Role of Essential Histidine Residues in L-α-Hydroxy Acid Oxidase from Rat Kidney†

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ABSTRACT: L-α-Hydroxy acid oxidase from rat kidney is rapidly inactivated by diethyl pyrocarbonate at pH 7 and 23 °C. The kinetics and pH dependence of inactivation as well as the absorbance spectrum of the inactivated enzyme indicated that histidine residues were modified. Quantification of the increase in 240-nm absorbance due to *N*-carbethoxyhistidine showed that two histidine residues per active site were modified during total inactivation. Studies with [^{14}C]diethyl pyrocarbonate showed that only 2 equiv per active site was incorporated on complete inactivation, so only histidine residues were modified. Only one of the two reactive histidines was essential for catalysis, however. Treatment of the inactivated enzyme with hydroxylamine led to only partial recovery of activity and incomplete removal of ethoxycarbonyl groups from the enzyme. The presence of substrates and competitive inhibitors decreased the rate of inactivation but did not decrease the number of histidine residues finally modified. Further analysis of the protection data strongly indicated that the reactive histidines are within the active site of the enzyme and are extensively protected from reaction with diethyl pyrocarbonate by substrate and competitive inhibitors. Prior inactivation of the enzyme with the suicide substrate 2-hydroxybutynoic acid, which gives a covalent adduct with the FMN coenzyme (Cromartie, T. H., & Walsh, C. T. (1975) *Biochemistry 14*, 3482), unexpectedly had no effect on the rate or extent of reaction of the enzyme with diethyl pyrocarbonate. It is suggested that a histidine residue participates in the removal of a proton from the α carbon of hydroxy acid substrates to generate a required substrate carbanion but that this histidine is not immediately adjacent to the flavine coenzyme and is probably not the base which first removes the substrate proton.

It has become generally accepted that those simple flavoproteins which contain no metals or other cofactors and which oxidize α-hydroxy or α-amino acids cause the formation of an anion at the α carbon of the substrate early in the catalytic sequence (Bright & Porter, 1975; Bruice, 1976; Walsh, 1978). This conclusion rests partially on observations that many of these enzymes catalyze the elimination of HCl from β-chloro substrates concomi ant with oxidation and that several can generate and react with carbanions from nitroalkane substrate analogues. Also the hydroxy acid oxidases are irreversibly inactivated on reaction with β,γ-acetylenic substrates by a mechanism which remains unproven but which has been proposed to involve propargylic rearrangement of an initially formed α carbanion (Cromartie & Walsh, 1975b; Ghisla et al., 1976). Since a substrate carbanion is produced as an intermediate or transition state in these oxidations, there must be a base at the catalytic site of these enzymes to remove the substrate α hydrogen. Several flavoenzymes, including lactate monooxygenase (Choong et al., 1977), arginine monooxygenase (Thomê-Beau et al., 1971), monoamine oxidase (Hiramatsu et al., 1975), and L-amino acid oxidase (Page & Van Etten, 1971), have been found to have an essential histidine residue at the active site. Choong et al. (1977) have proposed that histidine is the base which accepts the substrate α proton in lactate monooxygenase, although no experimental evidence requiring such a conclusion was obtained.

We have recently purified an FMN-dependent[1] L-α-hydroxy acid oxidase from rat kidney (Cromartie & Walsh, 1975a). The enzyme is unusual in being able to catalyze the oxidation of both α-hydroxy and α-amino acids at the same active site and in being a tetramer containing only two FMN coenzymes. Duley & Holmes (1976) have purified a similar enzyme with four FMN coenzymes per enzyme tetramer from rat kidney, but use of our purification procedure for the major soluble isozyme from rat kidney reproducibly gives the fully kinetically competent enzyme with 2 FMN/tetramer. Unlike some other hydroxy acid oxidizing enzymes, this L-α-hydroxy acid oxidase does not cause the elimination of HCl from the substrate β-chlorolactic acid. But like the other hydroxy acid utilizing flavoenzymes, it is irreversibly inactivated by the acetylenic suicide substrate 2-hydroxy-3-butynoic acid to give a covalent adduct of the inactivator with the flavine coenzyme (Cromartie & Walsh, 1975b). This work develops a new use for such acetylenic suicide substrates which makes use of the fact that such inactivators are known to be attached to the flavine coenzyme at the chemically reactive part of the FMN ring system. If the functional group on the side chain of an amino acid thought to be at the active site is positioned near the reactive part of the flavine coenzyme, as expected for a residue removing the substrate α proton, its chemical reactivity toward reagents in solution should be modified by the introduction of an irreversible inactivator in its vicinity at the flavine. In particular it is shown that L-α-hydroxy acid oxidase has two reactive histidine residues, one of which is essential for catalysis, but that this residue probably does not directly serve as the base which generates the substrate α carbanion required for oxidation.

Experimental Procedure

Materials. L-α-Hydroxy acid oxidase was prepared as previously described by Cromartie & Walsh (1975a) with the modification that the 35–60% fraction in the first ammonium sulfate precipitation was retained. The final specific activity

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[1] Abbreviations used: FMN, flavine mononucleotide; MES, 2-[*N*-morpholino]ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DCIP, dichloroindophenol; $NaDodSO_4$, sodium dodecyl sulfate.

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obtained in this work was 385 nmol min^{-1} mg^{-1} compared to 416 nmol min^{-1} mg^{-1} obtained earlier. The enzyme assay buffer was also changed to 0.1 M imidazole, pH 8.2, which resulted in slightly higher rates of reaction than the phosphate buffer employed previously. The enzyme was stored as a saturated ammonium sulfate suspension in 20 mM MES buffer at pH 7.

Imidazole, MES, Tris, β-mercaptoethanol, DL-α-hydroxybutyric acid, DTNB, and *N*-acetylhistidine were purchased from Sigma. Guanidine hydrochloride was recrystallized twice from methanol. 2-Hydroxy-3-butynoic acid was synthesized by the procedure of Cromartie et al. (1974) and was stored as the lithium salt. Diethyl pyrocarbonate from Aldrich was distilled under vacuum before use.

