DAAG 29-85-K-0062) and to Dr. S. N. Mehra (Camphor and Allied Products, Bareilly, India) for the gift of (+)-3-carene and to Dr. Sukh Dev (Malti-Chem, Baroda, India) for the gift of (+)-2-carene.

References and Notes

†Dedicated to Professor Nung Min Yoon on the occasion of his 60th birthday.

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Chemical Modification of Glycolate Oxidase from Spinach by Diethyl Pyrocarbonate. Evidence of Essential Histidine for Enzyme Activity†

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Received March 31, 1987

FMN-dependent glycolate oxidase from spinach is inactivated by diethyl pyrocarbonate at pH 7.0. Inactivation of both apo- and holoenzyme by diethyl pyrocarbonate follows pseudo-first-order kinetics and first order with respect to the reagent. A series of difference spectra of inactivated and native enzymes show a single peak at 240 nm, indicating the modification of histidyl residues. No decrease in absorbance at around 280 nm due to formation of O-carbethoxytyrosine is observed. The rate of inactivation is dependent on pH, and the data for pH dependent rates implicate the involvement of a group with a pKa of 6.9. The activity lost by treatment with diethyl pyrocarbonate could be almost fully restored by incubation with 0.75M hydroxylamine. The reactivation by hydroxylamine and the pH dependence of inactivation are also consistent with that the inactivation is due to modification of histidyl residues. Although coenzyme FMN is without protective effect, the substrate glycolate, the product glyoxylate, and two competitive inhibitors, oxalate and oxalacetate, provide marked protection against the inactivation of the holoenzyme. These results suggest that the inactivation of the oxidase by diethyl pyrocarbonate occurs by modification of essential histidyl residue(s) at the active site.

Introduction

Glycolate oxidase (glycolate: oxygen oxidoreductase, EC 1.1.3.1) catalyzes the conversion of glycolate to glyoxylate, with O_2 as electron acceptor. The oxidase is found in the peroxisomes of mammalian liver and kidney,[1] and also in the green leaves of plants.[2] The enzyme of mammalia is probably involved in the metabolic production of oxalate by the oxidation of glycolate through glyoxylate.[3] Inhibition studies of mammalian enzyme have been made extensively,[1,4,5] since inhibition of the enzyme might be useful for treatment of hyperoxaluria.

In green plants, glycolate oxidase is one of the key enzymes involved in the process of photorespiration.[2] The primary reaction of photorespiration is the production of glycolate from ribulose-1,5-diphosphate and O_2, and the first step in glycolate pathway is the oxidation of glycolate to glyoxylate. Since photosynthetic productivity is drastically reduced by active photorespiration in C-3 plants, an effective control of photorespiration might be one possible way to increase the efficiency of photosynthesis. It was shown that inhibition of photorespiration with biochemical inhibitors of glycolate synthesis[6] or oxidation[7] increase net photosynthesis by 50% or more for a short period.

It is necessary to elucidate the nature and role of the amino acid residues within the active site for rational design of specific inhibitors of the oxidase. Recently. Lee and Choi[8] confirmed that an arginyl residue at the active site is involved

† Dedicated to Professor Nung Min Yoon on the occasion of his 60th birthday.

in the binding of substrate to the enzyme. The present paper describes studies on the chemical modification of histidyl residues in glycolate oxidase with diethyl pyrocarbonate, which has been used widely as a histidine-acylating reagent for proteins. The results described herein demonstrate that the oxidase is inactivated by diethyl pyrocarbonate due to modification of histidyl residue(s) at or near substrate binding site.

Experimental

Materials. Diethyl pyrocarbonate (DEP), FMN, potassium glycolate, protamine sulfate, potassium oxalate, potassium oxalacetate were purchased from Sigma Chemical CO. and were used without purification. Sodium 2,6-dichlorophenol-indophenol was obtained from Aldrich Chemical CO.. Sephadex G-25 was supplied by Pharmacia, and DEAE-cellulose was from Whatman. Bio-gel was a product of Bio-Rad. All other chemicals were of the highest commercial grade.

