increase in absorbance of the final product at 420 nm ($\epsilon = 4.6 \times 10^3$ M^{-1} cm^{-1} for 4-hydroxy-3-nitrobenzyl alcohol at pH 9.0). The values of V_{max} and K_m were determined from a fit of the data to the eq 1 where v is the initial velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant, and A is the substrate concentration.

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

Determination of Partition Ratio. Phosphotriesterase (50 nM) was incubated at 25 °C in a solution containing 0.1 M PIPES, pH 7.0, 5 μM $CdSO_4$, 1 mg/mL BSA, and various concentrations (0−2.0 mM) of compounds **I−III**. Dibromide **III** was tested as an inactivator in 15% methanol (v/v). After an incubation period of 5 min, a 10 μL aliquot was removed and assayed for residual enzyme activity. The partition ratio was determined from a plot of the relative enzyme activity as a function of the mole ratio of the inhibitor to enzyme, as previously described (*21*).

Determination of Inactivation Constants. Phosphotriesterase (50 nM) was incubated at 25 °C in a solution containing 0.1 M CHES, pH 9.0, 5 μM $CdSO_4$, 20% CH_3CN, 1 mg/mL BSA, and various concentrations of the benzylic halides **IVa−c**. Aliquots were removed at various time intervals and assayed for residual enzyme activity as described above. Pseudo-first-order rate constants for inactivation (k_{obs}) at each inhibitor concentration (I) were determined and the values for k_{inact} and K_I were determined from a fit of the data to eq 2.

$$k_{obs} = k_{inact}\, I/(K_I + I) \quad (2)$$

Reactivation of Inactivated Enzyme. Phosphotriesterase (50 nM) was inactivated by preincubation for 5 min at 25 °C in a solution containing 0.1 M PIPES, pH 7.0, 5 μM $CdSO_4$, 1 mg/mL BSA, and 26 μM of bromide **I** ([I]/[E_o] = 520). A 200 μL aliquot was removed and added to 200 μL of a solution (either PIPES, pH 7.0; TAPS, pH 8.0; CHES, pH, 9.0; or CHES, pH 10.0) to a final concentration of 0.25 M buffer with 5 μM CdSO4 and 1 mg/mL BSA and then assayed for the regain of enzyme activity at various time intervals. A control reaction in which the enzyme was preincubated without inhibitor was conducted in the same manner. Attempts were made to reactivate the enzyme inactivated by the bromide **IVa**. The reaction conditions were identical to those described above, except that the buffer and concentration of the bromide **IVa** used for the inactivation of PTE were 0.1 M CHES (pH 9.0) and 8 mM ([I]/[E_o] = 160 400), respectively.

Protection from Inactivation by Azide or Hydroxylamine. Phosphotriesterase (50 nM) was incubated at 25 °C in a 0.5 mL solution containing 0.1 M PIPES, pH 7.0, 5 μM $CdSO_4$, and 1 mg/mL BSA in the presence of 26 μM bromide **I** while varying the concentration of either NaN_3 or NH_2OH. At various time intervals, aliquots were removed and assayed for catalytic activity. A control reaction in which the enzyme was preincubated without added inhibitor was run in the same manner. The relative catalytic activity was obtained from the ratio of activity of the control sample and the inactivated enzyme at identical time points. To test the protection of the enzyme from inactivation by bromide **IVa**, phosphotriesterase (50 nM) was incubated at 25 °C in a 0.5 mL solution containing 0.1 M CHES (pH 9.0), 5 μM $CdSO_4$, 1 mg/mL BSA, 20% CH_3CN in the presence of 2 mM inhibitor **IVa**, and either 50 mM NaN_3 or 50 mM NH_2OH. At various time intervals, aliquots (10 μL) were removed and assayed for residual enzyme activity.

