

## Chemical and Kinetic Evidence for an Essential Histidine in the Phosphotriesterase from *Pseudomonas diminuta*\*

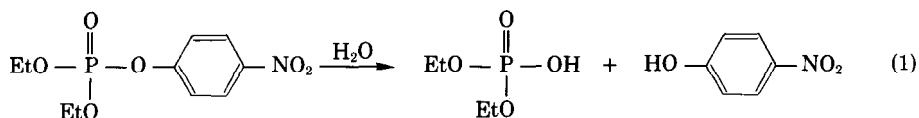
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The pH rate profile for the hydrolysis of diethyl-*p*-nitrophenyl phosphate catalyzed by the phosphotriesterase from *Pseudomonas diminuta* shows a requirement for the deprotonation of an ionizable group for full catalytic activity. This functional group has an apparent  $pK_a$  of  $6.1 \pm 0.1$  at 25 °C,  $\Delta H_{ion}$  of 7.9 kcal/mol, and  $\Delta S_{ion}$  of  $-1.4$  cal/K·mol. The enzyme is not inactivated in the presence of the chemical modification reagents dithiobis-(2-nitrobenzoate), methyl methane thiosulfonate, carbodiimide, pyridoxal, butanedione, or iodoacetic acid and thus cysteine, aspartate, glutamate, lysine, and arginine do not appear to be critical for catalytic activity. However, the phosphotriesterase is inactivated completely with methylene blue, Rose Bengal, or diethyl pyrocarbonate. The enzyme is not inactivated by diethyl pyrocarbonate in the presence of bound substrate analogs, and inactivation with diethyl pyrocarbonate is reversible upon addition of neutralized hydroxylamine. The modification of a single histidine residue by diethyl pyrocarbonate, as shown by spectrophotometric analysis, is responsible for the loss of catalytic activity. The  $pK_{inact}$  for diethyl pyrocarbonate modification is  $6.1 \pm 0.1$  at 25 °C. These results have been interpreted to suggest that a histidine residue at the active site of phosphotriesterase is facilitating the reaction by general base catalysis.

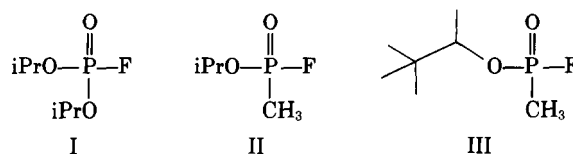
The phosphotriesterase from *Pseudomonas diminuta* catalyzes the hydrolysis of several organophosphate triesters (Lewis *et al.*, 1988; Donarski *et al.*, 1989; Dumas *et al.*, 1989) and phosphofluoridates (Dumas *et al.*, 1990). The fastest substrate found to date is diethyl-*p*-nitrophenyl phosphate (paraoxon) with a  $k_{cat}$  of  $2100$  s<sup>-1</sup> (Dumas *et al.*, 1989) (Equation 1). The hydrolysis of these substrates has been shown to occur



through the inversion of stereochemistry at the phosphorus center as a result of the direct attack of an activated water molecule (Lewis *et al.*, 1988). The most likely mechanism to

account for these observations is a general base mechanism in which there is the catalytic deprotonation of a water molecule by an active site base and the subsequent attack at the phosphorus center by the generated hydroxide.

There is a single zinc atom located at or near the active site of this phosphotriesterase (Dumas *et al.*, 1989). The removal of the zinc by addition of chelating agents, such as *o*-phenanthroline, causes the complete inactivation of the enzyme, but the inactivation is prevented by the addition of high concentrations of paraoxon. Based upon pH-rate data, Donarski *et al.* (1989) reported that a single ionizable group with an apparent  $pK_a$  of  $6.1 \pm 0.1$  is required for the hydrolysis of paraoxon. This value is lower than the expected value of 8–10 for a zinc-bound water molecule (Cleland, 1977) and has thus encouraged the investigation of the active site residues of phosphotriesterase to identify the catalytic base and other important amino acids within the active site. The inactivation of acetylcholinesterase and many other serine proteases by phosphotriesters and phosphofluoridates such as diisopropyl fluorophosphate (DFP, *I*),<sup>1</sup> isopropyl methylphosphonofluoridate (sarin, *II*), and 1,2,2-trimethylpropyl methylphosphonofluoridate (soman, *III*) has been demonstrated to take place as a result of



phosphorylation of a serine residue in the active site (Ohkawa, 1982). These compounds are all substrates for the phosphotriesterase from *P. diminuta* (Dumas *et al.*, 1990). Therefore, the mechanism involved in the hydrolysis of these powerful (and often deadly) modification reagents deserves a rigorous investigation through the identification of the active site

residues of the phosphotriesterase. The experiments presented in this study have provided evidence for the partici-

