Mechanistic Studies on the Rat Kidney Flavoenzyme L-α-Hydroxy Acid Oxidase†

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ABSTRACT: The flavoenzyme L-α-hydroxy acid oxidase from rat kidney [T. H. Cromartie and C. T. Walsh (1975), *Biochemistry 14,* 2588] fails to catalyze the elimination of HCl from D,L-β-chlorolactate, although this compound is a substrate for oxidation by the enzyme. Deuterium isotope effects demonstrate that proton removal from the α carbon of α-hydroxy acids is fully rate limiting, a finding in agreement with observations on L-lactate dehydrogenase from yeast [F. Lederer (1974), *Eur. J. Biochem. 46,* 393] which also does not promote elimination from D,L-β-chlorolactate. Both D-α-hydroxy acid dehydrogenase from rabbit kidney and L-α-hydroxy acid oxidase were found to be rapidly and irreversibly inactivated by the acetylenic substrate 2-hydroxy-3-butynoate. The partially purified dehydrogenase was observed to be inactivated within 10 min by 6.8×10^{-8} *M* hydroxybutynoate. For the more extensively studied oxidase, inactivation was found to occur after 25 catalytic events, inactivation occurring by covalent addition of the inactivator to the coenzyme. A stoichiometry of one molecule of hydroxybutynoate per flavine was found, and the time course of inactivation was unaffected by the presence of thiols. The oxidase could also be inactivated by prolonged incubation of the enzyme with 2-hydroxy-3-butenoate, an inactivation which could be completely prevented by the presence of thiols. Since the inactivation with hydroxybutenoate also left the flavine coenzyme unaltered, the inactivation was attributed to Michael addition of nucleophiles on the enzyme to the ketobutenoate product. Several 4-alkyl-substituted 2-hydroxy-3-butynoates were also observed to inactivate the oxidase by both coenzyme modification and random addition to the apoenzyme. It is proposed that the inactivation may occur by nucleophilic addition of a C_4 allenic carbanion to the oxidized flavine coenzyme.

Of the wide variety of flavoproteins known in nature the flavoenzymes which contain no metals or other cofactors and catalyze the dehydrogenation of α-hydroxy acids or α-amino acids would appear to be particularly suitable for mechanistic study of flavine-mediated oxidations. At the present time, however, there is no generally accepted mechanism for such dehydrogenations, although a number of possibilities have been mentioned in the literature (Palmer and Massey, 1968; Hemmerich et al., 1967; Neims et al., 1966; Hamilton, 1971; Hemmerich et al., 1970; Bruice et al., 1971; Walsh et al., 1971). Nevertheless, a body of evidence has begun to accumulate that these simple flavoenzymes catalyze the formation of an α-carbanion which interacts with the flavine undergoing two-electron reduction in some still undetermined fashion (Neims et al., 1966; Hamilton, 1971; Hemmerich et al., 1970; Walsh et al., 1971; Porter et al., 1973; Yokoe and Bruice, 1975). This conclusion rests on the results of experiments with three kinds of substrates. β-Chloro substrates have been observed to undergo elimination of HCl as well as oxidation. Thus the L-lactate oxidase from *Mycobacterium smegmatis* catalyzes the α,β elimination of HCl from β-chlorolactate as well as its four-electron oxidation to chloroacetate and CO_2 (Walsh et al., 1973), and pig kidney D-amino acid oxidase and snake venom L-amino acid oxidase will both oxidize and promote HCl elimination from the appropriate enantiomer of β-chloroalanine (Walsh et al., 1971). Product distributions from these reactions have been interpreted as arising from the partitioning of a common α-carbanionic species formed early in the catalytic sequence. The β-acetylenic substrate 2-hydroxy-3-butynoate also appears to generate a species which partitions between oxidation to product and inactivation of the enzyme when it is oxidized by either *M. smegmatis* L-lactate oxidase (Walsh et al., 1972a) or the *Escherichia coli* membrane bound D-lactate dehydrogenase (Walsh et al., 1972b). Rearrangements of α-acetylenic carbanions to allenes and subsequent inactivation of the catalyzing enzyme are well known (Bloch, 1971; Abeles and Walsh, 1973; Hevey et al., 1973; Holland et al., 1973), although such inactivation with the flavoproteins occurred with covalent addition of the substrate to the flavine coenzyme rather than to residues in the apoprotein (Walsh et al., 1972a,b). Finally, the inactivation of D-amino acid oxidase by nitroethane (a substrate) in the presence of cyanide has also been interpreted as involving a nitroethane carbanion (Porter et al., 1973).

Recently, Lederer (1974) has reported that the heme flavoprotein yeast cytochrome b_2, which has lactate dehydrogenase activity, is likewise inactivated by 2-hydroxy-3-butynoate. However, the enzyme does not carry out an α,β-elimination of HCl from β-chlorolactate even though it oxidizes this substrate to β-chloropyruvate. Lederer (1974) has suggested that the failure to observe HCl elimination during reaction with β-chlorolactate is due to rapid processing of the α-carbanion through the oxidative pathway before elimination can compete.

To further explore the relationship of β-chloro and β-acetylenic substrates to the mechanism of flavoenzyme catalysis, we have investigated some catalytic aspects of rat kidney L-α-hydroxy acid oxidase, an unusual flavoenzyme possessing both hydroxy acid and amino acid oxidase activity. We have recently reported that our preparations of the homogeneous enzyme, with full catalytic activity, are tetra-

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meric containing only two molecules of FMN coenzyme and that 2-hydroxy-3-butynoate can be used as a flavine coenzyme-specific active site titrant (Cromartie and Walsh, 1975). In addition, preliminary work on the enzyme D-α-hydroxy acid dehydrogenase from rabbit kidney reveals that it also is susceptible to inactivation with 2-hydroxy-3-butynoate.

Materials and Methods

Many of the materials and general methods for this study have been described (Cromartie and Walsh, 1975). The acetylenic α-hydroxy acids listed in Table I were prepared as previously described (Cromartie et al., 1974). D,L-2-Hydroxy-3-butenoate (vinylglycolate) was prepared by the procedure of Glatterfield and Hoen (1935). D,L-2-Amino-4-pentynoic acid (propargylglycine) was prepared according to Jansen et al. (1969). 2,3-Butadienoic acid was prepared by the alkaline isomerization of 3-butynoic acid as described by Elington et al. (1954). D,L-2-Hydroxy-3-chloro-3-butenoate was a gift of Dr. A. Maycock. Rabbit kidneys were purchased from Pel-Freez Biologicals, Inc. Polyethylene glycol 6000 was from the Baker Company; dithiothreitol was obtained from the Sigma Chemical Company. Membrane filters were purchased from Schleicher and Schuell, Inc.