Reactivation of Inactivated Enzyme by Hydroxylamine. Phosphotriesterase (50 nM) was inactivated by preincubation at 25 °C for 5 min in a 0.5 mL solution containing 0.1 M PIPES, pH 7.0, 5 μM $CdSO_4$, 1 mg/mL BSA, and either 26 μM inhibitor **I** or 2 mM inhibitor **IVa**. A 400 μL aliquot of this solution was diluted to a final concentration of 40 mM NH_2OH, 5 μM $CdSO_4$, and 1 mg/mL BSA. At various time intervals, a 15 μL aliquot was removed and assayed for

catalytic activity. Three control experiments in which the enzyme was incubated without NH_2OH in the presence or absence of inhibitor **I** and with NH_2OH in the absence of inhibitor **I** were also conducted. The data for the reactivation of inactivated enzyme and inhibition of the enzyme by NH_2OH were fitted to eqs 3 and 4, respectively. In these equations, k_1 and k_2 are the first-order rate constants, t is time, A is the amplitude factor, and C is a constant.

$$y = A[k_1/(k_1 - k_2)](e^{-k_1 t} - e^{-k_2 t}) + C \quad (3)$$

$$y = Ae^{-k_2 t} + C \quad (4)$$

UV−Vis Spectrum of Inactivated Phosphotriesterase. Phosphotriesterase (55 μM) was incubated at 25 °C in a 2.5 mL solution containing 0.1 M CHES (pH 9.0), 20 μM $CdSO_4$, 20% CH_3CN, and 10 mM of compound **IVa**. After a 3 h incubation, the solution was centrifuged and the reaction solution was eluted through a PD-10 gel filtration column (Pharmacia) equilibrated with the same buffer. To ensure complete removal of any unbound small molecules, the protein was reloaded and eluted from the PD-10 column once more. A control sample in which the enzyme was incubated without any inhibitor was conducted in the same manner. Spectra of the control and enzyme modified by inhibitor **IVa** were recorded on a Varian Cary 2200 UV−vis spectrophotometer.

Fluoride Elimination. The reaction of compound **IVc** with phosphotriesterase was monitored with an ATI Orion PerpHecT Model 370 pH/mV meter equipped with an Orion Model 96-09 fluoride selective electrode. The reaction mixture contained 1.0 mM inhibitor **IVc**, 50 nM Cd^{2+}-substituted enzyme, 5 μM $CdSO_4$, 1 mg/mL BSA, and 20% (v/v) CH_3CN in 0.1 M CHES buffer (pH 9.0) in a volume of 3 mL.

Trapping of the Quinone Methide. Cd^{2+}-substituted PTE (355 nM) was added to a 1.5 mL solution containing 0.1 M deuterated phosphate buffer (pD 7.0), 5 μM $CdSO_4$, 1 mg/mL BSA, 20% CD_3CN, and 3.0 mM or 6.0 mM of bromide **IVa** at room temperature. The ^1H NMR spectra at 300 MHz on a Varian Unity 300 spectrometer were recorded at various time intervals. The reaction was also performed with 1.0 mM of compound **IVc**, 355 nM Cd^{2+}-PTE, 5 μM $CdSO_4$, 1 mg/mL BSA, and 20% CD_3CN in 1.5 mL of 0.1 M deuterated phosphate buffer (pD 7.0). The reaction was monitored by ^1H and ^{19}F NMR spectroscopy at various time intervals.