Purification and Assay of the Oxidase. Glycolate oxidase was purified from spinach leaves essentially according to Kerr and Groves.[9] Glycolate oxidase apoenzyme was prepared by removing of FMN from holoenzyme as described previously.[8] For the rapid estimation of enzyme activity, the dye reduction method of Frigerio and Harbury[10] was used. Each assay solution contained: 1 mmole of potassium phosphate (pH 8.0), 6 μmoles of sodium glycolate, 0.1 μmole of 2,6-dichlorophenol-indophenol (DCIP), 0.2 μmole of FMN, enzyme and water to a volume of 3.0 m*l*. A decrease in absorbance at 610 nm was monitored in a Simadze Recording Spectrophotometer UV-240. One unit of activity is defined as 0.01 O.D change per minute. Protein concentrations were determined by Biuret method,[11] or that of Lowry et al.[12] using bovine serum albumin as the standard.

Inactivation of the Oxidase with Diethyl Pyrocarbonate. Diethyl pyrocarbonate was freshly diluted with anhydrous ethanol before use, and its concentration was determined spectrophotometrically using imidazole.[13] Inactivation reaction was initiated by adding DEP to the enzyme solution in 0.1 potassium phosphate buffer, pH 7.0, at 20°C. The ethanol concentration did not exceed 5% by volume and was found to have no effect on the activity and stability of the enzyme during incubation. Aliquots of reaction mixture were removed at time intervals and assayed for activity. In the protection experiments, the enzyme was preincubated with the substrate, product, or the competitive inhibitor for at least 10 min prior to the addition of DEP.

Spectroscopic Studies. The difference spectra of DEP-treated VS. native enzymes in 0.1M potassium phosphate buffer, pH 7.0, were obtained on the Simadze Recording Spectrophotometer UV-240. Diethyl pyrocarbonate did not contribute to the absorption spectra. Difference spectra were recorded between 300 nm and 235 nm before and at various time intervals after addition of DEP in ethanol to sample cuvette and an equal volume of ethanol to the reference cuvette. Both sample and reference cuvettes contained same amount of the enzyme in 0.1 M potassium phosphate buffer.

Reactivation of DEP-inactivated Enzyme by Hydroxylamine. The oxidase was incubated with DEP in 0.1M potassium phosphate buffer at pH 7.0 and 20°C. After 20 min, the reaction mixture was rapidly diluted 5-fold with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.9 M hydroxylamine. After incubation at 20°C for the indicated time, hydroxylamine was removed by Sephadex G-25 column chromatography, and then the enzymatic activity was assayed. The same manipulation for native enzyme without DEP treatment was done as a control.