[1-^{14}C]Diethyl pyrocarbonate was prepared by the procedure of Melchior & Fahrney (1970) from 0.25 mCi of [1-^{14}C]ethanol (California Bionuclear Corporation, 5.86 Ci/mol). The distilled diethyl pyrocarbonate [bp 68–70 °C (3.5 mm)] had a specific activity of 2.2 μCi/mmol and was stored in acetonitrile at −4 °C.

Inactivation with Diethyl Pyrocarbonate. To 75 μg of L-α-hydroxy acid oxidase in 20 mM MES buffer at pH 7.0 and 25 °C was added an ethanol solution of diethyl pyrocarbonate. The ethanol concentration did not exceed 2% by volume and was found to have negligible effects on the activity and stability of the enzyme. At appropriate times the modification was quenched by addition of aliquots of the inactivation solution to 90 volumes of 0.1 M imidazole buffer at pH 8.2. After several minutes DCIP and DL-α-hydroxybutyrate were added to 25 mM, and the enzyme activity remaining was determined as described by Cromartie & Walsh (1975a). The concentration of diethyl pyrocarbonate in ethanol or acetonitrile solution was determined by the absorbance at 242 nm in a Beckman 25K spectrophotometer after reaction with freshly prepared *N*-acetylhistidine (Choong et al., 1977). A molar extinction coefficient of 3900 was used.

The pH dependence of inactivation was similarly determined with the MES buffer adjusted to pH values between 5.90 and 7.50. It was determined that the pH did not change during the reactions. Although stable above pH 6.2 under these conditions in the absence of diethyl pyrocarbonate, the enzyme lost activity on standing at pH 6 or below. The rates of diethyl pyrocarbonate inactivation at pH 6.0 and 5.90 have been corrected for this loss of activity. Above pH 7 diethyl pyrocarbonate is hydrolyzed rather rapidly, and corrections for this loss of reagent were made for the pH studies above pH 7.0. Substrate and product protection experiments at pH 7 were identical except that the inhibitor was added to the enzyme solution just before the diethyl pyrocarbonate.

Reactivation of Diethyl Pyrocarbonate Treated Enzyme. Enzyme which retained less than 2% original activity before treatment with diethyl pyrocarbonate was dialyzed against several changes of pH 7 MES buffer at 4 °C or was passed through at 40 × 1 cm Sephadex G-25 column at 25 or 4 °C, using the same buffer, without recovering any activity. Native enzyme was stable to these treatments, and partially inactivated enzyme gave enzyme with the same activity after Sephadex chromatography.

Reaction of diethyl pyrocarbonate inactivated enzyme with hydroxylamine was explored under a variety of conditions. Enzyme treated with 1 mM diethyl pyrocarbonate for the time sufficient to give inactivation from 60 to 98% was added to a solution of hydroxylamine adjusted to pH 7. After a period of incubation at 4 or 25 °C, the solution was passed through the Sephadex G-25 column, and the protein was collected and assayed for activity. The Sephadex chromatography was necessary because hydroxylamine interferes with the DCIP assay for the enzyme. Reactivation conditions examined included 0.05–1.5 M hydroxylamine incubated for 1 to 48 h, 0.1 M sodium azide for 8 h at 4 °C, 0.01–0.2 M β-mercaptoethanol for 2 h at 25 °C or 24 h at 4 °C, and 0.1 M Tris or phosphate buffer at pH 7.5 for 24 h at 25 °C. Inclusion of 0.1 mM FMN at all steps in the reactivation experiment has no effect on the recovery of activity. Experiments with radiolabeled diethyl pyrocarbonate involved addition of 10 μL of a 75 mM solution of [1-^{14}C]diethyl pyrocarbonate to 0.5 mL of a 1.2 mg/mL solution of the enzyme followed by incubation at 25 °C for 20 min. To 0.25 mL of this solution was added hydroxylamine sulfate to give 0.1 M and to the other 0.25 mL was added hydroxylamine sulfate to give 0.5 M. After 2 h, the hydroxylamine solution was applied to a 40 × 1 cm Sephadex G-25 column equilibrated with 20 mM MES buffer, pH 7, and the fractions containing protein were collected and combined. The protein concentration was determined by A_{280}, and a 0.5-mL aliquot in 10 mL of Yorktown Research TT-21 cocktail was counted in a Beckman LS 2150 scintillation counter. After 8 h, the sample containing 0.5 M hydroxylamine was similarly treated. The experiment was repeated with 0.1 M hydroxylamine and 2% w/v sodium dodecyl sulfate for reactivation.

Spectroscopic Studies of Inactivation. A sample of enzyme that was 13.7 μM in active sites was treated with 1 mM diethyl pyrocarbonate for 20 min, and the UV spectrum was taken against a blank containing the same amount of native enzyme at 25 °C. This spectrum did not change for at least 1 h or after the addition of hydroxylamine sulfate to a final concentration of 0.1 M. Addition of hydroxylamine to 0.75 M denatured the enzyme and gave rise to featureless end absorption. A series of kinetic runs at 25 °C were made in which enzyme (0.8 mg/mL) was reacted with 0.5, 1.0, and 1.5 mM diethyl pyrocarbonate and the increase in absorbance at 242 nm monitored as a function of time. The same kind of kinetic experiment with 1.0 mM diethyl pyrocarbonate was conducted in the presence of 5 mM DL-α-hydroxybutyrate. A sample of enzyme was inactivated by treatment with hydroxybutynoic acid (Cromartie & Walsh, 1975b) and dialyzed against 20 mM MES buffer, pH 7, to give a completely inactive enzyme. This enzyme was then treated with diethyl pyrocarbonate under identical conditions as for the native enzyme, and the increase in A_{242} was measured as a function of time. A fluorescence difference spectrum between native and diethyl pyrocarbonate modified enzyme (8% original activity) was obtained at room temperature with an SLM-8000 spectrofluorometer with data acquisition by an HP-9815A calculator. The enzyme was excited at 280 nm and the emission monitored from 300 to 600 nm (peak at 337 nm).