RESULTS

1-Bromovinyl, Z-2-Bromovinyl, 1,2-Dibromoethyl, and 4-(Halomethyl)-2-Nitrophenyl Phosphate Esters as Substrates for PTE. 1-Bromovinyl (**I**), *Z*-2-bromovinyl (**II**), and 1,2-dibromoethyl (**III**) diethyl phosphates were tested as substrates for the phosphotriesterase. ^1H NMR spectroscopy indicated that the phosphotriesterase catalyzes the hydrolysis of compounds **I**−**III** to completion. The NMR experiments showed that acetic acid and diethyl phosphate were the major products of the enzyme-catalyzed hydrolysis of **I** while glyceraldehyde and diethyl phosphate were the major hydrolysis products from compounds **II** and **III**. These results are consistent with the intermediate formation of acetyl bromide or ketene from **I** and α-bromoacetaldehyde from compounds **II** and **III** in the reaction mechanisms. The initial rates of enzymatic hydrolysis were measured from the associated changes in the NMR spectra and the kinetic constants for the 1-bromide (**I**), *Z*-2-bromide (**II**), and dibromide (**III**) are presented in Table 1. The kinetic constants determined spectrophotometrically for the enzymatic hydrolysis of 4-(halomethyl)-2-nitrophenyl (**IVa**−**c**), 4-(hydroxymethyl)-2-nitrophenyl (**V**), 2-nitrophenyl (**VI**), and 4-nitrophenyl (paraoxon, **VII**) diethyl phosphates are also listed in Table 1. The progress of the enzymatic hydrolysis of compound **IVc** was followed by a fluoride ion selective electrode and ^{19}F NMR spectroscopy. At a substrate concentration of 1.0 mM, fluoride was produced with a turnover number of 16 s^{-1} at pH 9.0. When the reaction was followed with ^{19}F-NMR, the rate of appearance of F^- equaled that of the disappearance of the starting triester. No signals attributable to the fluorinated phenol (or phenolate) were detected.

The hydrolysis of the bromide **IVa** was followed by proton NMR spectroscopy. After the addition of enzyme to a sample of the bromide **IVa**, new pairs of singlets at δ = 7.97 and 7.89 ppm, pairs of doublets at δ = 7.52 and 6.95 ppm (J = 8.7 Hz) and at δ = 7.42 and 6.91 ppm (J = 8.7 Hz), and a doublet at δ = 4.66 ppm (J = 6.0 Hz) appeared while the resonances for the starting material decreased with time (data not shown). After approximately 35 h, the transient signals (singlet at δ = 7.97, pairs of doublets at δ = 7.52 and 6.95 ppm, and the doublet at δ = 4.66 ppm) completely disappeared and no further changes were observed. The aromatic resonances of the final product were identical to the ring protons assigned to 4-hydroxy-3-

Table 1: Kinetic Constants for the Reaction of Cd^{2+}-substituted Phosphotriesterase with Inhibitors **I**−**III**, **IVa**−**c**, and Other Substrates

	compound[a]	K_m (mM)	k_{cat} (s^{-1})
I	R−O−C(Br)=C(H)−H		230[b]
II	R−O−C(H)=C(H)−Br		1.3[b]
III	R−O−CH(Br)−CH$_2$(Br)		11.5[b]
IVa	R−O−(C_6H_3)(2-O_2N)−CH_2Br	5.1[c]	440[c]
IVb	R−O−(C_6H_3)(2-O_2N)−CH_2Cl	16[c]	920[c]
IVc	R−O−(C_6H_3)(2-O_2N)−CH_2F	10[c]	420[c]
V	R−O−(C_6H_3)(2-O_2N)−CH_2OH	6.6[c]	130[c]
VI	R−O−(C_6H_4)(2-O_2N)	3.0[c]	23[c]
VII	R−O−(C_6H_4)−NO_2	0.4[d]	4620[d]

[a] R = EtOP(O)(Et*O*). [b] Derived from initial velocity experiments at substrate concentration of 2.2−2.7 mM. [c] Derived from a fit of the data to eq 1. [d] Omburo *et al.* (*2*).

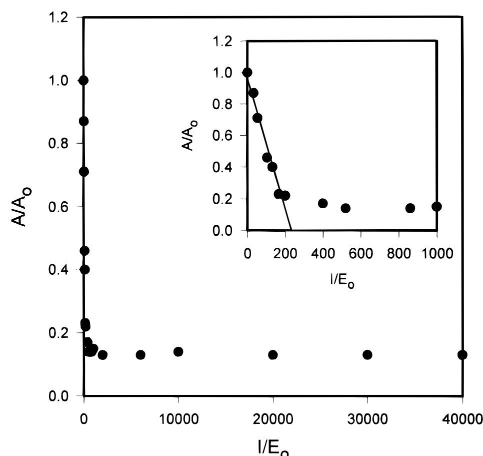


FIGURE 1: Inactivation of Cd^{2+}-substituted phosphotriesterase with 1-bromovinyl diethyl phosphate (**I**). Additional details are provided in the text.