[1-^{14}C]-2-Hydroxy-3-butynoate (35 Ci/mol) and [1-^{14}C]-2-hydroxy-3-butenoate (60 or 6.5 Ci/mol) were generously supplied by Dr. A. Lieberman of Hoffmann-La Roche, Inc. [4-^{3}H]-2-Hydroxy-3-butynoate (0.176 Ci/mol) prepared as described by Walsh et al. (1972) was a gift of Ms. A. Schonbrunn. D,L-[2-^{2}H]lactate was a gift of Dr. S. Ghisla. D,L-[2-^{2}H]Leucine was prepared by Mr. Rich Laura by the hydrolysis of ethyl α-acetamido-α-cyano-γ-methylpentanoate with ^{2}HCl as described by Albertson and Tullar (1945). D,L-[2-^{2}H]-3-Chlorolactate was prepared by reduction of chloropyruvic acid (Cragoe and Robb, 1960) with sodium borodeuteride (Merck Sharp and Dohme) in carbonate-buffered water (pH 8). After extraction of the acid with ether and chromatography on Dowex 1, the water was evaporated and the residue recrystallized from benzene (mp 67–70°). Nuclear magnetic resonance (NMR) was used to establish the position and extent of deuteration of all ^{2}H-labeled compounds. For isotope effect experiments samples of the corresponding protium compounds were prepared and purified by identical methods.

Purification and Enzyme Activity Analysis of D-α-*Hydroxy Acid Dehydrogenase from Rabbit Kidney.* The enzyme was partially purified from frozen rabbit kidneys as described by Cammack (1969). Protein was measured by the method of Warburg and Christian (1941), and enzyme activity assays were conducted with 0.002% DCIP, 2.5 m*M* $MgCl_2$, 25 m*M* D-lactate, and enzyme in 0.1 *M* Tris-HCl (pH 8.6) to a total volume of 1 ml. The crude cell extract with a specific activity of 0.15 nmol of DCIP[1] reduced per min per mg of protein was purified by treatment with acetic acid, ammonium sulfate precipitation, passage through DEAE-cellulose, and fractionation with polyethylene glycol 6000 to a specific activity of 20.7 nmol per min per mg in the presence of 1 m*M* potassium cyanide (Cammack, 1969). As previously noted by Cammack (1969) the activity of the enzyme varied more than sixfold from day to day during the early stages of purification, but the activity became stable after the DEAE column and standing for 3 days at 4°.

[1] Abbreviation used is: DCIP, dichloroindophenol.

Table I: Deuterium Kinetic Isotope Effects for L-α-Hydroxy Acid Oxidase.

Substrate	Isotope Effect
D,L-α-[^{2}H]Lactate	8.4
D,L-β-Chloro-α-[^{2}H]lactate	10.0
D,L-α-[^{2}H]Leucine	4.4

Purification and Enzyme Activity Analysis of L-*Hydroxy Acid Oxidase from Rat Kidney.* The enzyme was purified from the soluble fraction of rat kidney as previously described (Cromartie and Walsh, 1975) to a V_{max} of 714 nmol per min per mg as measured with the DCIP dye assay and D,L-α-hydroxybutyrate as standard substrate. The K_m values for the enzyme are independent of the assay procedure, and the V_{max} is identical for all concentrations of DCIP used. The DCIP assay was not affected by the presence or absence of oxygen. The rate measured by consumption of oxygen was found to be slightly less than that found using the DCIP assay (Cromartie and Walsh, 1975). Isotope effect experiments were conducted at 23° and the isotope effects were calculated from the V_{max} for the hydrogen and the deuterium compounds in double reciprocal plots.

Enzyme Inactivation Experiments. In most inactivation experiments, the enzyme was allowed to react with the substrate of interest in 0.1 *M* sodium phosphate at pH 8.4 and the reaction monitored by the reduction of DCIP or the utilization of oxygen. Because thiols readily reduce DCIP, all sets of inactivation experiments in which dithiothreitol was used were conducted with oxygen as the terminal electron acceptor. In some experiments, the enzyme was allowed to react with inactivator in a small volume (less then 150 μl) and, after a given time, assayed for residual enzymatic activity by the addition of a sufficient volume of 0.1 *M* sodium phosphate of pH 8.4 containing 0.002% DCIP and 25 m*M* sodium α-hydroxybutyrate to give 1.0 ml. The rate of reduction of DCIP was then determined to measure the activity of the enzyme. In a few experiments, the production of keto acids was determined with a 2,4-dinitrophenylhydrazine assay (Bohme and Winkler, 1954). Finally, those sets of experiments in which the time course for inactivation of L-α-hydroxy acid oxidase was measured in the presence and absence of dithiothreitol used a coupled assay in which the rate of turnover of the oxidase was measured by the rate of reduction of NADH in the presence of a large excess of rabbit muscle lactate dehydrogenase. Control experiments demonstrated that the rate of production of keto acids by the oxidase was being measured by the coupled assay system. Although the rate measurements made using the consumption of oxygen gave lower values than the DCIP assay, the lactate dehydrogenase coupled assay procedure, or the dinitrophenylhydrazine assay (all of which gave identical values under identical reaction conditions), all rate comparisons in this work are made on reactions using equivalent assay procedure.

Results

β-Chlorolactate as a Substrate for L-α-*Hydroxy Acid Oxidase.* D,L-β-Chlorolactate was found to be an acceptable substrate for L-α-hydroxy acid oxidase with a K_m of 28 m*M* and a V_{max} of 1150 nmol per min per mg, a rate 161% of that for the standard substrate D,L-2-hydroxybutyrate.

