Histidine-254 Is Essential for the Inactivation of Phosphotriesterase with the Alkynyl Phosphate Esters and Diethyl Pyrocarbonate†

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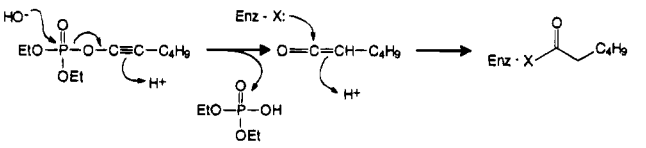
ABSTRACT: The alkynyl phosphate ester, 1-hexynyl diethyl phosphate (**I**), is a mechanism-based inhibitor of phosphotriesterase. It has been previously determined that a histidine residue in the wild-type phosphotriesterase is covalently modified by this compound. In order to identify which of the seven histidine residues in the native enzyme are required for inactivation, the kinetic properties of phosphotriesterase mutants with this suicide substrate were examined in detail. Six of the seven mutants (histidine to asparagine) were rapidly inactivated by **I**. The mutants H55N, H57N, and H230N also showed partition ratios that were lower than for the wild-type enzyme. The rate of inactivation of H201N was significantly slower than that of wild-type phosphotriesterase. The H254N mutant could not be inactivated; no more than 60% of the initial activity was lost, even at I/E_0 ratios of 4000:1. These results suggest that His-254 is essential for the inactivation of phosphotriesterase and is likely to be the primary target in the wild-type enzyme for modification by **I**. The inactivation of wild-type phosphotriesterase and the seven mutants was also studied using diethyl pyrocarbonate, a histidine selective reagent. The second-order rate constant for the inactivation of wild-type phosphotriesterase was determined to be 1.3 M^{-1} min^{-1}. The rate constants for the inactivation of the H55N, H57N, H201N, and H230N mutants were larger than for the wild-type enzyme. Thus, it appears that when these histidine residues are replaced by asparagine, other histidine residues in the active site become more susceptible to modification, resulting in a faster rate of inactivation. The mutant H254N was not inactivated in the presence of DEPC. The activity of this mutant increased within the first 2 min of incubation with DEPC and then gradually decreased. These studies demonstrate that His-254 is required for the inactivation of the phosphotriesterase and is likely to be the residue that is modified during the reaction of phosphotriesterase with either DEPC or 1-hexynyl diethyl phosphate.

Phosphotriesterase is a zinc metalloenzyme which catalyzes the hydrolysis of organophosphate triesters. We have previously demonstrated that alkynyl phosphate esters can act as potent mechanism-based inactivators of this enzyme. These suicide substrates are thought to be catalytically converted by the phosphotriesterase into highly reactive ketene intermediates, which subsequently react with an active site nucleophile. The modified enzyme has little or no catalytic activity, and the postulated reaction mechanism is presented below in Scheme 1.

Chemical and spectroscopic evidence suggests that an essential histidine residue at the active site is modified during the inactivation of phosphotriesterase with these alkynyl phosphate esters. The inactivated enzyme can be reactivated at alkaline pH values, demonstrating that the newly formed covalent bond is susceptible to hydrolysis at elevated pH. The UV difference spectrum between the modified and unmodified phosphotriesterase shows an absorption band centered at 245 nm. These properties are fully consistent with the formation of an *N*-acylimidazole derivative from an active site histidine residue and are inconsistent with the expected properties for any of the other nucleophilic groups associated with the 20 common amino acids.

Scheme 1



The seven histidine residues in phosphotriesterase have been previously mutated to asparagine, and the kinetic parameters for these proteins have been reported (Kuo & Raushel, 1994). The relative catalytic activity (V_{max}) of the Co-substituted mutants for the hydrolysis of paraoxon are as follows: the H55N and H201N mutants have less than 1% activity, the H57N, H230N, and H254N mutants have less than 10% activity, and the H123N and H257N mutants have at least 50% activity compared to the wild-type phosphotriesterase. The pH rate profiles of the Co-reconstituted mutants H254N, H55N, and H230N were significantly different from those for the wild-type enzyme, indicating that functional groups from other residues may become involved in the reaction mechanism upon mutation of these histidine residues to asparagine. The H123N mutant displayed catalytic and spectroscopic properties nearly identical to those of the wild-type phosphotriesterase, and thus it appears to have no involvement in the enzymatic activity or

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the structural integrity of the active site. We have previously postulated that the remaining six histidine residues are clustered together in the active site region (Kuo & Raushel, 1994)[1] such that the majority of these histidine residues are involved directly in the ligation of the two divalent cations required for structure and catalysis, with one histidine residue participating as a general base or proton shuttle group. In order to identify which of the seven histidine residues are critically modified by the alkynyl phosphate esters, the inactivation properties of these mutants with the suicide substrates were examined in detail.