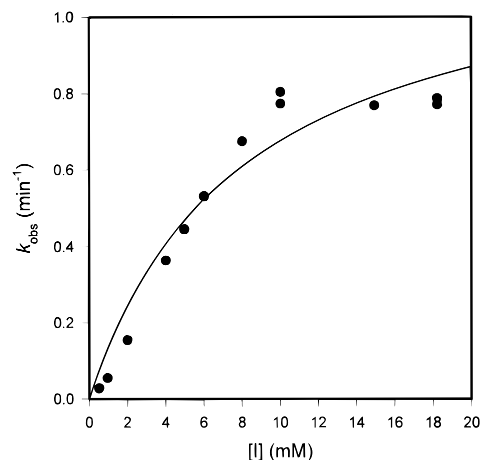


FIGURE 2: Plot of the rate of inactivation of phosphotriesterase with varying concentrations of 4-(bromomethyl)-2-nitrophenyl diethyl phosphate (**IVa**). The data were fit to eq 2 to give values for $k_{inact} = 1.2\ min^{-1}$ and $K_I = 7.9$ mM.

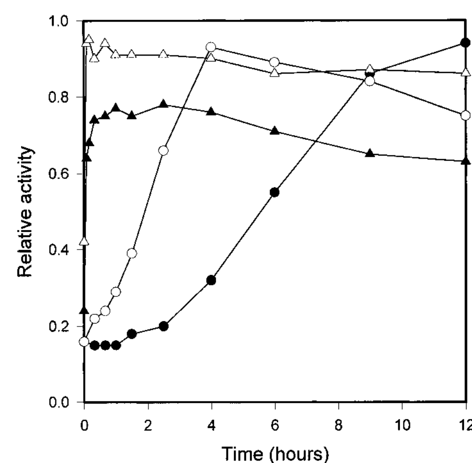


FIGURE 3: Time courses for the reactivation of phosphotriesterase inactivated by 1-bromovinyl diethyl phosphate (**I**) at various pH: pH 7.0 (●), pH 8.0 (○), pH 9.0 (▲), and pH 10.0 (△).

nitrobenzyl alcohol. The UV−vis spectrum of the product from the phosphotriesterase-catalyzed hydrolysis of **IVa** was also consistent with that of 4-hydroxy-3-nitrobenzyl alcohol. No evidence of an intermediate phenol or quinone methide was detected in these experiments.

1-Bromovinyl, Z-2-Bromovinyl, 1,2-Dibromoethyl, and 4-(Halomethyl)-2-Nitrophenyl Phosphate Esters as Enzyme Inactivators. 1-Bromovinyl (**I**), *Z*-2-bromovinyl (**II**), and 1,2-dibromoethyl (**III**) diethyl phosphates were tested as mechanism-based inactivators of phosphotriesterase. The extent of inactivation of phosphotriesterase upon incubation with 1-bromovinyl diethyl phosphate (**I**) is shown in Figure 1. A partition ratio of 230 for inhibitor **I** was determined by extrapolation of the linear portion of these data. However, the inactivation of the enzyme by **I** was not total and approximately 15% of the initial catalytic activity remained after incubation of the enzyme with inhibitor **I** up to an initial ratio of $[I]/[E_o]$ of 40000:1. Values of V_{max} and K_m for the inhibited and uninhibited enzymes were determined using steady-state kinetic assays with paraoxon as a substrate. The values of K_m for the inhibited and uninhibited enzymes were 400 μM and 580 μM, respectively. However, the value of V_{max} of the inhibited enzyme was found to be approximately 15% of the uninhibited enzyme. The rate of inactivation of the enzyme by 1-bromovinyl diethyl phosphate ester (**I**) was sufficiently fast that the rate constant for inactivation could not be determined by simple manual assays.