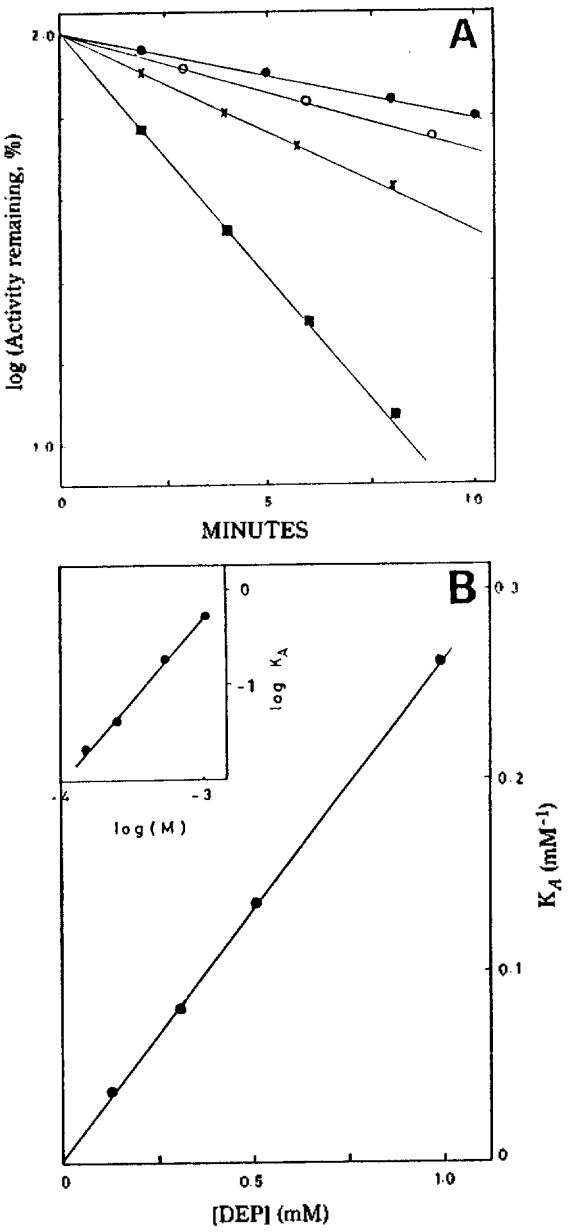

Figure 1. Inactivation of glycolate oxidase apoenzyme by diethyl pyrocarbonate. A. The oxidase (0.2 mg/m*l*) was incubated with 0.125 mM (●), 0.250 mM (○), 0.500 mM (×), and 1.00 mM (■) diethyl pyrocarbonate in 0.1 M potassium phosphate buffer, pH 7.0 at 20 °C. At time intervals, aliquots were removed for measurement of the residual enzyme activity. B. Plot of pseudo-first-order rate constants for inactivation (k_A) determined from the slopes of semilogarithmic plots of Figure 1A against concentrations of the reagent. Insert. plot of log (pseudo-first-order rate constant) vs. log[diethyl pyrocarbonate]. The native enzyme of the control retains full activity over this period of time.

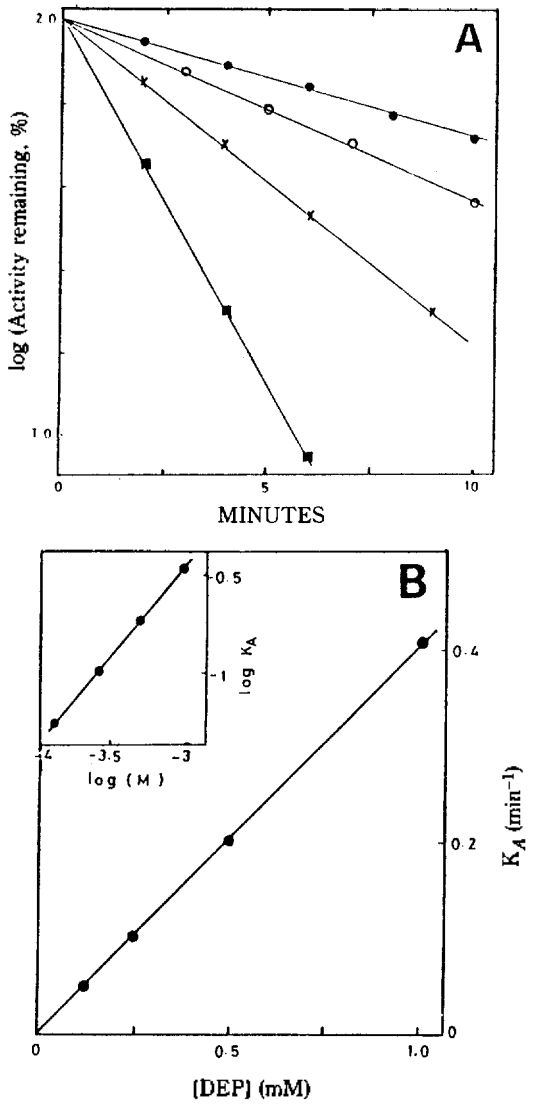

Figure 2. Inactivation of glycolate oxidase holoenzyme by diethyl pyrocarbonate. A. The oxidase (0.2 mg/ml) was incubated with 0.125 mM (●), 0.250 mM (○), 0.500 mM (×), and 1.00 mM (■) diethyl pyrocarbonate in 0.1 M potassium phosphate in the presence of 0.2 mM FMN, pH 7.0 at 20°C. B. Plot of pseudofirst-order rate constants (k_A) against concentrations of the reagent. Insert. plot of log (pseudo-first-order rate constant) vs. log [diethyl pyrocarbonate].