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<sup>1</sup> The abbreviations used are: DFP, diisopropyl fluorophosphate; CAPS, 3-(cyclohexylamino)-1-propanesulfonate; CHES, 2-(cyclohexylamino)ethanesulfonate; DEPC, diethyl pyrocarbonate; DMF, dimethylformamide; DTNB, dithiobis-(2-nitrobenzoate); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EPN, ethyl *p*-nitrophenylphenylthiophosphonate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; MMTS, methylmethanethiosulfonate; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonate); TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonate; MES, 2-(*N*-morpholino)ethanesulfonic acid.

pation of a histidine residue that is required for catalytic activity.

#### EXPERIMENTAL PROCEDURES

**Materials**—The phosphotriesterase from *P. diminuta* was purified to homogeneity from a baculovirus expression system as described previously (Dumas *et al.*, 1989). The concentration of enzyme was calculated from the absorbance at 280 nm using an extinction coefficient of 1.86 ml/(mg·cm). All chemicals used in this study were purchased from either Aldrich or Sigma.

**Enzyme Assays**—The activity of phosphotriesterase was determined spectrophotometrically using a Gilford Model 260 spectrophotometer in 150 mM 2-*N*-(cyclohexylamino)ethanesulfonic acid (CHES), pH 9.0, buffer containing 1.0 mM diethyl-*p*-nitrophenyl phosphate (paraoxon) by monitoring the increase in absorbance at 400 nm resulting from the release of *p*-nitrophenol ( $\epsilon = 17,000 \text{ M}^{-1}\text{cm}^{-1}$ ). One unit of enzymatic activity is defined as the amount of enzyme required to hydrolyze 1  $\mu\text{mol}$  of paraoxon/min at 25 °C. Kinetic data were analyzed with the aid of the computer programs of Cleland (1979).

**pH Rate Profiles**—The kinetic parameters from paraoxon hydrolysis ( $V$  and  $V/K_m$ ) were measured at different pH values by fitting the data to Equation 2.

$$v = \frac{VA}{K_m + A} \quad (2)$$

The buffers used were 2-*N*-morpholinoethanesulfonic acid (MES, pH 5.3–6.5), piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES, pH 6.6–7.2), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.3–8.2), 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid (TAPS, pH 8.3–8.9), CHES (pH 9.0–9.9), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, pH 10.0). Below pH 6.0, the rates of enzymatic hydrolysis were measured by monitoring the change in absorbance at 320 nm. The extinction coefficients at 400 and 320 nm at several pH values over the range of interest were determined for *p*-nitrophenol using Beer's Law. The exact extinction coefficient ( $\epsilon$ ) at any pH was then determined graphically from a plot of  $\epsilon$  versus pH. The kinetic  $pK_a$  values were determined by fitting the data to Equation 3:

$$\log k = \log \left( \frac{c}{1 + H/K} \right) \quad (3)$$

where  $k$  is either  $V$  or  $V/K$ ,  $c$  is the pH-independent value of  $V$  or  $V/K$ ,  $H$  is the hydrogen ion concentration, and  $K$  is the dissociation constant of the ionizable group. These experiments were repeated at 20, 25, 30, 35, and 40 °C. Extinction coefficients were determined for *p*-nitrophenol at each temperature as described above.