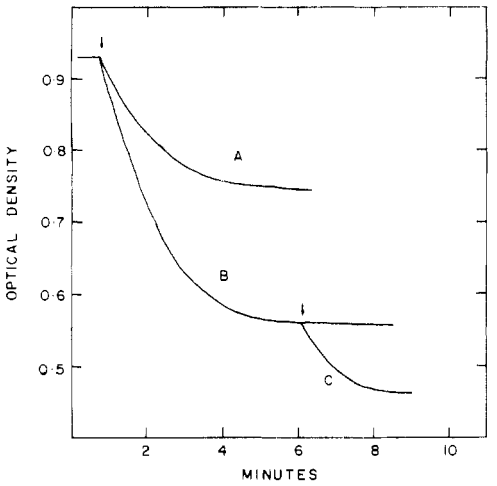


FIGURE 1: Inactivation of L-α-hydroxy acid oxidase with hydroxybutynoate. Each incubation contained 20 μg of DCIP, the reduction of which was monitored at 600 nm, and 1 μmol of D,L-2-hydroxy-3-butynoate in 0.1 *M* sodium phosphate (pH 8.4) to a total volume of 1 ml. Enzyme was added at the point indicated by the *upper arrow;* 34.3 μg was added for curve A and 68.6 μg for curve B. Curve A is the reduction of 8.19 nmol of DCIP, and curve B is the reduction of 16.38 nmol of DCIP. If another 17.1 μg of enzyme is added to the incubation of curve B at the *lower arrow,* another 4.09 nmol of DCIP is reduced (curve C).

The product of this reaction was identified as β-chloropyruvate by analysis of the uv spectrum of its 2,4-dinitrophenylhydrazone (Walsh et al., 1971). On the basis of the uv spectrum of the 2,4-dinitrophenylhydrazones, no pyruvate was formed. To test further for an elimination pathway, the oxygen independent production of pyruvate, 10 m*M* β-chlorolactate and 132 μg of enzyme were mixed under anaerobic conditions and pyruvate formation subsequently was assayed by either a coupled assay with NADH and lactate dehydrogenase or by reaction with either 2,4-dinitrophenylhydrazine or thiosemicarbazide (Walsh et al., 1971). Although the flavine 450 absorbance was bleached immediately, in no instance was the *catalytic* production of pyruvate detectable, even after 19 hr. A slow nonenzymatic formation of pyruvate from chlorolactate at the pH of 8.4 used in these studies was observed. The enzyme retained almost complete competence to oxidize β-chlorolactate in the presence of O_2 during this time. The inability of L-hydroxy acid oxidase to liberate HCl anaerobically from β-chlorolactate stands in contrast to the ready elimination of HCl from β-chlorolactate by *M. smegmatis* L-lactate oxidase (Walsh et al., 1973) or from β-chloroalanine by D-amino acid oxidase (Walsh et al., 1971). However, Lederer (1974) has observed that yeast L-lactate dehydrogenase, an FMN–heme enzyme, is incapable of releasing chloride from β-chlorolactate in the absence of a suitable electron acceptor although the yeast enzyme is also capable of oxidizing β-chlorolactate to β-chloropyruvate in the presence of a suitable electron acceptor.

Substrate Kinetic Isotope Effects. Deuterium kinetic isotope effects for three substrates of L-hydroxy acid oxidase are reported in Table I. In all cases the isotope effects were calculated from the V_{max} for the protio and deuterio compounds obtained from double reciprocal plots. The K_m's obtained from these plots were identical for the protio and deuterio compounds. The protio compounds were prepared and purified in a manner completely analogous to that for the deuterio compounds. The isotope effects clearly indicate that proton removal from the α carbon is rate limiting in these oxidations. In fact the unusually large values for the two hydroxy acids require that proton removal be completely rate limiting in the overall oxidation of these two substrates. Even the amino acid leucine, which has a V_{max} 12-fold less than that for lactate, exhibits a kinetic isotope effect at V_{max} of 5, suggesting that hydrogen abstraction is still important in the rate-limiting step. Interestingly, Lederer (1974) has reported a kinetic isotope effect of ca. 5 on the oxidation of lactate by yeast L-lactate dehydrogenase, an enzyme which does not eliminate chloride ion from β-chlorolactate in a nonoxidative process (the same behavior as L-hydroxy acid oxidase). In contrast the L-lactate oxidase from *M. smegmatis,* which does eliminate chloride from β-chlorolactate, has an isotope effect of 1.8 during the oxidation of D,L-[α-^{2}H]lactate (Walsh et al., 1973).

Reaction and Inactivation of L-Hydroxy Acid Oxidase with 2-Hydroxy-3-butynoate. In a recent paper (Cromartie and Walsh, 1975) on the properties of the homogeneous rat kidney L-α-hydroxy acid oxidase, we employed D,L-2-hydroxy-3-butynoate to probe two points. We established that it caused irreversible inactivation of both the hydroxy acid oxidase and the amino acid oxidase activities of the enzyme at identical rates, consistent with both activities being carried by a single enzyme. Secondly, we noted that hydroxybutynoate could be used as a titrant for the FMN coenzyme molecules, confirming the presence of two coenzymes per enzymatically active tetramer. The characteristics of the interaction of the enzyme with this molecule are elaborated here.

Assuming one active site per flavine (i.e., one per dimer), 2-hydroxy-3-butynoate is oxidized in the presence of DCIP at 25° for about 25 turnovers for each active site before irreversible loss of catalytic activity. An example of the experiments on which this conclusion is based is shown in the time dependent inactivation profiles of Figure 1, in which the reduction of DCIP is monitored. When the reduction of DCIP ceases, the enzyme is inactive; addition of a large excess of α-hydroxybutyrate, the substrate normally used to measure the activity of the enzyme, produces no further dye reduction. The holoenzyme cannot be reactivated by dialysis to remove excess hydroxybutynoate or by gel filtration on Sephadex G-25 columns. Since hydroxybutynoate functions as a substrate prior to inactivation and gives a linear rate of product formation during the initial period of reaction, the kinetic parameters K_m and V_{max} for this compound can be obtained and are listed in Table II. By these criteria, 2-hydroxy-3-butynoate is comparable to the best hydroxy acid substrates for the enzyme. When oxygen is used as the electron acceptor, identical results are obtained. As expected, the addition of catalase, which removes product hydrogen peroxide, has no effect on the inactivation, and 25 turnovers before loss of catalytic activity are still observed.