Results and Discussion

Incubation of glycolate oxidase with DEP in the absence and presence of coenzyme FMN in 0.1 M potassium phosphate buffer at pH 7.0 and 20°C, resulted in a time-dependent loss of enzyme activity (Figure 1A and 2A). Complete inactivation could be achieved by prolonged incubation with the reagent. Over the time necessary to completely inactivate the enzyme, the control enzyme sample retained full activity. When the inactivation was examined with respect to time, the plots of logarithm of remaining activities against reaction time showed straight lines for at least first 10 min of incubation. Prolonged incubation beyond 10 min resulted in a deviation from the straight line. Diethyl pyrocarbonate was reported to be rather unstable in aqueous solution due to a spontaneous hydrolysis. The rate of the hydrolysis is dependent on pH, the concentration and composition of the buffer.[14] However, the hydrolysis did not significantly affect on the inactivation for short reaction period under conditions, 0.1 M potassium phosphate, pH 7.0 and 20°C, used in the present investigation, as shown previously.[15] Inactivation of both apo- and holoenzyme follows pseudo-first-order kinetics, and the rate of inactivation is a function of reagent concentration (Figure 1A and 2A). When the pseudo-first-order rate constants obtained at several concentrations of DEP were plotted against reagent concentrations, straight lines going through the origin were obtained (Figure 1B and 2B). The apparent second-order rate constant for the inactivation of apo- and holoenzyme with DEP in 0.1 M phosphate buffer at pH 7.0 and 20°C was calculated to be 4.4 $M^{-1}S^{-1}$ and 6.8 $M^{-1}S^{-1}$, respectively. The reaction order with respect to DEP concentration for the inactivation of the oxidase was determined from the slope of a plot of log (pseudo-first-order rate constant, k_A) *vs.* log [DEP] as described by Levy *et al.*[16]:

$$k_A = k[DEP]^n$$
$$\log k_a = \log k + n\log[DEP]$$

Where k is the second-order rate constant, n is reaction order of DEP, reacting with amino acid residues in the enzyme to yield an inactive complex. From slope of Figure 1B and 2B (insert), the reaction order of DEP was determined to be 1.1 and 1.0 for apo and holoenzyme, respectively. The combination of pseudo-first-order kinetics and possible achievement of complete inactivation of the oxidase with DEP is consistent with that the inactivation proceeds in all-or-none fashion. Thus, the enzyme exists only either in fully active or in totally inactive state after treatment with DEP. First-order kinetics with respect to DEP indicated that a reaction of a single residue of the enzyme with one molecule of DEP could be resulted in total inactivation of the enzyme, and there is no reversible complex with DEP related to inactivation. In other words, the oxidase has at least one essential amino acid residue in the active site which is accessible to DEP.

Although the reaction of diethyl pyrocarbonate with amino acid residues in proteins is considerably specific for histidine residue at neutral or slightly acidic pH values, inactivation due to the modification of residues other than histidyl residues, such as tyrosyl, lysyl, and seryl residues, was reported in some enzymes.[17,18] Therefore, it appears to be critical to identify amino acid residues modified responsible for inactivation. In this connection, the modification of glycolate oxidase resulting in inactivation, was investigated by the changes of absorption spectra in the ultraviolet region upon DEP treatment, pH dependence of inactvation rate, and reactivation of DEP-inactivated enzyme with hydroxylamine. The reaction of diethyl pyrocarbonate with histidine residues gives on N-carbethoxyhistidyl moiety with an absorption maximum near 240 nm and with tyrosyl residues gives a decrease in absorption at around 280 nm due to formation of O-carbethoxytyrosine.[17] A series of difference spectra of DEP-treated and native enzymes, which were recorded in the ultraviolet region at various time intervals after addition of diethyl pyrocarbonate, showed a single peak with an absorption maximum at 240 nm, indicating the formation of N-carbethoxyhistidines (Figure 3). The absence of spectral change around 280 nm rules out modification of tyrosyl residues. The pH dependence of inactivation rate was

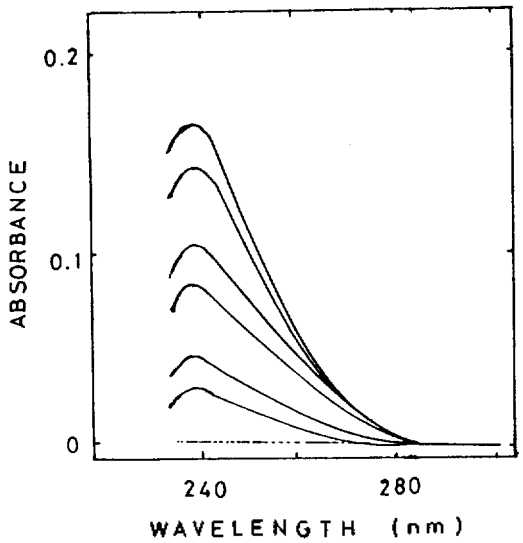

Figure 3. Ultraviolet difference spectra of glycolate oxidase treated with diethyl pyrocarbonate against untreated enzyme. Sample consisted of 2.0 m*l* of the enzyme (0.2 mg/m*l*) in 0.1 M potassium phosphate buffer, pH 7.0, and contained 0.75 mM diethyl pyrocarbonate. The difference spectra were recorded at 1,3,5,7.10, and 15 min after addition of the reagent.