**Survey of Chemical Modification Reagents**—The inactivation of phosphotriesterase by chemical modification reagents was explored by the addition of a large excess of reagent to an enzyme solution and incubated at 25 °C. Aliquots of these solutions were removed at various time intervals and assayed for enzymatic activity over a 3-h period. The modification of cysteinyl residues was investigated by the addition of either 20 mM methylmethanethiosulfonate (MMTS) or 50  $\mu\text{M}$  5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to a 3.2 nM solution of phosphotriesterase in 50 mM CHES, pH 9.0, buffer. Iodoacetamide (50 mM), which could react potentially with any nucleophilic group on the enzyme, was added to a 12.8 nM phosphotriesterase solution in 100 mM CHES, pH 9.0, buffer. The inactivation of phosphotriesterase by modification of lysyl residues was investigated by the addition of 100 mM pyridoxal to a 4.0 nM enzyme solution in 50 mM CHES, pH 9.0, buffer. Inactivation by modification of arginine residues was investigated by incubation of the enzyme with 100 mM butanedione in 500 mM borate, pH 9.0, buffer. Water-soluble carbodiimides have been shown to inactivate enzymes by reaction with carboxylate groups (Hoare and Koshland, 1966). A 4.0 mM solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) with 16.8 nM phosphotriesterase in 200 mM CHES, pH 9.0, buffer or 100 mM HEPES, pH 7.0, buffer was studied in the absence or presence of 40 mM glycine methyl ester. The reaction of 9.0 nM phosphotriesterase with histidine modification reagents was explored by photo-oxidation with 60  $\mu\text{M}$  methylene blue or 600  $\mu\text{M}$  Rose Bengal dye in 100 mM HEPES, pH 6.5, buffer. Photoinactivation was initiated by exposure to light 15 cm from an overhead projector bulb for 30 min at room temperature. Methyl-*p*-nitrophenylsulfonic acid was tested at a concentration of 5.0 mM with a 12.8 nM solution of phosphotriesterase

in 100 mM MES, pH 6.0, buffer containing 5% methanol. The chemical modification of phosphotriesterase with diethyl pyrocarbonate (DEPC) was screened by the addition of 5.0 mM DEPC to a 1.0 nM solution of phosphotriesterase in 500 mM PIPES, pH 6.5, buffer.

**Inactivation of Phosphotriesterase with Diethyl Pyrocarbonate**—DEPC stock solutions ( $\approx 600 \text{ mM}$ ) were made fresh the day of use in anhydrous ethanol and stored on ice. The exact concentrations of the stock solutions were calculated from the increase in absorbance at 230 nm when an aliquot was added to a solution of 10 mM imidazole and 100 mM phosphate, pH 6.8, buffer using an extinction coefficient of  $3000 \text{ M}^{-1}\text{cm}^{-1}$  for the ethoxyformylated imidazole product (Miles, 1977). The phosphotriesterase was incubated at 25 °C in 500 mM acetate buffer for the pH range of 5.4 to 6.0 and in phosphate buffer for the pH range of 6.0 to 7.3. Protection against inactivation was studied by the addition of 2.0 mM diethyl-*p*-fluorophenyl phosphate. The extent of histidine modification was determined using an extinction coefficient at the maximum absorbance of 245 nm of  $3200 \text{ M}^{-1}\text{cm}^{-1}$  (Miles, 1977). Spectra were obtained with a Gilford Model 2600 programmable recording spectrophotometer.

#### RESULTS

**pH Rate Studies**—The rate of paraoxon hydrolysis increases with pH to a limiting value at high pH, indicating the titration of a single base. The  $pK_a$  determined at 30 °C for the log  $V$  profile is  $6.0 \pm 0.1$  (Fig. 1). The log( $V/K$ ) versus pH rate profiles gave apparent  $pK_a$  values that were the same values obtained for the log  $V$  profile within experimental error. The measurement of the enthalpy ( $\Delta H_{\text{ion}}$ ) and entropy ( $\Delta S_{\text{ion}}$ ) of ionization for this single ionizable group was obtained by measurement of the apparent  $pK_a$  as a function of temperature (Equation 4).

$$pK_a = \frac{\Delta H}{2.303RT} - \frac{\Delta S}{2.303R} \quad (4)$$

In this equation,  $R$  is the ideal gas constant and  $T$  is the temperature in degrees Kelvin (Fig. 2). The enthalpy and entropy were determined to be 7.9 kcal/mol and  $-1.4 \text{ cal/K}\cdot\text{mol}$ , respectively.