Evidence that the rat kidney flavoprotein oxidase undergoes inactivation by covalent modification of the bound FMN coenzyme is provided by the spectroscopic data of Figure 2. The absorption spectrum of the enzyme was recorded under anaerobic conditions before (curve A) and after (curve B) addition of an excess of hydroxybutynoate. Although the long wavelength flavine absorbance is clearly significantly bleached, it is less reduced than when the normal substrates hydroxybutyrate and L-leucine are anaerobically added to the enzyme (see Figure 5 of Cromartie and Walsh, 1975). The steep increase in optical density starting

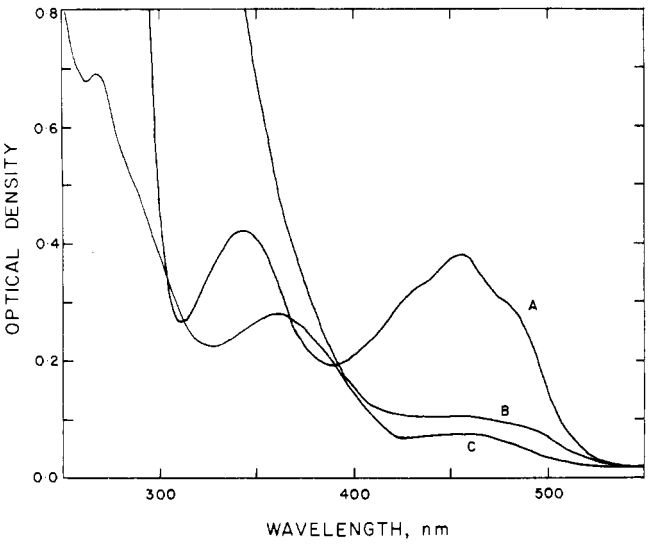


FIGURE 2: Spectroscopic changes of L-α-hydroxy acid oxidase on anaerobic inactivation with D,L-2-hydroxy-3-butynoate. Curve A is the spectrum of the native enzyme at a concentration of 2.87 mg/ml taken under anaerobic conditions. Curve B was obtained after the addition of 5 m*M* D,L-2-hydroxy-3-butynoate. After this treatment, the enzyme retained less than 1% of its original activity. Exposure to air caused no significant alteration of the spectrum from that of curve B. After dialysis of the enzyme, the inactivated coenzyme was liberated by addition of trichloroacetic acid and centrifugation of the denatured protein. After adjustment of the pH to ca. 9, the spectrum of curve C was obtained.

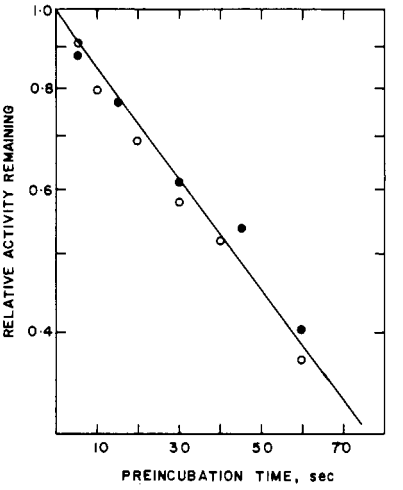


FIGURE 3: Time course for the inactivation of L-α-hydroxy acid oxidase with hydroxybutynoate in the presence and absence of dithiothreitol. For each point 258 μg of enzyme was incubated with 3.7 μmol of D,L-2-hydroxy-3-butynoate in 50 m*M* sodium phosphate (pH 8.4) to a final volume of 50 μl (○) or in 50 m*M* sodium phosphate (pH 8.4) and 2.2 m*M* dithiothreitol (●). After the preincubation time, the incubation solution was rapidly injected into 0.94 ml of solution containing 25 m*M* sodium α-hydroxybutyrate, 100 μg of NADH, and 16 μg of rabbit muscle lactate dehydrogenase in 50 m*M* sodium phosphate at pH 8.4. The high concentration of hydroxybutyrate and the 20-fold dilution of the hydroxybutynoate served to rapidly quench the inactivation and the rate of oxidation of NADH was monitored at 340 nm to determine the residual activity of L-α-hydroxy acid oxidase.

Table II: Reaction of L-α-Hydroxy Acid Oxidase with Unsaturated Substrates.

Substrate	K_m(m*M*)	V_{max} (% of Hydroxybutyrate Rate)	Turnovers before Inactivation
D,L-2-Hydroxy-3-butynoate	4	115	25
D,L-2-Hydroxy-3-pentynoate	9	218	4800
D,L-2-Hydroxy-3-hexynoate	7	65	8500
D,L-2-Hydroxy-3-heptynoate	0.38	86	18,000
D,L-2-Hydroxy-3-octynoate	0.14	70	15,000
D,L-Vinylglycolate	10	92	See text
2,3-Butadienoic acid			*a*
D,L-2-Hydroxy-3-chloro-3-butenoate			*a*
Propargylglycine			*a*

[a] No inactivation observed.

at ca. 400 nm is not due to any easily removable low molecular weight species, for dialysis of the inactivated enzyme does not significantly alter the spectrum. At the present time we have no explanation for this unusual increase in absorbance. When the inactivated enzyme is opened to the atmosphere and subsequently assayed for enzymatic activity toward hydroxybutyrate, it was found that less than 1% of the original activity remained. After exposure to air for 1 hr, the spectrum of curve B was not significantly changed. Oxygen no longer causes a return to the oxidized flavine spectrum and, in fact, causes some slight further reduction in the 450-nm region. If the inactivated enzyme is treated with a small crystal of trichloroacetic acid and the denatured protein removed by centrifugation, the spectrum of curve C is obtained after adjustment of the pH to about 9.

Depending on experimental conditions, inactivation of L-α-hydroxy acid oxidase results in attachment of the inactivator to the coenzyme or alternatively to the coenzyme and also to the apoenzyme. When [1-^{14}C]hydroxybutynoate (35 Ci/mol) is reacted in air with the enzyme to produce greater than 96% inactivation, and the incubation mixture submitted to gel filtration, a peak of radioactivity elutes with the enzyme as indicated in Figure 3 of Cromartie and Walsh (1975). In this particular experiment, the incubation was placed on the same Sephadex G-200 column used earlier to determine the molecular weight of the native holoenzyme (Cromartie and Walsh, 1975). The inactive, radioactive holoenzyme elutes at the same volume as the active tetramer, indicating that no dissociation of the active tetrameric structure occurs on inactivation. The radioactive, inactive enzyme peak was pooled and lyophilized, then redissolved in 1 ml of column buffer and the protein concentration and radioactivity were quantitated. A result of 1 mol of radioactive inactivator/102,000 g or 2 mol/enzyme tetramer, as we have earlier noted (Cromartie and Walsh, 1975), was observed.