Compounds **II** and **III** were also tested for their ability to inactivate the Cd^{2+}-substituted enzyme. Incubation of the enzyme with inhibitors **II** and **III** showed very slow inactivation (data not shown). After prolonged incubation (2 h) of the enzyme with the *Z*-2-bromide (**II**) at 12 mM, the enzyme was found to be partially inactivated and approximately 60% of the initial activity remained. Since α-bromoacetaldehyde was expected to be the reactive intermediate derived from the enzyme-catalyzed hydrolysis of inhibitors **II** and **III**, α-bromoacetamide and α-iodoacetamide were tested as analogues of α-bromoacetaldehyde. However, neither of these compounds were able to inactivate the enzyme at concentrations up to 10 mM.

When phosphotriesterase was incubated with varying concentrations of the bromide **IVa** (0−18.2 mM), the enzyme showed a loss of activity, resulting in a time-dependent inactivation. A fit of these data to eq 4 shown in Figure 2 provided a saturating inactivation rate constant of $k_{inact} = 1.2\ min^{-1}$ and an inactivation constant (K_I) for the inhibitor of 7.9 mM. Incubation of phosphotriesterase with either the chloride (**IVb**; 0−20 mM) or the fluoride (**IVc**; 0−20 mM) derivatives caused no significant time-dependent inactivation (data not shown).

Reactivation of Inactivated Phosphotriesterase. Banzon *et al.* (*16*) showed that the phosphotriesterase inactivated by alkynyl phosphate esters could be reactivated by increasing the pH of the solution. The wild-type phosphotriesterase was inactivated by preincubation with inhibitor **I** and then diluted into various buffer solutions at pH 7.0, 8.0, 9.0, and 10.0. The time courses for the reactivation of the inactivated enzyme at various pH values are shown in Figure 3. The partially inactivated enzyme (∼15% of the initial activity) regained over 80% of the initial catalytic activity after 12 h at pH 7.0, after 4 h at pH 8.0, after 20 min at pH 9.0, and after 5 min at pH 10.0. Attempts were made to reactivate the enzyme inactivated with bromide **IVa** by changing the pH of the enzyme solution. Incubation of phosphotriesterase (50 nM) with 8 mM of bromide **IVa** in 0.1 M CHES, pH 9.0, resulted in the rapid loss of activity. The inactivated enzyme was then diluted into buffers at pH 7.0, 8.0, 9.0, and 10.0. No recovery of activity was observed after 2 h.

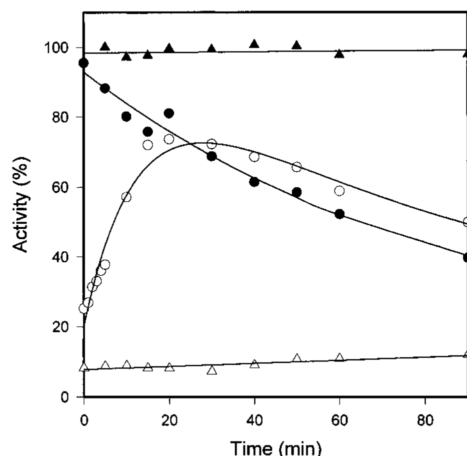


FIGURE 4: The time courses for the reactivation when phosphotriesterase inactivated by 1-bromovinyl phosphate ester (**I**) was incubated with NH_2OH. Control incubations of phosphotriesterase were run in the absence (▲) or presence (●) of NH_2OH and the incubations of inactivated enzyme were run in the absence (△) and presence (○) of NH_2OH.