**Survey of Chemical Modification Reagents**—The results of a survey of several chemical modification reagents for the inactivation of phosphotriesterase are summarized in Table I. No significant inactivation was observed with DTNB, MMTS, EDC, pyridoxal, butanedione, methyl-*p*-nitrophenylsulfonate, or iodoacetamide. Phosphotriesterase could be photoinactivated completely with 1.0 mM methylene blue ( $k_{\text{obs}} = 0.7 \pm 0.1 \text{ h}^{-1}$ ) or 600  $\mu\text{M}$  Rose Bengal dye ( $k_{\text{obs}} = 1.9 \pm 0.1 \text{ h}^{-1}$ ). Phosphotriesterase is also inactivated with 6.0 mM DEPC. The DEPC-inactivated enzyme is reactivated almost completely upon addition of 1 M neutralized hydroxylamine, and there is complete protection from inactivation when phosphotriesterase is incubated with 6.0 mM DEPC in the presence of 2.0 mM diethyl-*p*-fluorophenylphosphate (Fig. 3). The time-dependent loss in activity with DEPC, corrected for

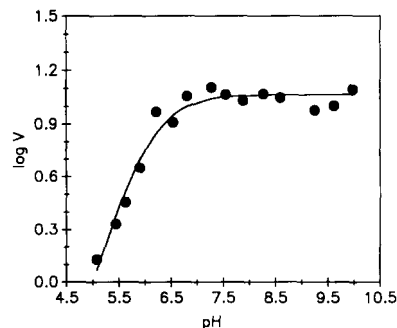


FIG. 1. The pH rate profile for paraoxon hydrolysis at 30 °C. The solid line is drawn from a fit of the data to Equation 3 using a  $pK_a$  of  $6.0 \pm 0.1$ . Additional details are given in the text.

the hydrolysis of DEPC in buffer, can be described by Equation 5.

$$\ln(A/A_0) = (-k/k')(1 - e^{-k't}) \quad (5)$$

The amount of activity remaining at time ( $t$ ) is represented by the ratio  $A/A_0$ ,  $k$  is the pseudo-first-order rate constant for inactivation of the enzyme, and  $k'$  is the pseudo-first order rate constant for the hydrolysis of DEPC in buffer (Gomi and Fujioka, 1983). The value of  $k'$  at 25 °C in 500 mM acetate or phosphate buffer ranges from 0.10 min<sup>-1</sup> at pH 7.4 to 0.07 min<sup>-1</sup> at pH 5.4. A plot of the pseudo-first-order rate constants for inactivation ( $k$ ) versus the DEPC concentration is linear indicating that no reversible complex between enzyme and DEPC is formed (Church *et al.*, 1985). The second-order rate constant for inactivation at pH 6.8 and 25 °C (the slope of this line) is  $40 \pm 5 \text{ M}^{-1} \text{ min}^{-1}$  (data not shown).

The pH dependence for inactivation of phosphotriesterase with DEPC at 25 °C was studied over the pH range of 5.4 to 7.3. The pseudo-first-order rate constants for inactivation with 6.0 mM DEPC were fit to Equation 3 to calculate the  $pK_a$  of the modified residue. The  $pK_a$  of the titratable group is  $6.1 \pm 0.1$  (Fig. 4).

The difference spectrum between 12.4  $\mu\text{M}$  native enzyme and 12.4  $\mu\text{M}$  enzyme 30 min after addition of DEPC (20% remaining activity) shows a large absorption peak at 245 nm (data not shown), indicating the modification of histidine residues (Miles, 1977). No absorbance decrease is observed around 278 nm. The number of histidine residues modified

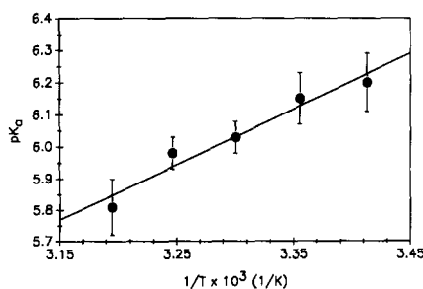


FIG. 2. Variation of the kinetic  $pK_a$  for paraoxon hydrolysis as a function of temperature. A fit of the data to Equation 4 gives the values for  $\Delta H_{\text{ion}}$  and  $\Delta S_{\text{ion}}$  as 7.9 kcal/mol and  $-1.4 \text{ cal}/^\circ\text{K}\cdot\text{mol}$ , respectively. Additional details are available in the text.