In a series of experiments, 0.5 mL of a 2.5 mg/mL solution of L-α-hydroxy acid oxidase was irreversibly inactivated by reaction with DL-2-hydroxybutynoic acid as described by Cromartie & Walsh (1975b) and then dialyzed against 20 mM MES buffer, pH 7, or passed through a Sephadex G-25 column equilibrated with the same buffer. This totally inactive enzyme was then treated with 1 mM diethyl pyrocarbonate under conditions identical with those used earlier for native enzyme, and the increase in absorbance at 242 nm was monitored as a function of time. In similar experiments, 0.5 mM or 1.5 mM diethyl pyrocarbonate was used.

Specificity of Inactivation. Thiol groups in native and diethyl pyrocarbonate treated enzyme were determined with DTNB by the procedure of Ellman (1959) with 1% NaDod-

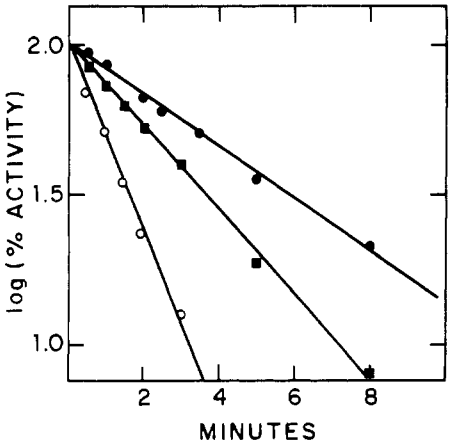


FIGURE 1: Inactivation of rat kidney L-α-hydroxy acid oxidase with varying concentrations of diethyl pyrocarbonate. Reaction mixtures contained 20 mM MES buffer (pH 7.0), 100 mg of enzyme, 1–2% ethanol, and the indicated concentrations of diethyl pyrocarbonate (●, 0.28 mM; ■, 0.44 mM; and ○, 1.0 mM).

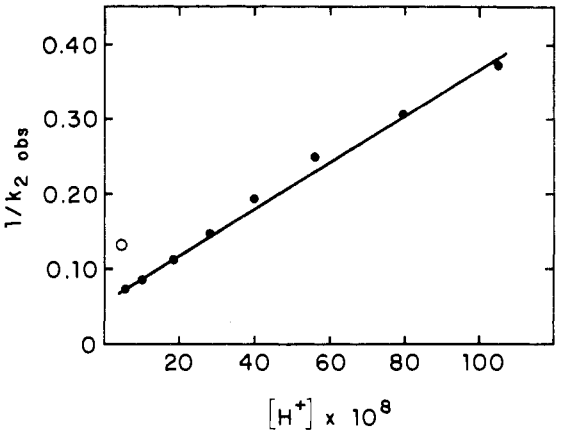


FIGURE 2: pH dependence of inactivation of L-α-hydroxy acid oxidase with diethyl pyrocarbonate. Reaction conditions were as in Figure 1 with 1 mM DEPC except that the pH was adjusted to the indicated value with HCl or NaOH. The open data point was not included in determination of the slope; see text.

SO_4 present. Tyrosine and tryptophan were estimated by the method of Bredderman (1974). Radiolabeling was done with [1-^{14}C]diethyl pyrocarbonate as previously described. After inactivation the enzyme was passed through a 1 × 40 cm Sephadex G-25 column, fractions were assayed for protein by spectrophotometry at 280 nm, and 0.5 mL was counted for radioactivity. Since the reagent is labeled in only one of two equivalent ethyl groups, the concentration of label incorporated, as measured by scintillation counting, was doubled to give the concentration of labeled diethyl pyrocarbonate actually taken up. As previously described, a sample of enzyme was inactivated by treatment with 10 mM 2-hydroxybutynoate and DCIP, dialyzed against 20 mM MES buffer, and radiolabeled in the same manner, and the incorporated radioactivity was determined as before.

Determination of Phosphate Binding. On the addition of phosphate to a solution of the enzyme in MES buffer, the 455-nm absorbance due to the flavine decreases, while the absorbances at 410 and 250 nm increase. These changes were used to measure the binding constant of phosphate to native and to diethyl pyrocarbonate inactivated enzyme as described by Lockridge et al. (1972).

Results

Treatment of L-α-hydroxy acid oxidase in 20 mM MES buffer at 25 °C with diethyl pyrocarbonate (0.2–2.0 mM) leads to a rapid loss of enzyme activity in a first-order process which was linear down to 10% residual activity (Figure 1). From these and other similar kinetic experiments, the inactivation was found to be first order in enzyme and in diethyl pyrocarbonate with a second-order rate constant of 11.5 M^{-1} s^{-1} at 25 °C and pH 7. Under the same conditions, diethyl pyrocarbonate was found to spontaneously hydrolyze with a half-life of 28 min. Berger (1975) has reported a $t_{1/2}$ of 9 min for diethyl pyrocarbonate hydrolysis in 27 mM sodium phosphate, pH 7, at 25 °C, so MES buffer appears to be especially suitable for studies of reaction of diethyl pyrocarbonate with proteins.

The pH dependence of the rate of inactivation of the enzyme with diethyl pyrocarbonate was determined over the range 5.9–7.5 in MES buffer. Above pH 7 the spontaneous hydrolysis of diethyl pyrocarbonate became significant ($t_{1/2}$ = 6 min at pH 7.4), and corrections to the observed rate of loss of activity due to this loss of reagent were made. It was also found that L-α-hydroxy acid oxidase lost activity on standing in MES buffer below pH 6.2 ($t_{1/2}$ = 23 min at pH 6.1), and this loss of activity was factored out from the observed rate of loss of activity with diethyl pyrocarbonate. With these corrections, the rate of inactivation could be correlated with eq 1.

$$\frac{1}{k_{2(\text{obsd})}} = \frac{1}{k_2} + \frac{[H^+]}{k_2 K_a} \qquad (1)$$

In eq 1, k_2 is the second-order rate constant for modification of an unprotonated residue with an apparent dissociation constant for the protonated form of K_a (Cousineau & Meighen, 1976), which can be derived from the assumption that a single type of amino acid reacts with diethyl pyrocarbonate substantially faster when unprotonated. For L-α-hydroxy acid oxidase at 25 °C, k_2 is 14 M^{-1} s^{-1} and K_a is 2.3 × 10^{-7} (pK_a = 6.6) (Figure 2). As the pH is raised above 7.2, there is a decrease in the rate of inactivation which is not due solely to the decreased stability of the reagent at higher pH. The origin of this decrease remains unclear. In the pH range of 5.9–7.2, the inactivation is dependent on the unprotonated form of a residue with an apparent pK_a of 6.6, a value consistent with histidine (Schneider, 1978). Furthermore, the rate constant of 14 M^{-1} s^{-1} for inactivation of the enzyme with diethyl pyrocarbonate is similar to the rate constant for reaction of diethyl pyrocarbonate with *N*-acetylhistidine (Holbrook & Ingram, 1973).