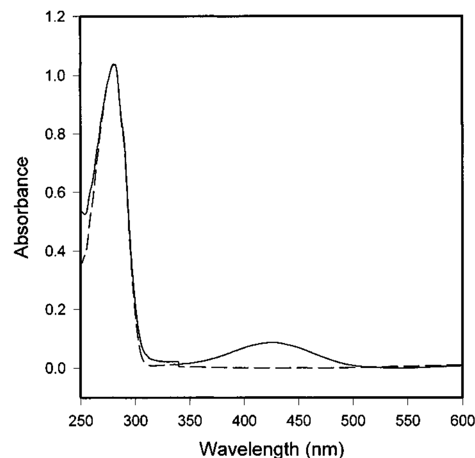


FIGURE 5: Normalized spectra of unlabeled phosphotriesterase (the dotted line) and phosphotriesterase inactivated by 4-(bromomethyl)-2-nitrophenyl diethyl phosphate (**IVa**) to 64% of its original activity (the solid line).

Protection Against Inactivation. The overall effect of adding various concentrations of azide and hydroxylamine on the extent of inactivation of phosphotriesterase with inhibitor **I** was determined at pH 7.0. Phosphotriesterase, inhibitor **I** (26 μM), and varying concentrations of the nucleophilic scavenger azide, or NH_2OH, were incubated together for 15 min and then aliquots were assayed for residual enzyme activity. The residual catalytic activity of the inactivated enzyme was not altered in the presence of sodium azide (0−20 mM). In contrast, an increase in the concentration of NH_2OH (0−20 mM) resulted in an increase in the residual enzyme activity and nearly complete protection against inactivation was observed at concentrations of NH_2OH above 10 mM. Neither NaN_3 nor NH_2OH protected the enzyme from inactivation with compound **IVa**. The inactivated enzyme was treated with NH_2OH to determine if any of the covalent adduct could be cleaved by NH_2OH and enzymatic activity recovered. The time courses for the reactivation of catalytic activity when the enzyme, preinactivated by the 1-bromide **I**, was incubated with NH_2OH are presented in Figure 4. Incubation of the unlabeled enzyme with NH_2OH resulted in a time-dependent inactivation. The observed first-order rate constant for the inactivation of the native enzyme by NH_2OH is 0.012 min^{-1}. However, NH_2-OH reactivated the enzyme inhibited by 1-bromide **I**; the recovery of enzyme activity reached a maximum after approximately 20 min and then decayed exponentially. Over 75% of the original activity was recovered with k_{obs} = 0.08 min^{-1}.

UV−Vis Spectrum of Inactivated Phosphotriesterase. The spectrum of phosphotriesterase, covalently modified by the bromide **IVa**, shows an increase in absorbance in the region between 350 and 500 nm (λ_{max} = 420 nm) when compared with a spectrum of the unmodified enzyme (Figure 5). A pK_a of 7.1 for the phenolic adduct of the modified enzyme was spectrophotometrically determined by measuring the change in absorbance at 420 nm as a function of pH.

DISCUSSION

Inactivation of PTE with Bromovinyl and Dibromoethyl Diethyl Phosphates. An essential criterion for any mechanism-based inhibitor is the direct involvement of a catalytic step, which converts the potential inhibitor to a species that is capable of blocking catalysis of the target enzyme. In order to test if compounds **I**, **II**, and **III** meet this criterion, phosphotriesterase was incubated with these compounds under catalytic conditions, and the reaction was monitored by ^{1}H NMR. The turnover rate of 230 s^{-1} for the enzymatic hydrolysis of compound **I** compares favorably with the k_{cat} for the hydrolysis of paraoxon (**VII**) by the Cd^{2+}-substituted enzyme (k_{cat} = 4620 s^{-1}; ref 2) and the hydrolysis of alkynyl phosphate esters with the Co^{2+}-substituted enzyme (k_{cat} = 200−2200 s^{-1}; ref 16). Inactivation of phosphotriesterase by inhibitor **I** was so fast that all of the inactivation occurred within 1 min after mixing with the enzyme. The partition ratio of 230 compares favorably to the partition ratios (500−1200) reported earlier using alkynyl phosphate esters as inactivators of the Co^{2+}-substituted phosphotriesterase (*16*). NMR assays showed that compounds **II** and **III** are hydrolyzed 180-fold and 20-fold more slowly than is inhibitor **I**. The low turnover rates for compounds **II** and **III** and the lack of a significant reduction in the catalytic activity indicates that the α-bromoacetaldehyde intermediate is a relatively poor inactivator of phosphotriesterase compared with either the ketene or the acetyl bromide.