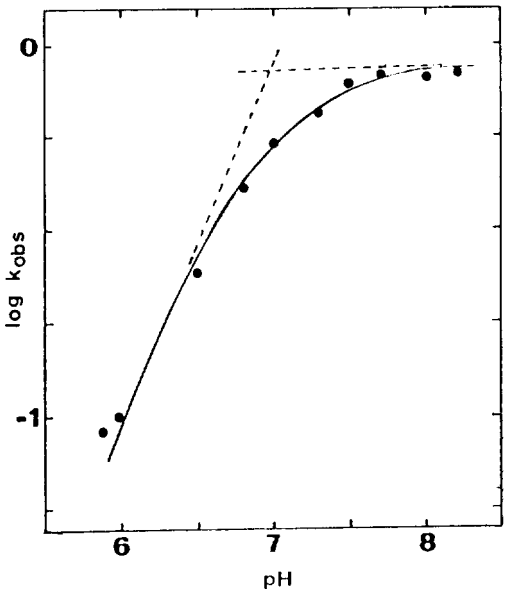

Figure 4. pH dependence of inactivation of glycolate oxidase with diethyl pyrocarbonate. The enzyme (0.2 mg/m*l*) was incubated with 1.0 mM diethyl pyrocarbonate in 0.1 M potassium phosphate buffer in the presence of 0.2 mM FMN. The pseudo-first-order rate constants for the inactivation were obtained as in Figure 1A. The points in the figure represent the experimental values, the curve in calculated from the equation $k(obs) = k[M]/(1+[H+]/Ka)$ with $k = 9.3 \times 10^2\ M^{-1}\ min^{-1}$ (the pH-independent value) and $Ka = 10^{-6.9}$.

studied in 0.1 M potassium phosphate buffer between pH 5.8 and 8.2. The pseudo-first-order rate constant (k_A) of inactivation by diethyl pyrocarbonate increased with the increase in pH up to 7.5. The plot of the rate constants of inactivation obtained at various pH vs. pH showed a typical titration curve (Figure 4). The plot of log k_A vs. pH gave a curve with

Table 1. Reactivation of DEP-inactivate Oxidase with Hydroxylamine

Incubation time with NH_2OH h	Enzyme activity %
0	5
0.5	77
1	82
2	90
3	93
5	94

The holoenzyme (1 mg/m*l*) was preincubated with 1.0 mM diethyl pyrocarbonate for 20 min in 0.1 M phosphate buffer at pH 7.0 and 20°C. Restoration of activity was obtained by incubation of treated enzyme with 0.75 M hydroxylamine. Other conditions were as described under "Experimental procedures."

two asymptotes whose slopes are +1 and 0 at acidic side and alkali region, respectively. From intersection of two asymptotes, the pKa value of the ionzing group was determined to be 6.9. The pH dependent rate of inactivation could be correlated with following equation.

$$k(obs) = k[M]/(1 + [H^+]/Ka)$$

Where M is diethyl pyrocarbonate, k is the second-order rate constant for modification of an unprotonated residue with an apparent dissociation constant of Ka[19], which can be derived from the assumption that a single type of amino acid reacts with diethyl pyrocarbonate substantially faster when unprotonated. The experimental values of k(obs) fit fairly well with a theoretical curve calculated using pKa = 6.9 and $k = 9.3 \times 10^2\ M^{-1}min^{-1}$ (Figure 4. solid curve). These data indicated that an ionzing group of the oxidase with a pKa value of about 6.9 is involved in inactivation reaction and that only unprotonated from of the residue is able to react with diethyl pyrocarbonate to inactivate the enzyme. Since diethyl pyrocarbonate reacts only with unprotonated form (nucleophile) of imidazole (p*K*a = 6.9) in model system and in proteins[19], and the pKa value found is within the range of that for a histidine residue in proteins, this result is consistent with the spectral data that are indicative of the inactivation being to the modification of essential histidyl residue(s). The modification of essential cysteine residue (s) related to the inactivation is unlikely, since pKa value of cysteine (pKa = 8.4) is significantly higher than observed pKa value of 6.9.