Extensive dialysis of the inactivated enzyme against 20 mM MES buffer at 4 °C or Sephadex chromatography with the same buffer led to only small (<5%) recoveries of activity. Similar treatment of partially inactivated enzyme (80 or 40% initial activity remaining) returned enzyme of the same partial activity. It has been noted by Miles (1977) that inactivation of an enzyme by diethyl pyrocarbonate probably occurs by modification of a histidine residue if enzymatic activity can be recovered by treatment with hydroxylamine. However, a large number of attempts at reactivating L-α-hydroxy acid oxidase with hydroxylamine led to only moderate recoveries of activity. The best recovery of 44% of the initial activity was obtained by treatment of fully inactivated enzyme with 0.5 M hydroxylamine for 1 h at 4 °C followed by dialysis at 4 °C to remove the hydroxylamine. A variety of other experiments with hydroxylamine at different concentrations for different lengths of time led to activity recoveries of 20–35%. Thomê-Beau et al. (1971) reported that arginine oxygenase inactivated with diethyl pyrocarbonate could be restored by

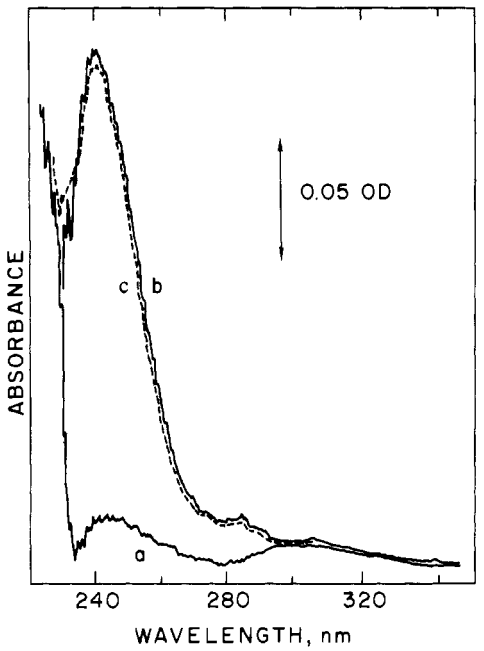


FIGURE 3: Differential absorption spectra of L-α-hydroxy acid oxidase. Against 3.0 mg/mL native enzyme was run an equivalent concentration of (a) native enzyme, (b) enzyme inactivated to less than 5% residual activity with diethyl pyrocarbonate, and (c) inactivated enzyme treated with 0.5 M hydroxylamine.

a high concentration of thiols, but L-α-hydroxy acid oxidase could not be reactivated by even high concentrations of β-mercaptoethanol. Human prostatic acid phosphatase (McTigue & Van Etten, 1978) and bovine plasma amine oxidase (Tsurushiin et al., 1975) also do not show full recovery of activity with hydroxylamine, even though histidine residues are implicated as the reactive group. For L-α-hydroxy acid oxidase, part of the failure to reactivate with hydroxylamine is that the enzyme is slowly inactivated by large concentrations of NH_2OH at pH 7 and 25 °C. The second-order rate constant for this inactivation was found to be 3×10^{-4} M^{-1} at pH 7, but the origin of this loss of activity was not further explored.

The loss of enzymatic activity on treatment of L-α-hydroxy acid oxidase with diethyl pyrocarbonate could arise from a change of substrate binding affinity on acylation rather than from interference with a catalytic event. However, a sample of enzyme reacted with diethyl pyrocarbonate to 10% residual activity had a K_m of 2.2 mM for DL-α-hydroxybutyrate compared with 2.1 mM for the native enzyme in 20 mM MES buffer at pH 7. The double-reciprocal plot for the K_m value of the inactivated enzyme was linear to substrate concentrations of $20K_m$, and it is estimated that the K_m value of the inactivated enzyme would have had to increase by greater than 75-fold to escape detection. At the same time, the K_I value for phosphate, determined from the perturbation of the flavin absorbance by the method of Lockridge et al. (1972), was found to be 63 mM for the native enzyme and 70 mM for the completely inactivated enzyme. A change in binding of substrate without a similar change in binding of phosphate is unlikely, so it can be concluded that acylation of the enzyme blocks a step in the catalytic sequence. The uncorrected fluorescence emission of completely inactivated enzyme (excitation 290 nm) showed an emission maximum at 337 nm identical with that of native enzyme, so no changes which affect the environment or chemical nature of tyrosine or tryptophan groups occur on inactivation.

The reaction of diethyl pyrocarbonate with histidine gives an *N*-carbethoxyhistidine with an absorbance maximum near

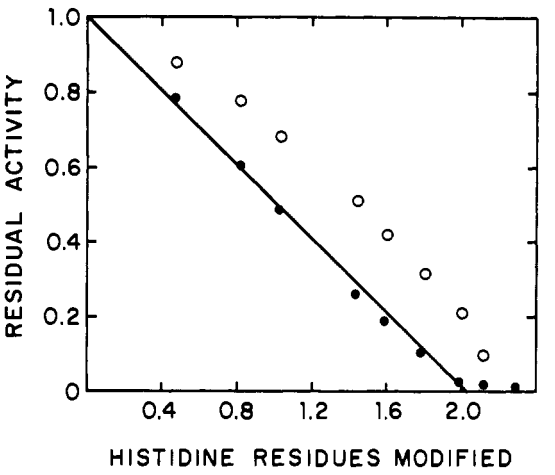


FIGURE 4: Correlation between enzyme activity and histidine residues modified by diethyl pyrocarbonate. Inactivation experiments were conducted as in Figure 1 with 1 mM DEPC while histidine residues modified were determined spectrophotometrically at 240 nm on a 50-fold larger sample. (●) Activity to the first power; (○) activity to the one-half power.