Banzon *et al.* (*16, 17*) have previously reported the mechanism-based inactivation of phosphotriesterase by a series of alkynyl phosphate esters. These studies have demonstrated that the species leading to enzyme inactivation was a reactive ketene intermediate formed during the enzyme catalyzed hydrolysis of these esters. The most probable mechanism for the inactivation of the enzyme by compound **I**, as shown in Scheme 1, utilizes the formation of a ketene or an acetyl halide as the reactive intermediate. This mechanism predicts the formation of a covalently modified residue containing a carbonyl group, which is the same covalent adduct derived from inactivation by the alkynyl phosphate esters. Banzon *et al.* (*16*) have also demonstrated that phosphotriesterase inactivated by acylation of a nucleophilic amino acid residue could be reactivated by increasing the pH or adding hydroxylamine. Similar experiments were conducted to determine whether the enzyme inactivated by compound **I** could be reactivated at alkaline pH or by the

addition of NH_2OH. The results reported here demonstrated that the covalent adduct formed during the inactivation of phosphotriesterase is susceptible to hydrolysis at elevated pH and dissociation by NH_2OH. Therefore, the residue covalently modified by compound **I** behaves identically to the one that is labeled by 1-hexynyl diethyl phosphate (*16*). In experiments using mutants of phosphotriesterase, it was demonstrated that His-254 was crucial for the inactivation of the Co^{2+}-substituted phosphotriesterase by 1-hexynyl diethyl phosphate (*17*). The fact that inactivation of the enzyme by compound **I** was not complete indicates that the residue chemically modified by this inhibitor may not be absolutely essential for full enzyme activity.

Inactivation of Phosphotriesterase with 4-Halomethylphenyl Phosphates. It has recently been reported that 4-halomethylphenyl phosphate esters are mechanism-based inhibitors of prostatic acid phosphatase, calcineurin, phosphotyrosine phosphatase, and ribonuclease A (*18, 20, 22*−*24*). Enzyme-catalyzed cleavage of the P−O bond of these compounds generates a 4-hydroxybenzyl halide. This intermediate has the potential to be subsequently converted through the elimination of the halide to a quinone methide. The quinone methide can irreversibly inactivate the enzyme by the rapid formation of a covalent adduct with an active site residue. However, in order to use 4-halomethylphenyl phosphate triesters as potential inactivators of phosphotriesterase, the acidity of the phenolic leaving group must be sufficiently high to support enzyme hydrolysis of the parent triester (*6, 13*). 4-Halomethylphenyl diethyl phosphates were synthesized and tested for efficacy as mechanism-based inhibitors of phosphotriesterase. However, these compounds were shown to be poor substrates and ineffectual inactivators of the enzyme. These observations are most likely due to the poor leaving group ability of the phenols derived from the 4-halomethylphenyl diethyl phosphate. A recent study has described the introduction of an electron withdrawing group on the departing phenol of an inhibitor of prostatic acid phosphatase that appears to enhance the leaving group ability (*20*). We therefore anticipated that the addition of a nitro group attached to the ortho position of the phenyl ring would reduce the pK_a of the phenol and thus enhance the hydrolysis of the potential inactivator by phosphotriesterase. Kinetic analyses of the 4-(halomethyl)-2-nitrophenyl diethyl phosphate triesters (**IVa**−**c**) showed them to be respectable substrates of phosphotriesterase (Table 1). The higher K_m values for compounds **IVa**−**c**, relative to paraoxon (**VII**) can best be attributed to steric hindrance by the ortho substituent of the phenyl ring that induces less favorable binding for catalysis.