MATERIALS AND METHODS

Materials. Paraoxon, diethyl pyrocarbonate, and all buffers were purchased from Sigma Chemical Co. Anhydrous acetonitrile was obtained from Aldrich Chemical Co. The mechanism-based inactivator of phosphotriesterase, 1-hexynyl diethyl phosphate, was prepared as previously reported (Stang et al., 1986, 1989).

Purification of Phosphotriesterase Mutants. The mutants H55N, H57N, H123N, H201N, H230N, H254N, and H257N were purified as previously described (Kuo & Raushel, 1994). The Co-substituted proteins were used in all experiments.

Efficiency of Inactivation by 1-Hexynyl Diethyl Phosphate. The partition ratios of the seven phosphotriesterase mutants were determined with 1-hexynyl diethyl phosphate (**I**). The

(I)

phosphotriesterase mutants were incubated at 25 °C in 0.1 M PIPES[2] buffer, pH 7.0, containing 3% CH_3CN in a final volume of 100 μL with various concentrations of **I**. An aliquot of the enzyme solution was assayed with 1.0 mM paraoxon in 0.1 M CHES[2] buffer, pH 9.0. In some cases the assay solution was supplemented with excess $CoCl_2$ (10–500 μM). The partition ratios were calculated as described by Knight and Waley (1985) by plotting the fraction of remaining enzyme activity ($[E]_r/[E]_0$) versus the initial ratio of inhibitor to enzyme ($[I]/[E]_0$).

Rate of Inactivation of H201N Phosphotriesterase by 1-Hexynyl Diethyl Phosphate. The mutant H201N (500 nM) was incubated in 0.1 M PIPES buffer, pH 7.0, containing 3% CH_3CN with various concentrations of **I** (0–0.54 mM) at 25 °C. Aliquots were removed and assayed with 1.0 mM paraoxon in 0.1 M CHES buffer, pH 9.0, containing 200 μM $CoCl_2$ at various times from 30 s to 30 min. The rate of inactivation was calculated by plotting ΔA_{400}/min versus time and fitting the curve to a single exponential decay.

DEPC Inactivation of the Phosphotriesterase Mutants. Diethyl pyrocarbonate (DEPC) was used to modify the histidine residues in the wild-type and mutant enzymes. DEPC stock solutions were made in anhydrous ethanol immediately before use and stored on ice. The concentration of the stock solution was determined by measuring the change in the absorbance at 230 nm when an aliquot was added to a solution of 10 mM imidazole/0.1 M potassium phosphate, pH 6.8, and using an extinction coefficient of 3000 M^{-1} cm^{-1} for the resulting *N*-carbethoxyimidazole product (Miles, 1977). The rate of hydrolysis for DEPC in 0.1 M potassium phosphate, pH 6.8 buffer was determined by incubating DEPC in the buffer at 25 °C and removing aliquots at various time intervals to measure the remaining DEPC concentration by reaction with 10 mM imidazole as described above. Wild-type phosphotriesterase was exchanged through Pharmacia PD-10 columns into 0.1 M potassium phosphate, pH 6.8 buffer and incubated at 25 °C. Inactivation of the enzyme was initiated by adding DEPC to the protein solution in a ratio of 60 mM DEPC to 2 μM enzyme. Aliquots were removed at regular time intervals and assayed for remaining enzyme activity. The seven histidine mutants were also exchanged into 0.1 M potassium phosphate, pH 6.8 buffer prior to inactivation with DEPC. The H123N and H257N (5–8 μM) mutant enzymes were inactivated in the same manner as the wild-type enzyme. The other five mutant enzymes (6–13 μM) were inactivated with 17 mM DEPC, except for the H57N mutant, which needed no more than 0.3 mM DEPC for complete inactivation in a reasonable length of time. The H254N (13 μM) mutant was inactivated with varying amounts of DEPC (3–60 mM).