Table I: Cysteine, Tryptophan, and Tyrosine Analysis of Native and Diethyl Pyrocarbonate Modified Enzyme[a]

enzyme	activity (%)	cysteine	trypto-phan	tyrosine
native	100	2.2	30.2	37.6
modified	7	2.0	32.4	37.4

[a] Inactivation with 1 mM diethyl pyrocarbonate as described in Figure 1. Content determined as described in the text.

240 nm. The UV difference spectrum obtained on complete inactivation of L-α-hydroxy acid oxidase with 1 mM diethyl pyrocarbonate at pH 7.0 is shown in Figure 3. There is the expected increase at 240 nm for the modification of histidine but only a small increase in absorbance at 280 nm. Reaction of tyrosine residues with diethyl pyrocarbonate results in a decrease in absorbance around 280 nm (Muhlrad et al., 1967), so no tyrosine residues in L-α-hydroxy acid oxidase are modified with 1 mM diethyl pyrocarbonate. Identical spectra were obtained with 0.5 and 1.5 mM diethyl pyrocarbonate. Use of a value of 3900 for the molar extinction coefficient for *N*-carbethoxyhistidine (Choong et al., 1977) and a molecular weight of 95 000 per FMN coenzyme (Cromartie & Walsh, 1975a) allowed the determination of the equivalents of histidine modified per FMN as a function of time. When this rate was measured under the same conditions as the rate of loss of enzymatic activity, the correlation between the equivalents of histidine modified per FMN coenzyme and rate of loss of enzymatic activity shown in Figure 4 can be obtained. Extrapolation of the least-squares line to zero enzymatic activity indicates that the reaction of *two* histidines per flavine occurs on complete inactivation. As complete inactivation is approached, reaction with more than the first 2 equiv of histidine can be noted in Figure 4. However, as noted under Discussion, only one of these residues is essential for activity. The data in Table I show that there is no reaction of diethyl pyrocarbonate with sulfhydryl, tyrosine, or tryptophan residues of L-α-hydroxy acid oxidase.

The specificity of diethyl pyrocarbonate for histidine residues in L-α-hydroxy acid oxidase was further demonstrated with [1-^{14}C]diethyl pyrocarbonate. Incubation of 0.9 mg of enzyme with 1.5 mM radioactive diethyl pyrocarbonate for 20 min followed by Sephadex chromatography and determination of the protein concentration and radioactivity of the fractions

Table II: Incorporation of [1-^{14}C]Diethyl Pyrocarbonate into Native and 2-Hydroxybutynoate-Inactivated Enzyme[a]

enzyme	activity (%)	nmol of active sites	cpm above background	labels/ active site
native	100	1.04	4914	2.1
native	100	1.60	6749	1.9
native[b]	100	0.88	5783	2.95
inactivated	<1	1.45	6554	2.04

[a] See text for experimental details. [b] Same as others except 3.6 mM diethyl pyrocarbonate for 30 min.

Table III: Deacylation of [1-^{14}C]Diethyl Pyrocarbonate Inactivated Enzyme with Hydroxylamine[a]

NH_2OH (M)	time of incubation (h)	$NaDodSO_4$	labels remaining/ active site
0.1	2	0	1.61
0.5	8	0	0.10
0.1	2	2% w/v	0.52

[a] See text for experimental details. Labeled enzyme retained 2% activity.

showed that 2 equiv of diethyl pyrocarbonate is incorporated per FMN coenzyme under these conditions, as shown in Table II. Reaction of the enzyme with a higher concentration of diethyl pyrocarbonate for a longer period of time increased the degree of labeling. However, total enzymatic activity is lost in 20 min with 1 mM reagent (Figure 1), and reaction of diethyl pyrocarbonate with the histidines is also complete under these conditions (see below, Figure 6). The radiolabeling data suggest that only 2 equiv of diethyl pyrocarbonate reacts under conditions where total activity is abolished and two histidine residues are ethoxyformylated. The effect of hydroxylamine on the labeled inactivated enzyme was studied (Table III). Treatment with 0.5 M hydroxylamine sulfate denatured the enzyme and removed essentially all of the ethoxyformyl groups from the enzyme, but 0.1 M hydroxylamine removed only a part of the label. Addition of $NaDodSO_4$ to denature the labeled enzyme along with 0.1 M hydroxylamine increased the amount of label removed but did not cleave it completely.

When compared to the visible absorbance spectrum of native enzyme, the spectrum of enzyme inactivated to 6% residual activity shows only very minor differences such as a 5% decrease at 405 nm and a 3% increase at 280 nm. The 455-nm flavin absorbance, however, is unaltered in shape or magnitude. If the inactivated enzyme solution is made anaerobic and 10 mM DL-α-hydroxybutyrate is added, there is no change in the visible absorbance over a 2-h period. However, addition of sodium dithionite to give 0.5 mM instantly bleached both native and diethyl pyrocarbonate enzyme to the same featureless spectrum.