The above experiments were carried out in the presence of 2 m*M* dithiothreitol to scavenge the 25 or so product molecules of 2-keto-3-butynoate which are produced prior to enzyme inactivation. This unsaturated keto acid would be expected to undergo ready Michael addition with nucleophilic groups on the apoprotein part of the holoenzyme, a feature we have utilized elsewhere (Walsh and Kaback, 1973). When [1-^{14}C]hydroxybutynoate is allowed to inactivate the enzyme in the absence of dithiothreitol, and the reaction allowed to stand for 30 min, the inactive holoenzyme after gel filtration has an increase of labeling to 1.3 mol of inactivator/100,000 g of protein. Dithiothreitol has

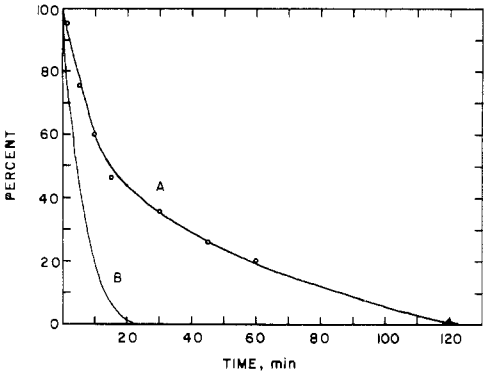


FIGURE 4: Inactivation of L-α-hydroxy acid oxidase by vinylglycolate. For each point of curve A 60 μg of enzyme was incubated with 16 μmol of vinylglycolate and 0.02 mg of DCIP in a total volume of 50 μl of sodium phosphate (pH 8.4) for the indicated time. The percent of enzyme activity remaining was assayed by the addition of 0.95 ml of a solution of 25 mM sodium α-hydroxybutyrate in the same buffer. Curve B is the time course for the oxidation of the vinylglycolate in the incubation mixture determined by reduction of the corresponding keto acid produced with NADH and lactate dehydrogenase.

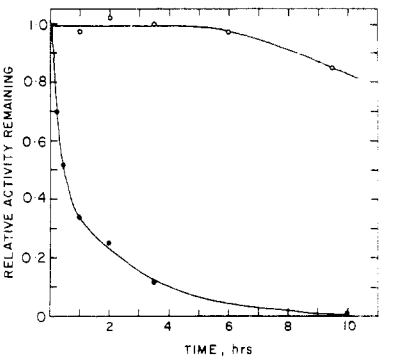


FIGURE 5: Dithiothreitol prevention of the inactivation of L-α-hydroxy acid oxidase with vinylglycolate. For each point on the graph 167 μg of enzyme was incubated for the indicated time with 30 μmol of vinylglycolate in air in 50 mM sodium phosphate (○) or in 50 mM sodium phosphate and 5 mM dithiothreitol (●). The residual enzymatic activity was assayed by the standard assay with hydroxybutyrate after Sephadex G-25 filtration to remove thiols.

no effect on the rate at which hydroxybutynoate inactivates the enzyme (Figure 3), an observation which demonstrates that the process of inactivation occurs completely on the enzyme prior to product release and that just two molecules of inactivator per tetramer, both bound exclusively to the coenzyme molecules, are required for loss of activity.

When the radioactive, inactive enzyme after Sephadex filtration is treated with trichloroacetic acid at 0° for 10 min, followed by centrifugation to remove denatured protein, at least 98% of the initial radioactivity is released in the supernatant. The same result is obtained if the enzyme is denatured in boiling water for 2 min and the protein removed by filtration through a 45-μ membrane filter. Both these treatments quantitatively release FMN from the native holoenzyme. In the absence of trichloroacetic acid or heat treatment, the radioactivity is not released by prolonged dialysis or by ammonium sulfate precipitation of the enzyme. Therefore, inactivation occurs by covalent modification of only the coenzyme and not of any amino acids in the apoenzyme.

To demonstrate that the C-4 proton of hydroxybutynoate was retained in the inactivated enzyme, 4.4 mg of enzyme was incubated with 27 μmol of [4-^{3}H]hydroxybutynoate (0.176 Ci/mol) in the presence of 5 mM dithiothreitol. After Sephadex G-25 filtration and ammonium sulfate precipitation, the inactive enzyme was found to have retained about 2.4 molecules of inactivator/tetramer. That more than the expected two labels were found on the enzyme is most likely a reflection of experimental uncertainty, since the specific activity of the [4-^{3}H]hydroxybutynoate was quite low.

If the enzyme is incubated with 2-hydroxy-3-butynoate under anaerobic conditions, the 450-nm flavine absorbance is rapidly bleached. If the enzyme is then assayed for residual enzymatic activity by the DCIP assay procedure, less than 10% of activity is lost regardless of the length of the anaerobic incubation.

Reaction and Inactivation with Vinylglycolate. It has been reported that lactate oxidase from *M. smegmatis* (Walsh et al., 1972a) and D-lactate dehydrogenase from *E. coli* (Walsh et al., 1972b), two flavoenzymes which suffer irreversible inactivation on oxidation of 2-hydroxy-3-butynoate, utilize 2-hydroxy-3-butenoate (vinylglycolate) as a substrate without apparent inactivation. Yet vinylglycolate has been found to be an effective inhibitor of vectorial phosphorylation and transport of glucose in *E. coli*, by a process proposed to involve irreversible alkylation of a specific enzyme critical to active transport, by the highly reactive product of the enzymatic oxidation of vinylglycolate, 2-keto-3-butenoate (Walsh and Kaback, 1973). Vinylglycolate has been found to be a good substrate (DCIP assay) for the rat kidney L-α-hydroxy acid oxidase (Table II), and slight if any inactivation was observed after 10,000 turnovers. Nevertheless, when 700 μg of L-α-hydroxy acid oxidase was allowed to react with 1.0 μmol of [1-^{14}C]vinylglycolate (60 mCi/mol) in air and stand at room temperature for 6 hr, 3.6 radioactive labels per tetramer were found after Sephadex G-25 filtration. The enzyme so treated retained 58% of its initial activity, a surprising result which implies a marked insensitivity to random multiple alkylations. Other similar experiments gave results that seemed to indicate that the extent of inactivation was related not only to the number of turnovers of vinylglycolate, but also on the time the enzyme was allowed to stand after all the substrate had been oxidized. This observation was confirmed by the experiment of Figure 4 in which the time courses for the oxidation of vinylglycolate by L-α-hydroxy acid oxidase and for the inactivation of the enzyme are presented. This experiment demonstrates that much of the inactivation of L-α-hydroxy acid oxidase can occur after the catalytic process, probably by nonspecific addition of 2-keto-3-butenoate to nucleophiles on the apoprotein. Further support for this postulate was provided by the observation, shown in Figure 5, that the presence of 5 mM dithiothreitol almost completely prevents the loss of catalytic activity caused by the oxidation of and continued incubation with vinylglycolate in air. In other experiments (not shown) in which the production of keto acid was monitored with 2,4-dinitrophenylhydrazine assay (Bohme and Winkler, 1954), the presence of 5 mM dithiothreitol resulted in the continued production of keto acid for many hours after the enzyme in an identical reaction without dithiothreitol had been inactivated. These protection data are in marked contrast to the inactivation seen above with hydroxybutynoate, suggesting a different process.