UV Difference Spectroscopy. The mutant phosphotriesterase, H254N (24 μM), was incubated with 1-hexynyl diethyl phosphate (I/E_0 = 1000:1) in 50 mM PIPES, pH 7.0/3% CH_3CN in a total volume of 1 mL at 25 °C for 15 min. After incubation, the solution was centrifuged for 10 min at 14 000 rpm to remove any precipitated protein. The reaction solution (1 mL) was then loaded onto a PD-10 gel filtration column (Pharmacia) equilibrated with 50 mM PIPES, pH 7.0/3% CH_3CN, and the protein fraction was eluted with the same buffer. The protein fraction was passed through the PD-10 column a second time to ensure that all unbound small molecules were completely removed from the protein fraction. A control reaction was run in which the inhibitor was omitted but was otherwise treated identically to the inactivation reaction. UV spectra of the inactivation and control reactions were recorded on a Cary 2200 UV–visible spectrophotometer between 220 and 340 nm and stored digitally. The difference spectrum was obtained by normalizing the two spectra at 280 nm and then subtracting the control spectrum of the unmodified mutant enzyme from the spectrum of the inactivated enzyme.

RESULTS

Partition Ratio Determination. The seven histidine mutants of phosphotriesterase were tested for their ability to be inactivated by the alkynyl phosphate esters. With one exception all of the mutants were rapidly inactivated (<5% of initial activity) by these compounds. The partition ratios of the phosphotriesterase mutants H55N, H57N, H123N, H201N, H230N, H254N, and H257N using 1-hexynyl diethyl phosphate as the mechanism-based inhibitor were found to be 130, 20, 1020, 1030, 400, 1200, and 980, respectively. The partition ratio of the wild-type enzyme with this same

[1] The X-ray crystal structure of the apo-phosphotriesterase indicates that the tertiary structure of the enzyme is an α/β barrel. The six histidine residues implicated in the mechanism and in the binding of the divalent cations are clustered at the C-terminal end of the barrel (Matt M. Benning, Frank M. Raushel, and Hazel M. Holden, unpublished experiments).

[2] Abbreviations: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

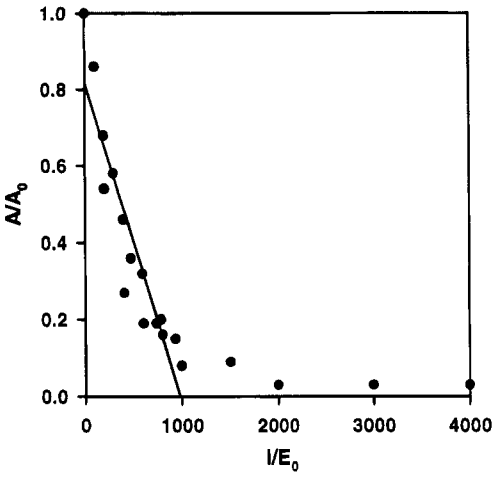


FIGURE 1: Inactivation of the H257N phosphotriesterase with variable amounts of 1-hexynyl diethyl phosphate. The partition ratio is 980. Additional details are presented in the text.

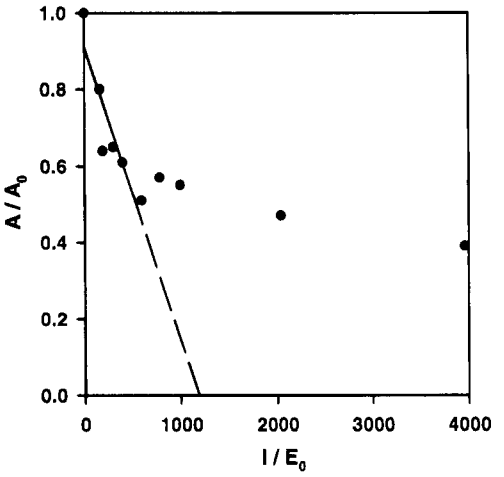


FIGURE 2: Inactivation of the H254N phosphotriesterase with variable amounts of 1-hexynyl diethyl phosphate. The partition ratio is 1200.

compound was 500. Representative data for the inactivation of H257N are shown in Figure 1. The H254N mutant was not completely inactivated by the alkynyl phosphate ester. At least 40% of the initial activity remained when this mutant was incubated with the ester at an I/E_0 ratio of 4000:1, as shown in Figure 2.