Inactivation of the enzyme in the presence of the substrate DL-hydroxybutyrate or the product pyruvate caused a decrease in the rate of inactivation (Figure 5). This protection appears to be specific for compounds which can bind to the active site, for the presence of 100 mM D-lactic acid, which is neither a substrate or an inhibitor of the enzyme, had no effect on the rate or extent of histidine modification by diethyl pyrocarbonate. From data not shown, it was also found that *trans*-cinnamic acid provided substantial protection from inactivation. The rate of inactivation of L-α-hydroxy acid oxidase by diethyl pyrocarbonate was measured in the presence of various concentrations of DL-α-hydroxybutyrate, pyruvate, and cinnamic acid, and the results were treated according to the

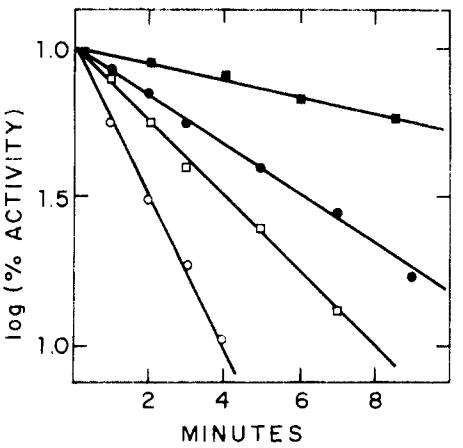


FIGURE 5: Effect of substrate and inhibitor on the rate of inactivation of L-α-hydroxy acid oxidase with diethyl pyrocarbonate. Experiments were conducted as in Figure 1 (○) or in the presence of 50 mM sodium pyruvate (□), 5 mM DL-hydroxybutyrate (●), or 50 mM DL-hydroxybutyrate (■).

Table IV: Kinetic Parameters for the Protection of L-α-Hydroxy Acid Oxidase from Diethyl Pyrocarbonate by Substrate, Product, and Competitive Inhibitor[a]

protective ligand	K^b (mM)	K_d (mM)	k_2/k_1	correlation coefficient[c]
α-hydroxybutyrate	2.1	2.2	0.027	0.9989
pyruvate	nd[d]	44	0.096	0.9999
trans-cinnamate	0.48	0.71	0.020	0.9680

[a] By the procedure of Scrutton & Utter (1965); see the text for definitions. [b] K_m for hydroxybutyrate; K_I for *trans*-cinnamate. [c] For the least-squares line in V_{PL}/V_0 vs. $(1 - V_{PL}/V_0)/[PL]$. [d] Not determined.

procedure of Scrutton & Utter (1965). If the rate of inactivation of an enzyme with excess inactivator is determined in the presence (V_{PL}) and absence (V_0) of a protective ligand, the following correlation can be derived between the ratio of these rates and the concentration of protective ligand [PL]:

$$\frac{V_{PL}}{V_0} = \frac{k_2}{k_1} + K_d\left[\frac{(1 - V_{PL})/V_0}{[PL]}\right] \qquad (2)$$

In this equation, K_d is the dissociation constant for the enzyme-protective ligand complex, and k_1 and k_2 are the rate constants for the inactivation reaction of the free enzyme and the enzyme-protective ligand complex, respectively. The slopes (K_d) and intercepts (k_2/k_1) from this type of plot with hydroxybutyrate, pyruvate, and cinnamic acid are collected in Table IV. The K_m value for hydroxybutyrate and the K_I value for cinnamic acid (a competitive inhibitor) determined under identical experimental conditions in the usual manner are also recorded. There is good agreement between these last values and the K_d values determined by the Scrutton & Utter procedure, demonstrating that protection from inactivation arises from binding to the same site, the active site. Furthermore, the agreement between K_d and K_m for hydroxybutyrate shows that the protective effect of this substrate arises from occupation of the active site rather than from conversion of the enzyme to the reduced form, which might be less suceptible to inactivation. In all three cases, the presence of the protective ligand at the active site fails to fully protect the enzyme from diethyl pyrocarbonate. Also, the extent of such protection varies with the structure of the protective ligand.

As expected, the presence of substrate also decreases the rate of reaction of diethyl pyrocarbonate with the histidine residues, as measured spectroscopically. This protection ex-

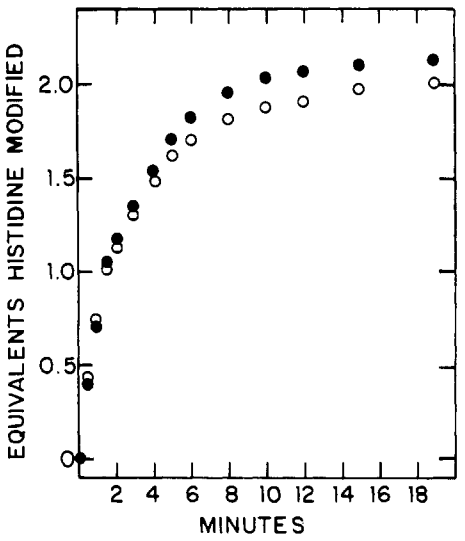


FIGURE 6: Effect of inactivation of L-α-hydroxy acid oxidase with hydroxybutynoic acid on the rate of reaction with diethyl pyrocarbonate. The increase in absorbance at 240 nm on reaction of a 2.5 mg/mL solution of native enzyme (●) or enzyme inactivated with 2-hydroxybutynoic acid (○) was converted to equivalents of (ethoxyformyl)histidine formed and plotted against time. The diethyl pyrocarbonate concentration was 1 mM and the reaction was conducted at 25 °C and pH 7.0.

actly parallels the decrease in loss of activity so that when the loss of activity is plotted against the rate of increase of absorbance at 242 nm as in Figure 4, the same correlation was obtained in the presence of 5 mM hydroxybutyrate as in its absence. This finding further strengthens the conclusion that histidine modification is the source of loss of activity and also shows that both histidine residues which are modified are equally protected by the presence of substrate.

L-α-Hydroxy acid oxidase was irreversibly inactivated by reaction with 2 mM DL-α-hydroxybutynoate at pH 7 in 20 mM MES buffer with DCIP as electron acceptor (Cromartie & Walsh, 1975b). After Sephadex chromatography and concentration of the inactivated enzyme or after dialysis at 4 °C, it was treated with 1 mM diethyl pyrocarbonate as before, and the increase in absorption at 242 nm was measured as a function of time as shown in Figure 6. The absorbance reached in 15 min under these conditions did not change appreciably over the next 15 min, and this value was taken to represent complete reaction. Another sample of enzyme similarly inactivated with hydroxybutynoate was treated with 1.5 mM [1-^{14}C]diethyl pyrocarbonate, and the extent of labeling with respect to FMN was determined (Table II). In both experiments irreversibly inactivated enzyme behaved identically with native enzyme.