Hydroxylamine has been reported to be able to remove the carbethoxy group from N-carbethoxyhistidine and O-carbethoxytyrosine, and several DEP-inactivated enzymes could be reactivated by treatment of hydroxylamine.[18,20-23] In glycolate oxidase, enzyme activity lost by treatment with DEP could be recovered from 5% to 94% of control after incubation with 0.75 M hydroxylamine for 5 h at pH 7.0 and 20°C (Table 1). Difference absorption spectrum with maximum at 240 nm between DEP-treated and untreated enzyme gradually disappeared during incubation with 0.75 M hydroxylamine (not shown). Consequently, removal of carbethoxy groups from histidine residue(s) was correlated with reactivation of the inactivated enzyme. For several enzymes, such as, human prostatic acid phosphotase[24], bovine plasma amine oxidase[25], and L-α-hydroxy acid oxidase[26], enzymatic activity was not fully recovered with hydroxylamine, even

Table 2. Effect of Substrate and Inhibitors on the Rate of Inactivation of the Oxidase with Diethyl Pyrocarbonate

System	Diethylpyrocarbonate mM	Activity %
Oxidase	0	100
	0.5	17
+10 mM glycolate	0.5	17
+10 mM glyoxylate	0.5	96
+10 mM glyoxylate	0.5	95
+15 mM oxalate	0.5	97
+15 mM oxalacetate	0.5	99

The holoenzyme (0.2 mg/ml) was incubated in 0.1 M potassium phosphate buffer, at pH 7.0 and 20 °C for 10 min with diethyl pyrocarbonate in the absence and presence of substrate, product, and competitive inhibitors.

though histidine residues were implicated as the reactive group. The failure to reactivate the enzyme with hydroxylamine is probably due to formation of disubstituted histidyl derivative with excess diethyl pyrocarbonate, as shown in model systems.[17] Treatment of the disubstituted derivative with hydroxylamine results in imidazole ring cleavage and the product also has absorbance around 240 nm.[17] With glycolate oxidase, the reversibility of the inactivation and the decrease in the absorption at 240 nm with hydroxylamine indicates that reactions of histidyl residues with DEP led to from mainly monosubstituted histidyl derivative.

Diethyl pyrocarbonate has been successfully used for chemical modification of the histidyl residue essential for catalytic activity in a number of enzymes, including several flavoproteins.[11,19,22,26-29] Although DEP has a high reactivity toward histidyl residue at neutral pH value,[17] other nucleophilic residues such as tyrosine, serine, lysine and cystein might also be modified in addition to histidine.[18] In the inactivation of glycolate oxidase, the difference spectra between the inactivated and native enzyme indicate modification of histidine residues and rule out tyrosine modification. Modification of serine residue(s) by DEP is readily reversible in neutral aqueous solutions.[30] However, the kinetic data of the present investigation eliminate the formation of such a reversible complex related to the inactivation. Modification of amino group of lysine could lead to inactivation of enzymes, and which is not reversed by treatment of hydroxylamine.[30] Since rectivation of DEP-inactivated oxidase by hydroxylamine was observed, modification of essential lysine residues with DEP can be ruled out. The reversibility with hydroxylamine also excludes the possible interaction of DEP with essential cysteine residues,[17] and confirms that modification of essential histidine residue(s) is fully responsible for the inactivation. The conclusion is also supported by pH dependence of the inactivation which indicates the involvement of a functional group with a pKa of 6.9, most likely pKa value of a histidine in protein.