Rate of Inactivation of H201N Phosphotriesterase by 1-Hexynyl Diethyl Phosphate. The rate of inactivation of the H201N phosphotriesterase by inhibitor **I** was slow enough to be determined by manual removal of aliquots from the enzyme/inhibitor incubation mixture (the rest of the mutants were inactivated at a rate that was too fast to be determined in this way). Each aliquot was assayed for catalytic activity using paraoxon as a substrate, buffered at pH 9.0, and supplemented with $CoCl_2$. The remaining enzyme activity (ΔA_{400}/min) was plotted versus time, and the rate constant for inactivation was calculated by fitting the data to eq 1 for a single exponential decay. The rate constants for inactivation of the H201N phosphotriesterase by inhibitor **I** at I/E_0 ratios of 102:1, 258:1, 500:1, and 1000:1 were determined to be 0.11 min^{-1}, 0.14 min^{-1}, 0.32 min^{-1}, and 0.52 min^{-1}, respectively, as shown in Figure 3. A fit of the data presented in Figure 3 to eq 2 provides values for k_{inact} = 1.5 ± 0.9 min^{-1} and K_i = 1.1 ± 0.8 mM.

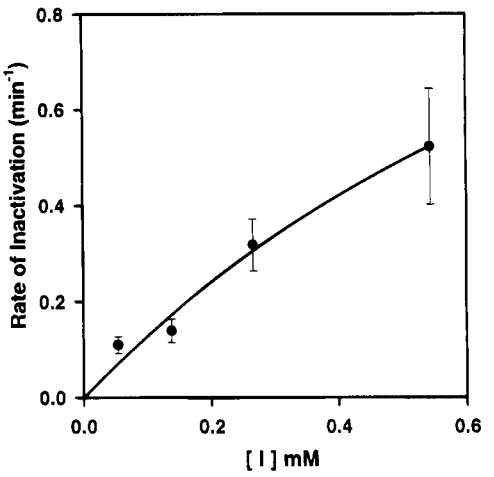


FIGURE 3: Variation in the rate of inactivation of the H201N phosphotriesterase with varying amounts of 1-hexynyl diethyl phosphate. A fit of the data to eq 2 gives values for k_{inact} = 1.5 ± 0.9 min^{-1} and K_i = 1.1 ± 0.8 mM.

Table 1: Effect of Diethyl Pyrocarbonate on Wild-Type and Mutant Enzyme Activities[a]

enzyme	k_{cat} for Co-substituted enzyme (s^{-1})	enzyme concn (μM)	DEPC concn (mM)	k (M^{-1} min^{-1})[b]
wild-type	7800	2	60	1.3
H55N	0.16	6	17	9
H57N	390	7	0.3	440
H123N	5600	5	59	1.5
H201N	25	7	17	9
H230N	500	13	17	24
H257N	7000	8	62	1.2

[a] Inactivation reactions were done in 0.1 M potassium phosphate, pH 6.8 at 25 °C. [b] The bimolecular rate constants for the inactivation of enzyme by DEPC.

$$y = a(e^{-kt}) + b \quad (1)$$

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

DEPC Inactivation of Phosphotriesterase Mutants. The inactivation of phosphotriesterase with DEPC followed pseudo-first-order kinetics. The loss of activity over time, corrected for the hydrolysis of DEPC in buffer, is described by eq 3, where A/A_0 is the fraction of activity remaining at

$$\ln(A/A_0) = -(k/k')I_0(1 - e^{-k't}) \quad (3)$$

time t, I_o is the initial concentration of DEPC, k is the bimolecular rate constant for the loss of enzyme activity, and k' is the first-order rate constant for the hydrolysis of DEPC in buffer (Gomi & Fujioka, 1983). The value of k' at 25 °C in 0.1 M potassium phosphate, pH 6.8 buffer was determined to be 0.07 min^{-1}. Table 1 summarizes the results for DEPC modification of the wild-type and mutant enzymes. A representative plot for the inactivation of the wild-type phosphotriesterase is shown in Figure 4. The H254N mutant demonstrated quite unusual behavior in that the initial catalytic activity was significantly enhanced within the first 2 min of incubation with DEPC. The time course for the change in activity of H254N upon incubation with various concentrations of DEPC is shown in Figure 5. The second-