under these conditions, based upon the maximal absorbance of the difference spectrum, is calculated as 2.9 using an extinction coefficient of  $3200 \text{ M}^{-1}\text{cm}^{-1}$  (Miles, 1977). When the inactivation is investigated in the presence of 2.0 mM diethyl-*p*-nitrophenyl phosphate at 25 °C with 12 mM DEPC, only 2.1 residues per enzyme are modified after 30 min.

#### DISCUSSION

The identity of the functional amino acids within the active site of the phosphotriesterase from *P. diminuta* has been examined with a combination of kinetic and chemical probes. No significant loss in catalytic activity of the enzyme was observed when the phosphotriesterase was incubated with dithiobis-(2-nitrobenzoic acid), methyl methane thiosulfonate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, pyridoxal, butanedione, or iodoacetamide. These results suggest that the side chains of cysteine (Kenyon and Bruice, 1977), aspartate (Hoare and Koshland, 1966), glutamate (Hoare and Koshland, 1966), lysine (Egorov *et al.*, 1982), and arginine (Riordan, 1973) are not required for catalytic activity or that some or all of these groups are totally inaccessible to the modification reagents. Since this phosphotriesterase has been shown to catalyze the hydrolysis of ethyl-*p*-nitrophenylthiophosphonate (EPN) (Lewis *et al.*, 1988), di-*s*-butyl-*p*-nitro-

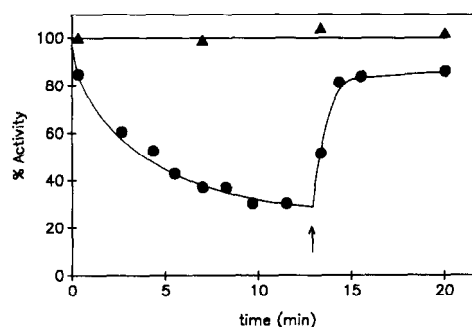


FIG. 3. The inactivation of phosphotriesterase with DEPC as a function of time. The inactivation of phosphotriesterase with 6.0 mM DEPC (circles) is reversed upon addition of 1.0 M neutralized hydroxylamine (arrow). There is complete protection against inactivation by 6.0 mM DEPC when the inactivation mixture contains 2.0 mM diethyl-*p*-fluorophenyl phosphate (triangles). Additional details are given in the text.

TABLE I  
Survey of chemical modification reagents

Targeted residue	Reagent	Conditions	Inactivation rate $h^{-1}$
Cysteine	50 $\mu\text{M}$ DTNB	pH 9.0 <sup>a</sup>	<0.02
Cysteine	20 mM MMTS	pH 9.0	<0.02
Aspartate or glutamate	4 mM EDC	pH 9.0 and 7.0	<0.07
Aspartate or glutamate	4 mM EDC	pH 9.0 and 7.0 with 40 mM glycine methyl ester	<0.06
Lysine	100 mM pyridoxal	pH 9.0	<0.09
Arginine	100 mM butanedione	pH 9.0 <sup>b</sup>	<0.01
Histidine	1 mM methylene blue	pH 6.5 <sup>c</sup>	0.72
Histidine	0.6 mM Rose Bengal	pH 6.5	1.92
Histidine	6 mM DEPC	pH 6.5 <sup>d</sup>	8.7
Histidine	5 mM methyl- <i>p</i> -nitro- phenylsulfonate	pH 6.0 <sup>e</sup>	<0.01
Histidine or cysteine	50 mM iodoacetamide	pH 9.0 <sup>a</sup>	<0.01

<sup>a</sup> 100 mM CHES buffer.

<sup>b</sup> 100 mM borate buffer.

<sup>c</sup> Photoinactivation as described in the text, 100 mM HEPES buffer.

<sup>d</sup> 500 mM phosphate buffer.

<sup>e</sup> 100 mM MES in 5% methanol.