Two other experiments demonstrated that the inactivation of L-α-hydroxy acid oxidase by vinylglycolate occurred by Michael addition of nucleophiles on the apoenzyme to

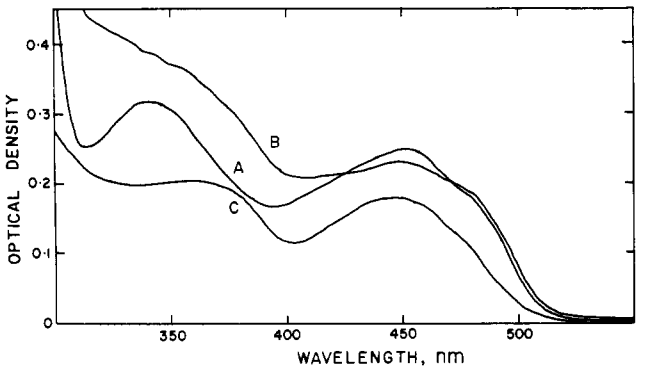


FIGURE 6: Absorption spectrum of L-α-hydroxy acid oxidase after inactivation with vinylglycolate. Curve A is the spectrum of the fully active holoenzyme. Curve B is obtained after aerobic inactivation of the enzyme with vinylglycolate. The spectrum of curve C results after the removal of the coenzyme from the enzyme by denaturation with trichloroacetic acid and centrifugation to remove the protein. The pH was adjusted to ca. 8 for curve C.

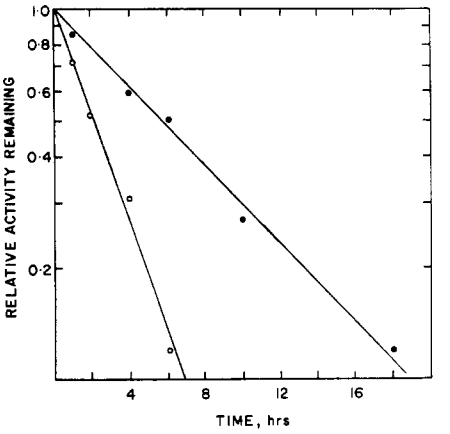


FIGURE 7: Effect of dithiothreitol on the inactivation of L-α-hydroxy acid oxidase by D,L-2-hydroxy-3-pentynoate. For each point 68.6 μg of enzyme was incubated for the appropriate time with 10 μmol of D,L-2-hydroxy-3-pentynoate in 450 μl of 0.1 *M* sodium phosphate (○) or 0.1 *M* sodium phosphate containing 2 m*M* dithiothreitol (●). Residual enzymatic activity was assayed with D,L-2-hydroxy-3-butyrate after Sephadex G-25 chromatography.

2-keto-3-butenoate rather than by alteration of the FMN coenzyme during oxidation. In the first it was found that with 11 μmol of [1-^{14}C]vinylglycolate (6.5 Ci/mol) and a 6-hr incubation, 166 μg of enzyme incorporated as many as 40 radioactive labels/tetramer. On the addition of trichloroacetic acid, only 3.15 of these were released into the supernatant under conditions where all the FMN was released. Furthermore, when the spectrum of a sample of L-α-hydroxy acid oxidase that had lost greater than 95% of its original activity after aerobic incubation with vinylglycolate was taken (Figure 6), the 450-nm peak of oxidized flavine was observed. Despite the loss of catalytic activity, the enzyme retained an oxidized flavine spectrum in contrast to the results of inactivation with hydroxybutynoate.

Inactivation of L-α-*Hydroxy Acid Oxidase by Other Acetylenic Hydroxy Acids.* A number of analogs of hydroxybutynoate with alkyl groups attached to C-4 were prepared and tested for reactivity and possible inactivator properties with L-α-hydroxy acid oxidase. As evidenced by the data in Table II, all these compounds were acceptable substrates for the enzyme. They were all also found to inactivate the enzyme, but only after very many more turnovers than required by hydroxybutynoate. These observations raised the question of whether the inactivation by these C-4 substituted acetylenic hydroxy acids was of the type observed with hydroxybutynoate (coenzyme modification) or that observed with vinylglycolate (apoenzyme alkylation). When 65 μg of the enzyme was incubated with 10 μmol of D,L-2-hydroxy-3-pentynoate in the presence and absence of 4 m*M* dithiothreitol, the results of Figure 7 were obtained. Although the rate of inactivation is decreased in the presence of dithiothreitol, it is not eliminated. From these results, we conclude that hydroxypentynoate, and by inference, the other acetylenic hydroxy acids of Table II, inactivate the enzyme by two processes: one, by active site reaction with the flavine coenzyme as seen for hydroxybutynoate and the other, by alkylation of the enzyme by the product acetylenic keto acids in a manner similar to inactivation by vinylglycolate. This result was not unexpected since even hydroxybutynoate was found to react nonspecifically with the enzyme after oxidation. Because so few product molecules are produced from hydroxybutynoate before loss of catalytic activity, this type of inactivation is not as important as for the higher analogs where more product is produced.

Inactivation of Rabbit Kidney D-*Hydroxy Acid Dehydrogenase by Hydroxybutynoate.* Tubbs and Greville reported the detection and subsequent solubilization from rabbit kidney mitochondria of an α-D-hydroxy acid dehydrogenase (Tubbs and Greville, 1961). This enzyme was subsequently purified to homogeneity by Cammack and shown to be an FAD containing enzyme with weak oxidase activity (Cammack, 1969). The enzyme displays no activity toward the L isomer. Since synthetic 2-hydroxy-3-butynoate is a racemic mixture, we tested partially purified preparations of the D-hydroxy acid dehydrogenase for inactivation by the acetylenic hydroxy acid. The rabbit kidney enzyme used in these preliminary experiments was purified 138-fold from crude extracts and had a specific activity of 21 nmol per min per mg, much lower than reported by Cammack (1969), but in the range listed by Tubbs and Greville. It did not oxidize L-lactate.