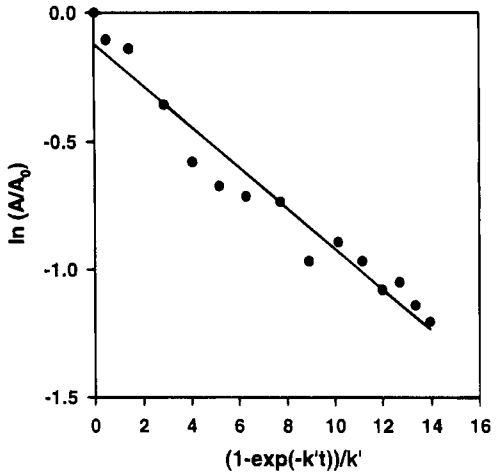


FIGURE 4: Inactivation of the wild-type phosphotriesterase by diethyl pyrocarbonate. The enzyme (2 μM) was incubated with 60 mM DEPC. The second-order rate constant, k, was determined to be 1.3 M^{-1} min^{-1} from a fit of the data to eq 3. Additional details are given in the text.

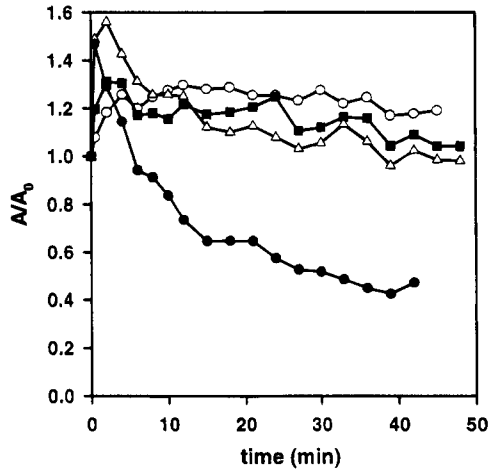


FIGURE 5: Inactivation of the H254N phosphotriesterase by diethyl pyrocarbonate. The mutant enzyme (13 μM) was incubated in the presence of 3 mM DEPC (○), 10 mM DEPC (■), 40 mM DEPC (△), and 60 mM DEPC (●). The activity of the mutant enzyme increased within the first 2 min of incubation with DEPC and then gradually decreased.

order rate constants for inactivation of the wild-type enzyme and the more active mutant enzymes, H123N and H257N, were quite similar, with k values of 1.2−1.5 M^{-1} min^{-1}. The less active mutant enzymes, H230N, H201N, H57N, and H55N, were inactivated at much faster rates; the second-order rate constants ranged in value from 9 M^{-1} min^{-1} to 440 M^{-1} min^{-1}.

UV Difference Spectroscopy. The UV spectrum of the H254N mutant phosphotriesterase after modification with the alkynyl phosphate ester shows an increase in absorption in the 235−270 nm range compared to the unmodified H254N phosphotriesterase (Figure 6). An absorption band centered at 249 nm (ϵ = 4100 M^{-1} cm^{-1}) is observed in the difference spectrum.

DISCUSSION

Inactivation of Phosphotriesterase Mutants with 1-Hexynyl Diethyl Phosphate. The partition ratios exhibited by 1-hexynyl diethyl phosphate (**I**) were measured with the individual phosphotriesterase mutants in order to determine which one

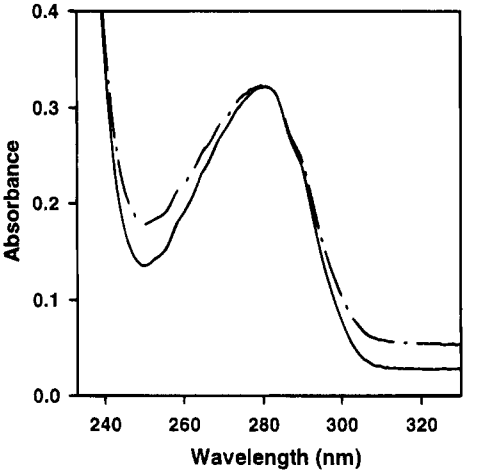


FIGURE 6: UV−Vis spectra of unmodified H254N phosphotriesterase (—) and H254N phosphotriesterase after incubation with 1-hexynyl diethyl phosphate (— • —). Additional details are given in the text.