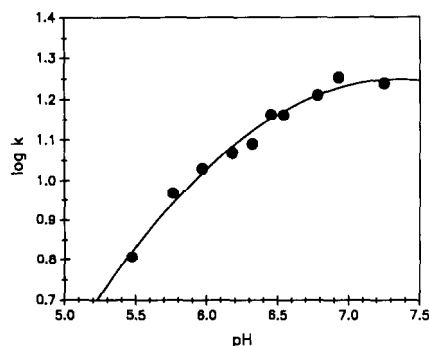


FIG. 4. The pH versus inactivation rate from DEPC modification of phosphotriesterase. The solid line is drawn from a fit of the data to Equation 3 for a  $pK_a$  of  $6.1 \pm 0.1$ . The values for  $\log k$  are taken as the pseudo-first order rate constant for inactivation of phosphotriesterase by DEPC. Additional details are given in the text.

phenylphosphate, and other phosphate triesters with rather large side chains (Donarski *et al.*, 1989) and can also accommodate the pesticide coumaphos (Dumas *et al.*, 1989) with a large coumarin leaving group then it would appear reasonable that only the largest modification reagents would be excluded physically from the active site.

The inactivation of phosphotriesterase by chemical modification has only been achieved by reaction with the histidine reagents methylene blue, Rose Bengal, and diethyl pyrocarbonate. The photo-oxidation of proteins with methylene blue or Rose Bengal dye has been shown in some proteins to result in the modification of methionine and tryptophan residues and sometimes tyrosine, serine, or threonine in addition to the most commonly modified histidine groups (Ray and Koshland, 1961). Whereas incubation with either of these dyes with light led to the complete inactivation of phosphotriesterase, the broad reactivity of these reagents weakens the value of these experiments toward the identification of an essential active site histidine. A more selective modification of histidyl residues has been made possible with the development of DEPC (Miles, 1977). DEPC inactivates phosphotriesterase with a second-order rate constant of  $40 \text{ M}^{-1}\text{min}^{-1}$ . This inactivation reaction requires the use of a large molar excess of reagent (approximately 1000 times) to counter the relatively rapid hydrolysis of DEPC in aqueous buffer. In spite of this large excess of reagent, very little disubstitution of the modified imidazole occurs because the inactivation is reversed almost completely upon addition of neutralized hydroxylamine (87% recovered). A dicarboxyethylated imidazole would not be reactivated with hydroxylamine, because ring scissions would be expected to occur (Miles, 1977). Modification of lysyl groups by DEPC is unaltered with hydroxylamine (Melchoir and Fahrney, 1970), therefore lysine can be ruled out as the critical residue because any inactivation at a lysine would not have been rapidly reversed with hydroxylamine. Modification at tyrosine is, however, reversible with hydroxylamine. A reaction of a tyrosyl group can be identified by a characteristic decrease in absorbance at 275–280 nm after reaction with DEPC (Burnstein *et al.*, 1974). There is no decrease in absorbance observed in the difference spectrum for phosphotriesterase in this wavelength range. There is, however, a large increase in absorbance at 245 nm as would be expected with the modification of histidine residues. This observed increase corresponds to the modification of 2.9 residues of the 7 total histidines present in phosphotriesterase after 80% of the original catalytic activity is lost. The paraoxon analog, diethyl-*p*-fluorophenyl phosphate, has been shown to be a competitive inhibitor *versus* paraoxon ( $K_i = 0.02 \text{ mM}$ ), but this

compound is not hydrolyzed at a significant rate (Donarski *et al.*, 1989). When phosphotriesterase is incubated with an amount 20 times greater than the  $K_i$  of diethyl-*p*-fluorophenyl phosphate, essentially all of the enzyme exists in the enzyme-inhibitor complex. The active site of the enzyme would therefore be protected by the inhibitor. When incubated with DEPC in the presence of diethyl-*p*-fluorophenyl phosphate, only 2.1 histidine residues are modified, and the enzyme is fully active. The difference between the inhibitor-protected enzyme and the inactivated enzyme of 0.8 histidines corresponds exactly to the percent activity lost. Presumably, the remaining histidines are less reactive toward DEPC and are therefore not modified under these conditions.