In order to examine protective effect of coenzyme FMN and substrate analogues on the inactivation of the oxidase by DEP, the enzyme was incubated with DEP in the absence and presence of these compounds. The second-order rate constant of 6.8 $M^{-1}S^{-1}$ for the inactivation of holoenzyme is slightly larger than the constant of 4.4 M^{-1} S^{-1} for apoenzyme, indicating that coenzyme FMN has no protective effect on the inactivation with DEP. In other words, essential histidyl residue(s), of which interaction with DEP could cause complete loss of activity, does not exist at coenzyme binding site. That binding of coenzyme enhances the rate of the inactivation by DEP implicates that conversion of apo- to holoenzyme probably exposes the reactive essential histidyl residue(s). The similar phenomenon with modification of isocitrate dehydrogenase[31] and pyridoxamine-5'phosphate oxidase[32] was reported. The substrate glycolate, product glyoxylate, and substrate competitive inhibitors oxalate and oxalate almost completely protected the holoenzyme against the inactivation by DEP (Table 2). However, the substrate and analogues showed no significant protective effect on the inactivation of the apoenzyme; this is consistent with the fact that they do not bind well to the apoenzyme. One could presume that conformational change, aggregation or dissociation to subunits induced by modification of histidyl residues might cause inactivation of the oxidase, but the kinetic data coupled with the protection effects of substrate analogues eliminates such possibility. The possibility that a conformation change induced by binding of substrate analogues causes the essential histidyl residue(s) to become inaccessible is unlikely, because the all analogues with different structure can completely protect the holoenzyme. The result from protection experiments coupled with spectral data and reactivation with hydroxylamine clearly demonstrates, therefore, that the inactivation of glycolate oxidase by diethyl pyrocarbonate is only due to modification of essential histidyl residue(s) at or near substrate binding site.

It has become generally accepted that those simple flavoproteins involved in the oxidation of α-hydroxy and α-amino acids cause the formation of an anion at the α-carbon of substrate in early stage of the catalytic sequence.[33-35] Since a substrate carbanion is produced as an intermediate in the oxidations, there must be a functional base at the active site of these enzymes to remove α-hydrogen from substrate. Several flavoenzymes, including D-amino acid oxidase[22], L-amino acid oxidase[22], monoamine oxidase[27], lactate oxidase[28], have been found to have an essential histidyl residue at the active site, the essential histidyl residue(s) of glycolate oxidase may function as a binding site for substrate or as a general acid base catalyst.

Acknowledgement. This research was supported by a research grant from the Korea Science and Engineering Foundation.

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Reducing Characteristics of Potassium Triethylborohydride

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The approximate rates, stoichiometries and products of the reaction of potassium triethylborohydride (KEt_3BH) with selected organic compounds containing representative functional groups under the standard condition (0°C, THF) were examined in order to explore the reducing characteristics of this reagent as a selective reducing agent. Primary alcohols, phenols and thiols evolve hydrogen rapidly whereas secondary and tertiary alcohols evolve very slowly. n-Hexylamine is inert to this reagent. Aldehydes and ketones are reduced rapidly and quantitatively to the corresponding alcohols. Reduction of noncamphor gives 3% *exo*-and 97% *endo*-norboneol. Anthraquinone is cleanly reduced to 9,10-dihydro-9,10-dihydroxyanthracene stage. Carboxylic acids liberate hydrogen rapidly and quantitatively but further reduction does not occur. Anhydrides utilize 2 equiv of hydride to give an equimolar mixture of acid and alcohol. Acid chlorides, esters and lactones are rapidly and quantitatively reduced to the corresponding alcohols. Epoxides are reduced at moderate rates with Markovnikov ring opening to give the more substituted alcohols. Primary amides liberate 1 equiv of hydrogen rapidly. Further reduction of caproamide is slow whereas benzamide is not reduced. Tertiary amides are reduced slowly. Benzonitrile utilizes 2 equiv of hydride in 3 h to go to the amine stage whereas capronitrile takes only 1 equiv. The reaction of nitro compounds undergo rapidly whereas azobenzene and azoxybenzene are reduced slowly. Cyclohexanone oxime rapidly evolves hydrogen without reduction. Phenyl isocyanate utilizes 1 equiv of hydride to proceed to formanilide stage. Pyridine N-oxide and pyridine is reduced rapidly. Disulfides are rapidly reduced to the thiol stage whereas sulfoxide, sulfonic acid are practically inert to this reagent. Sulfones and cyclohexyl tosylate are slowly reduced. Octyl bromide is reduced rapidly but octyl chloride and cyclohexyl bromide are reduced slowly.

Introduction

The discovery of the unique characteristics of lithium trialkylborohydrides in 1972 in the course of a study of the carbonylation of organoboranes aroused considerable interest in the exploration of their utility for organic functional group reductions.[1] As a result, a number of trialkylborohydrides have been developed in recent years as highly attractive reducing agents to achieve chemo-, regio- and stereoselective transformation in organic synthesis.[2] Lithium triethyl-