Discussion

Although it has been shown to react preferentially with histidine residues, diethyl pyrocarbonate is also known to react with the free N-terminal amino group of a protein and with a variety of amino acid side chains, including lysine, tyrosine, serine, arginine, and cysteine (Melchior & Fahrney, 1970; Miles, 1977). However, the inactivation of L-α-hydroxy acid oxidase from rat kidney by diethyl pyrocarbonate at pH 7 appears from a number of experiments to arise solely from the ethoxyformylation of histidine. The rate constant for inactivation is that expected for the reaction of diethyl pyrocarbonate with a histidine residue, and the pH dependence of inactivation indicates the inactivation depends on the modification of a residue with an apparent pK_a of 6.6, a value typical for the modification of histidine residues in other proteins (Cousineau & Meighen, 1976; Holbrook & Ingram, 1973). At pH 7 and 1 mM diethyl pyrocarbonate, quantitation of the increase in absorbance at 242 nm due to the formation of (ethoxyformyl)histidine showed that 2 equiv of diethyl pyrocarbonate per equiv of FMN coenzyme was incorporated. The correlation between the rate of loss of activity and the rate of reaction, measured spectroscopically, of diethyl pyrocarbonate with the enzyme could be extrapolated to show that complete loss of activity correlated with the reaction of two histidine residues per FMN. With the use of [1-^{14}C]diethyl pyrocarbonate, it was also found that 2 equiv of diethyl pyrocarbonate was incorporated under conditions which led to complete loss of activity and the reaction of 2 equiv of diethyl pyrocarbonate with histidine. Thus reaction with histidine completely accounts for the inactivation of L-α-hydroxy acid oxidase with diethyl pyrocarbonate. This conclusion was further documented by determining that inactivation did not cause any change in the cysteine, tyrosine, or tryptophan content of the enzyme.

Although 2 equiv of histidine per active site reacts at identical rates on complete inactivation of the enzyme, only one of these is "essential" for catalysis. This is most easily shown by analysis of the data in Figure 4 by the method of Tsou (1962). With this approach the number of essential residues of a given type modified by a particular amino acid specific reagent can be determined from the relationship between the number of such groups reacted and the activity remaining in the partially modified enzyme. Certain limitations to this method have been noted by Rakitzis (1978), and it must be assumed that the modification of essential residue(s) leads to complete inactivation, an assumption which proves valid in this work (Figure 4). Since all the histidine residues modified by diethyl pyrocarbonate react at the same rate, relationship 3 can be formulated as

$$a^{1/i} = (p - m)/p \qquad (3)$$

where p is the number of groups which can be modified by the reagent, i is the number of these which are catalytically essential, and a is the fractional activity remaining when m groups have reacted (Paterson & Knowles, 1972). The number of essential residues is then determined by the value of i which best fits a plot of $a^{1/i}$ vs. m. From Figure 4, this is clearly one histidine residue. Although only one of the two reactive histidine residues is essential for catalysis, their behavior is identical kinetically, in pH dependence, and in spectroscopy.

An unsettling feature of the inactivation reported in this work is that it can be only partially reversed by treatment with hydroxylamine. In the best case 44% of the initial activity could be recovered by hydroxylamine, and recovery was usually less than 30%. Part of this failure to reactivate is probably due to the slow inactivation of the native enzyme on treatment with high concentrations of hydroxylamine. However, hydroxylamine did not cause a significant change in the difference UV absorption spectrum, which showed the 240-nm peak characteristic of *N*-carbethoxyhistidine. Addition of 0.1 M hydroxylamine also did not remove appreciable radioactivity from enzyme inactivated with [1-^{14}C]diethyl pyrocarbonate. Addition of 0.5 M hydroxylamine denatured the inactivated enzyme and released 95% of the radioactivity, and addition of 0.1 M hydroxylamine to inactivated enzyme, treated with sodium dodecyl sulfate to denature it, removed 74% of the radiolabel. According to Miles (1977) the failure of hydroxylamine to fully reactivate a modified enzyme implies either reaction of diethyl pyrocarbonate with other than histidine residues or reaction of 2 equiv of diethyl pyro-

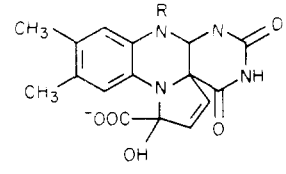
carbonate/equiv of histidine followed by a Bamberger reaction to open the imidazole ring. In the present case, reaction with residues other than histidine can be ruled out. The reaction of one histidine residue with 2 equiv of diethyl pyrocarbonate is unlikely because there is no marked increase in absorbance in the 220–230-nm region during inactivation characteristic of the Bamberger reaction (Loosemore & Pratt, 1976) and because the large extinction coefficient of the stable products of this cleavage would require reaction of diethyl pyrocarbonate with only a fraction of a histidine residue to give inactivation. Denaturation of the inactivated enzyme with high concentrations of hydroxylamine, or with detergent, does lead to removal of radioactivity from enzyme inactivated with radiolabeled diethyl pyrocarbonate, suggesting that these *N*-carbethoxyhistidines are only partially accessible to external nucleophiles in the intact, modified enzyme. Similar, limited recovery of enzymatic activity on incubation with hydroxylamine has been reported with other enzymes (Tsurushiin et al., 1975; McTigue & Van Etten, 1978). In view of the results with L-α-hydroxy acid oxidase, the suggestion that the loss of absorbance at 240 nm on treatment of diethyl pyrocarbonate inactivated enzymes with hydroxylamine is the best indicator of the number of histidine residues reacted (Burstein et al., 1974; Loosemore & Pratt, 1976) should be viewed with some caution.