The  $pK_a$  for the imidazole moiety of the single histidine modified with DEPC was determined by measuring the rate of inactivation as a function of pH. Only an unprotonated imidazole can act as a nucleophile and thus the reaction of the phosphotriesterase DEPC is pH-dependent. The  $pK_a$  for the imidazole that appears critical for enzyme activity is 6.1 at 25 °C.

The inactivation studies of the phosphotriesterase with a variety of modification reagents were augmented with an analysis of the change in the kinetic parameters  $V$  and  $V/K$  with pH. For both  $V$  and  $V/K$  there is observed the titration of a single ionizable group with a  $pK_a$  of 6.1 at 25 °C that must be unprotonated for catalytic activity. Because the substrate paraoxon has no ionizable groups in the pH range 5–10, this group must be of enzyme origin.

This  $pK_a$  value of 6.1 is within the range typically reported for histidine residues, but could possibly represent a carboxylate or a zinc-bound water (Auld *et al.*, 1986). The enthalpy ( $\Delta H$ ) of ionization (7.9 kcal/mol) falls within the range of values expected for histidine, cysteine, tyrosine residues, or zinc-bound water but is much lower than the value expected for a lysyl or arginyl residue (about 10–12 kcal/mol) and much higher than the value expected for carboxylate groups (0–1 kcal/mol). Only histidyl and lysyl residues have been reported to have entropies as low as  $-1.4 \text{ cal/K}\cdot\text{mol}$  (Auld *et al.*, 1986). Consideration of the enthalpy and entropy together are consistent only with the titration of a histidine (Fig. 5).

In summary, the apparent  $pK_a$  of 6.1 and the related thermodynamic components  $\Delta H_{\text{ion}}$  (7.9 kcal/mol) and  $\Delta S_{\text{ion}}$  ( $-1.4 \text{ cal/K}\cdot\text{mol}$ ) determined from pH rate profiles and rates of inactivation with DEPC are most consistent with the ionization of a critical histidine residue. Upon addition of histidine modification reagents, phosphotriesterase is inactivated completely, and incubation of phosphotriesterase with other residue-specific reagents causes no loss in enzymatic activity. These results are evidence for an essential histidine in the

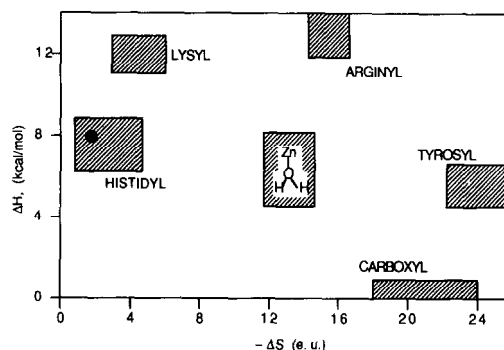


FIG. 5. Comparison of thermodynamic parameters for phosphotriesterase to literature values. The  $\Delta H_{\text{ion}}$  and  $\Delta S_{\text{ion}}$  values for phosphotriesterase (filled circle) are consistent with the ionization of a histidine. Adapted from Auld *et al.* (1986).

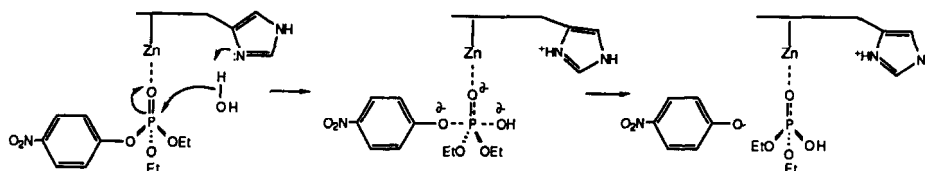


FIG. 6. The proposed mechanism of action for phosphotriesterase. An imidazole abstracts a proton from a water molecule with the subsequent attack of the hydroxyl on the phosphorus center. The active site zinc functions in the polarization of the phosphate making the phosphorus more susceptible to attack. The intermediate complex collapses to the products, *p*-nitrophenolate and diethyl phosphate.

active site of phosphotriesterase. The function of this histidine in the catalysis may be defined better in the course of studies of the active site zinc.