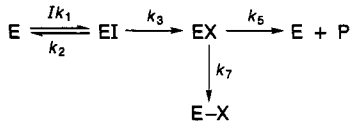
of the seven histidine residues in the wild-type enzyme is the fatal target for the suicide substrate. The histidine mutants H55N, H57N, and H230N showed partition ratios with **I** that were lower than that of the wild-type enzyme, which showed a partition ratio of 500. All of the enzymatic activity was, nevertheless, rapidly lost. Therefore, it appears that none of these residues is the site for reaction with the ketene intermediate. However, the change in partition ratio, relative to the wild-type enzyme, suggests that the active site is perturbed enough within these three mutants to enable more efficient inactivation by the alkynyl phosphate ester. This enhanced efficiency could be due to a number of factors: increased nucleophilicity of the active site histidine, a change in the release rate of the ketene intermediate, or a difference in solvent accessibility to the active site.

The partition ratios for the reaction of the alkynyl phosphate ester with the H123N, H201N, and H257N mutants are within the range previously observed for the wild-type phosphotriesterase. It has been determined by an earlier investigation (Kuo & Raushel, 1994) that His-123 does not appear to be critical for catalytic activity.[3] Thus, it is not surprising that the H123N mutant phosphotriesterase shows kinetic parameters for the inactivation process which are nearly identical to those for the wild-type enzyme.

Interesting results were, however, obtained with the H201N and H254N mutants. In the case of H201N, the rate of inactivation of the mutant enzyme is significantly slower than that of the wild-type phosphotriesterase. In fact, the rate of inactivation is slow enough to be determined by manual removal of aliquots from the reaction solution, in contrast with the wild-type enzyme which required a stopped-flow instrument to determine the rate of inactivation. The apparent decrease in the inactivation rate is due, in part, to an increase in the K_i for the inhibitor. Within the concentration range available for these investigations, saturation was barely observed (see Figure 3). However, a fit of these data to eq 2 provides an estimate for k_{inact} of approximately 1.5 min^{-1} at saturating inactivator. The working model for the

[3] The X-ray crystal structure of the apoenzyme indicates that His-123 is at the N-terminal end of the barrel, away from the putative active site (Matt M. Benning, Frank M. Raushel, and Hazel M. Holden, unpublished experiments).

Scheme 2



inactivation of phosphotriesterase by the mechanism-based inactivators is shown in Scheme 2, where I is the alkynyl phosphate ester, X is the ketene intermediate, and E−X is the inactivated complex.

The expression for k_{inact} in terms of the rate constants presented in this model is given by the following equation (Knight & Waley, 1985):

$$k_{inact} = k_3k_7/(k_3 + k_5 + k_7) \quad (4)$$

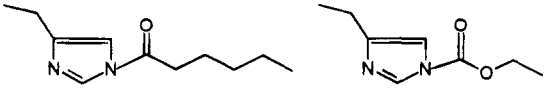
Since $k_5/k_7 = 1030$ for H201N, the minimal value for k_3 is 1550 min^{-1}, which is close to the k_{cat} value for the hydrolysis of paraoxon by this mutant (1500 min^{-1}; Kuo & Raushel, 1994). Therefore, it appears that the rate constant for the initial cleavage of the alkynyl phosphate ester (k_3) is similar to the observed k_{cat} value for the hydrolysis of paraoxon by the mutant H201N. In the previous investigation the minimum value for either k_3 or k_5 was reported to be 850 s^{-1} (or 51 000 min^{-1}) for the inactivation of the wild-type phosphotriesterase by 1-hexynyl diethyl phosphate. It has been proposed previously (Kuo & Raushel, 1994) that His-201 acts as a general base or proton shuttle group to activate the water molecule that directly attacks the substrate. The significant decrease in the rate of inactivation when His-201 is replaced with a nonbasic group is consistent with this working model.