Insufficient amounts of enzyme were available to demonstrate that D-2-hydroxy-3-butynoate functioned as a substrate. However, it did inactivate this flavoenzyme. Separate incubations were conducted with 105 μg of D-hydroxy acid dehydrogenase with D-lactate; D,L-vinylglycolate; and D,L-hydroxybutynoate at 2 m*M* concentrations for 1.5 hr, followed by gel filtration with Sephadex G-25 and assay for enzymatic activity in the effluent. The enzymes from incubations with lactate and vinylglycolate were still active and had similar specific activities, indicating that vinylglycolate does not inactivate the enzyme over this time period. The enzyme from the hydroxybutynoate incubation, on the other hand, was completely inactive. When hydroxybutynoate was added to enzyme solutions actively oxidizing D-lactate, loss of lactate oxidizing ability was time dependent. At 1.1×10^{-8} *M* D,L-hydroxybutynoate, the half-time for enzymatic inactivation was about 12 min at 25°, at 6.8×10^{-8} *M*, it was 5 min. Conversely, while 9×10^{-7} *M* vinylglycolate gave a 51% inhibition of the initial rate of D-lactate oxidation, no time-dependent inactivation occurred.

Discussion

Our previous postulate that flavoenzyme hydroxy acid and amino acid oxidases may oxidize their substrates by initial proton abstraction at the α carbon and subsequent two electron transfer steps involving the flavine coenzyme is primarily based on experiments with β-halo substrates. *M.*

Table III: Flavoenzymes Inactivated by Hydroxybutynoate.

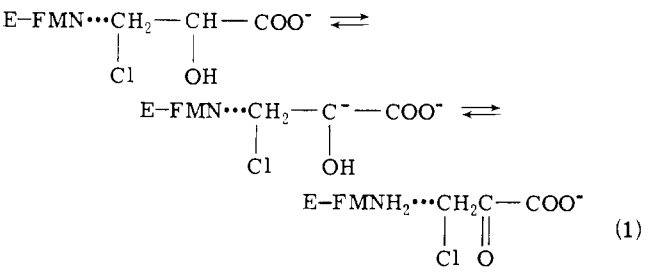
Enzyme	Source	Coenzyme	Adduct Involving Modified Coenzyme	Ref
L-Lactate oxidase	*M. smegmatis*	FMN	Yes	Walsh et al., 1972a
D-Lactate dehydrogenase	*E. coli*	FAD	n.d.	Walsh et al., 1972b
L-Lactate dehydrogenase	*E. coli*	n.d.[a]	n.d.	Walsh et al., 1972b
D-Lactate dehydrogenase	*P. elsdenii*		Yes	*b*
Cytochrome b_2	Yeast	FMN	Yes	Lederer, 1974
L-α-Hydroxy acid oxidase	Rat kidney	FMN	Yes	This work
D-α-Hydroxy acid oxidase	Rabbit kidney	FMN	n.d.	This work

[a] n.d., not determined. [b] Personal communication, Dr. C. Whitfield.

smegmatis L-lactate oxidase (Walsh et al., 1972a) and D- and L-amino acid oxidases (Walsh et al., 1971) carry out catalytic elimination of chloride from β-chlorolactate and β-chloroalanine, respectively, as well as normal oxidation of these substrates. Since the ratio of elimination to oxidation depended on the oxygen concentration for the L-lactate oxidase and D-amino acid oxidase, partitioning of an intermediate α-carbanion between elimination of chloride ion and electron transfer to flavine to give normal oxidation was suggested.

Recently, Lederer (1974) has reported the yeast heme flavoprotein L-lactate dehydrogenase (cytochrome b_2) is capable of oxidizing β-chlorolactate but incapable of catalyzing an elimination of HCl from this substrate. In this paper we report that the flavoprotein L-hydroxy acid oxidase from rat kidney is also unable to catalyze the elimination of HCl from D,L-β-chlorolactate although this β-chloro substrate can be rapidly oxidized in the presence of O_2. Lederer (1974) has postulated that the lack of the elimination pathway is a kinetic consequence of a slow α-hydrogen abstraction followed by an electron transfer from the incipient α-carbanion to the FMN coenzyme too rapid to allow elimination to compete. An α-deuterium isotope effect of ca. 5 for D,L-[^{2}H]lactate agreed with this hypothesis. The isotope effect data in Table I further substantiate this conclusion for L-hydroxy acid oxidase. The very large isotope effects of 8 and 10 for D,L-lactate and D,L-β-chlorolactate strongly imply that proton removal is fully the rate-limiting step and that subsequent electron transfer to the flavine is much faster. Since the rat kidney oxidase has the unusual capacity to oxidize amino acids, at slow but detectable rates, e.g., L-leucine at 3% the rate of D,L-hydroxybutyrate, we could determine that hydrogen abstraction was rate limiting for amino acid oxidation as well. The data on these two enzymes contrast with magnitude of deuterium isotope effects for similar reactions with D,L-[^{2}H]lactate (k.i.e. = 1.8) with L-lactate oxidase or with D,L-[α-^{2}H]-β-chloroalanine (k.i.e. = 1.7–2.0) with both D- and L-amino acid oxidases, the three flavoenzymes which do carry out elimination reactions. Clearly, the extent to which proton removal is rate determining in these reactions correlates with whether or not elimination can be observed from β-halo substrates.

That α-hydrogen abstraction from chlorolactate by the rat kidney oxidase or the yeast L-lactate dehydrogenase is much slower than any subsequent step in the oxidation is not in itself sufficient to vitiate any chance for elimination. Rather, it is required that the electron transfer steps leading to substrate oxidation be much faster than the slowest step for elimination to suppress the elimination pathway completely. The observation that under anaerobic conditions there is flavine reduction but no catalytic conversion of chlorolactate to pyruvate suggests three possible explanations for the observed failure of the rat kidney oxidase to eliminate chloride from β-chlorolactate. The equilibrium of eq 1 may lie so far to the right that no finite concentration



(1)

of carbanion accumulates to break-down by the elimination pathway. A second possibility is that the enzyme binds chlorolactate such that the carbanion once formed has such an unfavorable geometry for elimination by either a syn or an anti mechanism that elimination does not occur. This explanation would be surprising in view of the fact that the oxidase accepts both hydroxy acids and amino acids of wide structural diversity (Cromartie and Walsh, 1975) suggesting a large active site. Finally, it may be that a mechanism involving a carbanion of any finite lifetime is not used by this enzyme but rather a transfer of electrons into the flavine is concerted with proton removal.