There are at least two catalytic roles a zinc ion may play in a metalloenzyme. First, the substrate may displace a water ligand and bind directly to the zinc. The zinc, as a Lewis acid, polarizes the substrate making it more susceptible for nucleophilic attack. The second possible role would be for a zinc-bound hydroxide as the actual nucleophile for the hydrolysis of a substrate. Both of these mechanisms have been proposed for many zinc metalloenzymes, including carbonic anhydrase (Lindskog, 1983), carboxypeptidase A (Vallee *et al.*, 1983), and thermolysin (Holmes and Matthews, 1981). Similarly, the zinc ion may function in either of these capacities in phosphotriesterase.

Pocker and Strom (1968) proposed a histidine as the catalytic group in carbonic anhydrase based upon thermodynamic data. However, the <sup>1</sup>H-NMR spectroscopy studies by Campbell *et al.* (1974) have shown that none of the histidines titrate near the apparent *pK<sub>a</sub>* of the catalytic group. Unlike phosphotriesterase, the chemical modification of histidine residues in carbonic anhydrase results in only a partial inactivation of the enzyme. The crystal structure of the complex of carbonic anhydrase with imidazole (a competitive inhibitor *versus* CO<sub>2</sub>) has been solved by Kannon *et al.* (1975). This inhibitor is located near the zinc ion without displacing the metal-bound water. This result suggests the zinc may act as a Lewis acid in addition to providing the site for the nucleophilic hydroxide.

The chemical modification of carboxypeptidase A with carboxylate-modifying reagents results in the complete inactivation of the enzyme, but the *pK<sub>inact</sub>* for carboxypeptidase with Woodward's reagent *K* is 7.0, a value greater than the apparent *pK<sub>a</sub>* of 6.0 determined from pH rate data (Petra, 1971). The apparent *pK<sub>a</sub>* is also perturbed significantly in metal replacement studies (Auld and Vallee, 1970). The modified Glu-270 of carboxypeptidase A is within hydrogen bond distance to the zinc-bound water (Bauer *et al.*, 1979) and the *pK<sub>a</sub>* at 6.0, therefore, could be regarded as a cooperative ionization of these two groups (Bauer *et al.*, 1983). The enthalpy of ionization for the catalytic group in carboxypeptidase has been shown to be 7.2 ± 0.1 kcal/°K · mol (Makinen *et al.*, 1979). This value compares favorably to the observed  $\Delta H_{ion}$  for a water on model pentacoordinate zinc ions (Woolley, 1975). Solvent perturbation studies for carboxypeptidase A have indicated a cationic acid (Vallee *et al.*, 1983). The similarities between carboxypeptidase A and phosphotriesterase include thermodynamic evidence and parity in chemical modification studies.

Thermolysin has been shown to be completely inactivated by the modification of Glu-143 (Holmes *et al.*, 1983) or by modification of histidine residues (Pangburn and Walsh, 1975), and the bell-shaped pH rate profile is consistent with the ionization of either a carboxylate or zinc-bound water and the deprotonation of a histidine (Stauffer, 1971). Further-

more, the x-ray crystal structure for a thermolysin-phosphonamidate complex has shown the phosphonamidate inhibitor to be ligated to the zinc (Christianson and Lipscomb, 1988). Therefore the role of zinc in thermolysin appears to be in the polarization of the carbonyl group. As shown in the comparison between phosphotriesterase and carboxypeptidase A described above, the results of thermodynamic and chemical modification studies for thermolysin and phosphotriesterase are analogous. The hydrolysis of organophosphates by phosphotriesterase most likely occurs by the deprotonation of a water molecule by histidine followed by attack of the hydroxyl on the phosphorus center that has been polarized by binding to the zinc ion (Fig. 6).

Despite many years of study, the molecular mechanisms of carbonic anhydrase, carboxypeptidase A, and thermolysin are still under investigation. The zinc ion has been implicated as a Lewis acid and as a general base (as zinc-bound water) for each of these enzymes. Like these enzymes, phosphotriesterase is a zinc metalloenzyme for which either mechanism may be proposed. The distinction between these mechanisms for phosphotriesterase may become more clear with the measurement of primary and secondary oxygen isotope effects and metal replacement experiments which are currently in progress.

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