Completely different results were observed during the attempt to inactivate H254N with the alkynyl phosphate ester. With this mutant, the partition ratio with **I** was more difficult to determine due to the fact that the enzyme could not be inactivated, even at I/E_0 ratios of 4000:1 (Figure 2). No more than 60% of the initial activity was lost even at relatively high concentrations of inactivator to enzyme. This type of behavior is not observed with the wild-type enzyme nor with any of the six other histidine mutants. These results therefore support the conclusion that His-254 is essential for complete inactivation of phosphotriesterase by 1-hexynyl diethyl phosphate, and it is likely to be the primary target in the wild-type enzyme for the putative ketene intermediate. However, some activity is lost, and since it is unlikely that a carboxamide group of an asparagine side chain would react with the intermediate, then it appears that an alternate residue is modified in the mutant H254N upon the hydrolysis of the hexynyl phosphate ester. Figure 6 shows the spectra of the modified and unmodified H254N phosphotriesterase. It has been shown in the previous investigation that the acylation of the imidazole side chain of a histidine residue will result in an increase in absorption between 235 and 270 nm in the UV spectrum of the enzyme. The increase in absorption seen in this region upon modification of H254N indicates that a histidine residue is acylated. It appears likely that the mutant H254N is modified at an alternate histidine residue, resulting in an adduct that is only half as active as the unlabeled mutant enzyme.

Diethyl Pyrocarbonate Inactivation of Phosphotriesterase Mutants. The inactivation of the seven mutants was also studied using DEPC, a chemical reagent which specifically modifies histidine residues. This reagent is not active site specific like the mechanism-based inhibitor discussed above, but comparison of the time courses for reaction with DEPC of each of the mutants should give complementary information about the reactivity of each histidine. The second-order rate constant for the inactivation of wild-type phosphotriesterase with DEPC was determined to be 1.3 M^{-1} min^{-1}. The rate constants for the inactivation of the H55N, H57N, H201N, and H230N mutants were larger than for the wild-type enzyme. It appears that when these histidine residues are replaced by asparagine, other histidine residues in the active site become more susceptible to modification by DEPC, resulting in a faster rate of inactivation.

Modification of the mutant H254N with DEPC gave surprising results. Within the first 10 min of incubation with DEPC, the enzyme activity increases and then decreases back to its initial activity. At high concentration of DEPC (60 mM), the activity of this mutant enzyme decreases to a minimum of 40% remaining activity; however, H254N does not become inactivated in the presence of lower concentrations of DEPC. It appears that His-254 plays a key role in the inactivation of phosphotriesterase by DEPC, since its replacement with asparagine results in a protein that is unable to be inactivated like the wild-type phosphotriesterase.

Conclusion. Histidine-254 is essential for the inactivation of phosphotriesterase with either DEPC or the mechanism-based inhibitor 1-hexynyl diethyl phosphate and is most likely the primary site of modification. These studies have shown that when six of the seven histidines in phosphotriesterase are changed individually to asparagine, the resulting protein is equally or more efficiently inactivated by 1-hexynyl diethyl phosphate or DEPC compared to the wild-type protein. The exception, the mutant H254N, lost a maximum of 60% activity in the presence of the mechanism-based inhibitor and lost only a small amount of activity upon incubation with DEPC. The structures of the modified histidine residue with these two reagents are illustrated below.



REFERENCES

Dumas, D. P., & Raushel, F. M. (1990) *J. Biol. Chem. 265,* 21498.

Gomi, T., & Fujioka, M. (1983) *Biochemistry 22,* 137.

Knight, G. C., & Waley, S. G.. (1985) *Biochem. J. 225,* 435.

Kuo, J. M., & Raushel, F. M. (1994) *Biochemistry 33,* 4265.

Miles, E. W. (1977) *Methods Enzymol. 47,* 431.

Stang, P. J., Boehshar, M., & Lin, J. (1986) *J. Am. Chem. Soc.* 108, 7832.

Stang, P. J., Kitamura, T., Boehshar, M., & Wingert, H. (1989) *J. Am. Chem. Soc. 111,* 2225.