The reaction of histidine residues of the enzyme with diethyl pyrocarbonate appears to occur at or near the active site of the enzyme. The presence of the substrate DL-α-hydroxybutyric acid or the competitive inhibitors pyruvic acid or *trans*-cinnamic acid reduces the rate of inactivation, whereas D-lactic acid, which is not an inhibitor of hydroxy acid oxidation and thus does not bind at the active site, caused essentially no decrease in the rate of inactivation. Kinetic analysis of this protection by the method of Scrutton & Utter (1965) indicated that the protection arises from the binding of these protective compounds to the active site of the enzyme. The analysis further indicated that complete occupation of the active site of the enzyme with either of the three protective compounds studied did not completely prevent inactivation. The reactivity toward inactivation remaining in the protected enzyme varies with the structure of the protective compound. This observation is significant in the following sense. Protection of enzymatic activity from an external inactivating reagent by substrates, products, or competitive inhibitors is generally regarded as evidence that the target residues for that reagent reside within the active site (Miles, 1977). But it is possible that binding of these compounds causes a conformational change which renders a target residue outside the active site less reactive. In the present case there is no evidence from fluorescence studies on the enzyme for any significant conformational changes on binding substrates or keto acid products. Furthermore, it is reasonable to expect that such a conformational change altering the reactivity of a residue distant from the active site would be similar for binding of similar substrates. The data in Table IV, however, show that pyruvic acid is threefold less effective in protecting the enzyme than is hydroxybutyrate. Both hydroxybutyrate and cinnamate are quite effective at protecting the activity of the enzyme, and it appears simpler to regard the data in Table IV as being indicative of protection of residues at the active site of the enzyme. Of course, this has not been proven, and cannot be with kinetic arguments, but the weight of evidence favors the usual interpretation of the substrate and competitive inhibitor protection results. The presence of DL-α-hydroxybutyric acid also slowed the rate of reaction of diethyl pyrocarbonate with histidine, as measured by the increase in absorbance at 242 nm, by exactly the same amount as the loss of activity was slowed.

The loss of activity of L-α-hydroxy acid oxidase on reaction with diethyl pyrocarbonate is not due to a change in affinity for substrates. Enzyme inactivated to 10% residual activity gave the same K_m value for α-hydroxybutyrate as native enzyme, and the binding of inorganic phosphate at the active site was not significantly altered by inactivation. The inactive enzyme was still instantaneously reduced by sodium dithionite. However, addition of α-hydroxybutyrate to the inactivated enzyme under anaerobic conditions caused no reduction of the flavine, even on long incubation. Thus the acylation of a histidine residue in the active site of the enzyme blocks a step following substrate binding but prior to flavine reduction. This step must be removal of a proton from the α carbon of the substrate (Bruice, 1976; Bright & Porter, 1975).

In a study very similar to the present work, Choong et al. (1977) reported the presence of an essential histidine residue in the flavoenzyme L-lactate monooxygenase. This enzyme catalyzes the four-electron oxidation of lactate to acetate, reducing dioxygen to water, by the initial conversion of lactate to pyruvate through a mechanism closely related to that of the flavoenzyme hydroxy acid oxidases. With that enzyme also, modification of histidine with diethyl pyrocarbonate prevented a step after substrate binding but before flavin reduction. Although they did not obtain convincing evidence that the essential histidine residue was at the active site, they made the reasonable suggestion that histidine participates in the reaction as the initial proton acceptor. The effect of irreversible inactivation of the enzyme with acetylenic suicide substrates on the reactivity of histidines toward diethyl pyrocarbonate was explored to obtain further information on the role of the histidine residue in L-α-hydroxy acid oxidase.

It has previously been shown that L-α-hydroxy acid oxidase is irreversibly inactivated on reaction with α-hydroxybutynoic acid through the formation of a covalent bond between the inactivator and the FMN coenzyme (Cromartie & Walsh, 1975b). The exact structure of this adduct has not been proven, but it has an absorbance spectrum virtually identical with that of L-lactate monooxygenase irreversibly inactivated in the same way (Walsh et al., 1972). The structure of the initially formed lactate monooxygenase–hydroxybutynoate complex has been shown by Schonbrunn et al. (1976) to be



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Because of the sensitivity of the absorbance spectra of such adducts to structural variations (Gartner et al., 1976), it is very likely that the adduct for L-α-hydroxy acid oxidase has an identical structure. Like the L-lactate monooxygenase case, the hydroxybutynoate adduct with L-α-hydroxy acid oxidase undergoes slow spectral changes on standing (T. Cromartie, unpublished observations). However, the experiments on the adduct cited in this paper were carried out before any appreciable change in modified flavin spectrum occurred. If the essential histidine residue which reacts with diethyl pyrocarbonate functions to remove the α proton of the substrate, it must be located near the flavin ring system. Substrate and competitive inhibitors provide quite effective protection of this residue from diethyl pyrocarbonate. It was expected that the

presence of a modified flavin such as **1** should also show substantial protection of this histidine from diethyl pyrocarbonate. As measured by the increase in 240-nm absorbance, however, the hydroxybutynoate-inactivated enzyme is ethoxyformylated to almost the same extent and at the same rate as the native enzyme. Also, [1-^{14}C]diethyl pyrocarbonate is incorporated to the same extent in inactivated and in native enzyme.

The marked contrast between the efficient protection of the critical histidine residue by substrate and product and the almost complete lack of protection of this residue by the hydroxybutynoate–flavin adduct indicates that, in the adduct, the hydroxybutynoate moiety is placed so differently from normal substrates that it fails to alter the reactivity of the critical histidine. Regardless of how it fits in the active site, a flavin modifed at the C_{4a}-N_5 region, known to be the site of entry of substrate electrons during substrate oxidation, should provide some protection for the base which directly generates the substrate α carbanion. The simplest and most likely interpretation of these results is that the histidine residue is involved in some manner with the production of the substrate carbanion required for catalysis but that it is placed at some distance from the C_{4a}–N_5 bond of the flavine coenzyme. This would easily be possible if the histidine operated in concert with another basic group located quite near the flavin, which actually generates the carbanion. There is yet no other evidence for such a cooperative arrangement of bases in a flavoenzyme, and other explanations for the failure of the flavin–hydroxybutynoate adduct to protect the critical histidine from diethyl pyrocarbonate are possible. These results point out that caution must be taken before a role for the histidine residues found in a variety of flavoenzymes is assigned.

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