Formation of a quinone methide is largely dependent on deprotonation of the departing phenol (*25*) and thus inactivation experiments for the 4-(halomethyl)-2-nitrophenyl diethyl phosphate esters (**IVa**−**c**) were performed at pH 9.0. When phosphotriesterase was incubated with inhibitor **IVa** at pH 9.0, the loss of enzyme activity was time and concentration dependent. The inactivation process was first-order and exhibited saturation kinetics with the calculated values of $k_{inact} = 1.2\ min^{-1}$ and $K_I = 7.9$ mM. The partition ratio which demonstrates the efficiency of inactivation is equivalent to k_{cat}/k_{inact}. The partition ratio calculated from the turnover number and inactivation parameters for bromide **IVa** is 21 700. Myers *et al.* (*20*) have reported the inactivation of prostatic acid phosphatase by 4-(bromomethyl)-2-nitrophenyl phosphomonoester with a partition ratio of 5700. The lack of prevention by exogenous nucleophiles indicates that the inactivation process is active site directed. The failure to reactivate the inactivated enzyme by changing the pH of the incubation solution or by exogenous nucleophiles is consistent with an inactivation arising from the formation of stable covalent bond to the enzyme. Further evidence for covalent modification of phosphotriesterase resulting from the mechanism-based inactivation of the enzyme by bromide **IVa** is provided by the UV−vis absorption spectrum of the modified enzyme. The spectrum of the modified enzyme shows the appearance of a band centered at 420 nm at pH 9.0 that must be due to the ionization of the covalently attached nitrophenol.

X-ray crystallographic analysis of phosphotriesterase has revealed that the leaving group of a substrate is positioned in the hydrophobic pocket which consists of Trp-131, Phe-132, Leu-271, Phe-306, and Tyr-309 (*12*). The relative leaving group abilities of bromide, chloride, and fluoride ions from carbon are 1.0:0.02:0.0001 (*26*). The partitioning of halide ion elimination to give a bound quinone methide and the direct release of the ionized phenol into solution should be modulated by the energy barrier for the loss of aromaticity and the relative leaving group abilities of the three halide ions. Since inhibitors **IVa**−**c** have similar kinetic constants for substrate hydrolysis, the very efficient inactivation by bromide **IVa** but slow inactivation by the chloride (**IVb**) and fluoride (**IVc**) derivatives suggests that the rate of elimination of halide ion from the parent phenol largely dictates the observed partition ratio. Quinone methide formation is expected to occur much faster from the bromine-substituted phenol intermediate than from the either the chlorine- or the fluorine-substituted intermediate. Therefore, quinone methide formation is more likely to occur within the active site with **IVa** than with either **IVb** or **IVc**. The failure to detect a quinone methide intermediate using NMR spectroscopy demonstrates that the highly reactive quinone methide reacts rapidly with either a solvent water molecule (or hydroxide ion) or a phosphate buffer once the halide ion has been eliminated from the intermediate phenol. The phosphorylated benzyl alcohol was found to slowly solvolyze to yield the final product 4-hydroxy-3-nitrobenzyl alcohol. Additional evidence for a stoichiometric labeling of inactivator to the enzyme awaits the synthesis of a radiolabeled derivative.

Summary. Mechanism-based inactivation studies with bromovinyl, dibromoethyl, and 4-(halomethyl)-2-nitroaryl phosphotriesters were carried out on phosphotriesterase in order to investigate their efficacy as mechanism-based inactivators of the enzyme. 1-Bromovinyl and 4-(bromomethyl)-2-nitrophenyl phosphate esters have been shown to be efficient inactivators of the enzyme. Inactivation of the enzyme by these compounds is accompanied by covalent modification of the enzyme. The species leading to covalent modification of the enzyme by 1-bromovinyl phosphate ester is a reactive ketene or acetyl bromide formed by enzymatic hydrolysis of the inhibitor. To the best of our knowledge, this is the first reported example of a mechanism-based inactivation of a phosphatase by a halo enol phosphate. The reactive quinone methide generated when the 4-(bromomethyl)-2-nitrophenyl phosphate ester is hydrolyzed results in formation of a covalent adduct with the enzyme.

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