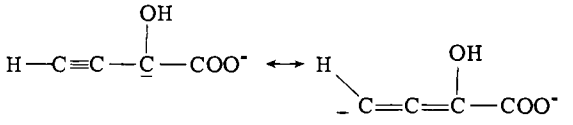
The second group of experiments in this paper concern the substrate behavior of the unsaturated hydroxy acids 2-hydroxy-3-butynoate and 2-hydroxy-3-butenoate (vinylglycolate) and their relevance to an understanding of flavoenzyme catalytic mechanisms. L-Hydroxy acid oxidase, and in preliminary experiments D-hydroxy acid dehydrogenase from rabbit kidney, are irreversibly inactivated by 2-hydroxy-3-butynoate. The inactivation occurs after 25 catalytic events for the oxidase and results in the formation of an adduct between the inactivator and the flavine coenzyme. All the flavoenzymes which have been reported to use 2-hydroxy-3-butynoate as a substrate are rapidly irreversibly inactivated (Table III). Inactivation occurs with either FMN or FAD as coenzyme, whether the enzymes are soluble or membraneous, whether or not they are insensitive to reoxidation by oxygen, and whether they are microbial or mammalian in origin. In all the cases so far examined, irreversible inactivation results in covalent attachment to the isoalloxazine nucleus of the coenzyme rather than to a nucleophilic active site amino acid residue. On the other hand, acetylenic substrates which cause irreversible inactivation of nonflavoenzymes do so by labeling amino acids in the protein (Bloch, 1971; Abeles and Walsh, 1973; Hevey et al., 1973; Holland et al., 1973). Either there is no suitably placed amino acid moiety at the active site of these flavoenzymes which can compete with addition by the coenzyme or a fundamentally different process causes inactivation of flavoenzymes than that for the other kinds of enzymes.

The actual mechanism by which hydroxybutynoate reacts with and inactivates flavoenzymes is still unknown, but two possibilities have been proposed (Walsh et al.,

1972a). The propargylic rearrangement of an initial C_2 carbanion with protonation to an allene followed by nucleophilic attack of the *oxidized* flavine on the allene would appear to be unlikely since oxidized flavine has no sites of high nucleophilicity.

Alternatively, the oxidized product 2-keto-3-butynoate could be attacked in a Michael addition by a nucleophilic center on the reduced coenzyme. To explore the possibility of inactivation of the enzyme by Michael addition to an unsaturated keto acid, the reaction of the alkenoic hydroxy acid 2-hydroxy-3-butenoate (vinylglycolate) with L-hydroxy acid oxidase was studied. The reactive product of oxidation of vinylglycolate, 2-keto-3-butenoate, does finally inactivate L-α-hydroxy acid oxidase but only on long standing and by quite nonspecific multiple alkylations. This type of inactivation leaves the flavine coenzyme unaltered and can be completely prevented by the presence of thiols in the reaction mixture to trap the unsaturated keto acid. The fact that 2-keto-3-butenoate is not alkylated by the reduced flavine coenzyme may result from much faster release of this product than of 2-keto-3-butynoate so that the alkenoic keto acid remains at the active site insufficiently long to suffer alkylation. The structural similarity of the alkenoic and acetylenic keto acids make this explanation unlikely and we feel that the results with vinylglycolate suggest that nucleophilic attack of reduced flavine on 2-keto-3-butynoate may not be the mechanism of inactivation. In contrast to *M. smegmatis* lactate oxidase (Walsh et al., 1973) and L-lactate dehydrogenase from yeast (Lederer, 1974), L-α-hydroxy acid oxidase is not inactivated on anaerobic reaction with 2-hydroxy-3-butynoate although the flavine coenzyme is reduced. The 2-keto-3-butynoate produced in this single turnover may fail to inactivate the enzyme because it is too rapidly released into solution to react with the reduced coenzyme or, more probably, because it is not the inactivating species.

A third heretofore unmentioned possibility is that inactivation of flavoenzymes occurs by nucleophilic attack on the oxidized coenzyme by a C_4 allenic anion. An initial C_2 carbanion or carbanionic species is a resonance hybrid with contributions from a C_4 allenic carbanion:



Attack of the form with C_2 carbanionic character would undergo normal oxidation while attack by the C_4 form could lead to irreversible reduction of the coenzyme. This last alternative differs from previous proposals (Walsh et al., 1972a) in postulating the inactivating species as a nucleophile rather than as an electrophile; this would explain why the coenzyme, an electron sink while oxidized, and not amino acids in the apoprotein, is modified in reactions with flavoenzymes. Table III also shows that the C_4 alkyl substituted hydroxybutynoates inactivate only after thousands of catalytic events. While steric bulk could control partitioning ratios, an alkyl group at C_4 could also exert a significant inductive effect in destabilizing an incipient allenic anion at this carbon, thus favoring a greater amount of oxidation prior to inactivation. Whereas the L-lactate oxidase of *M. smegmatis* undergoes 25 turnovers before inactivation with hydroxybutynoate, it undergoes *at least* 1300 turnovers (limited by the total consumption of oxygen in the system) with 2-hydroxy-3-pentynoate before inactivation.[2] With L-hydroxy acid oxidase the inactivation by the C_4 alkyl substrated acetylenic acids occurs by both the flavine modification pathway observed with hydroxybutynoate and by the nonspecific labeling and inactivation from the unsaturated keto acid seen for vinylglycolate. Even 2-keto-3-butynoate could be observed to nonspecifically label the enzyme but this was only a minor path in this case since so few product keto acid molecules were produced from 2-hydroxy-3-butynoate. This nonspecific process could be completely prevented by the addition of thiols. In any case a salient chemical difference between the acetylenic hydroxy acids and vinylglycolate is that only the former can partition to an allenic species and only the former cause inactivation of the enzyme by coenzyme modification. The failure to observe chloride elimination from β-chlorolactate does not mean that no (even transient) carbanionic species is formed in the case of hydroxybutynoate. As discussed earlier, a carbanionic species may be formed in the chlorolactate case, and even if such a species is not formed, there is sufficient structural difference between chlorolactate and hydroxybutynoate to allow a slightly different reaction sequence and the formation of a species capable of rearrangment to an allenic carbanion in the case of the acetylene.

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Malate Dehydrogenase, Anticooperative NADH, and L-Malate Binding in Ternary Complexes with Supernatant Pig Heart Enzyme†

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ABSTRACT: Supernatant malate dehydrogenase from pig heart, a dimeric protein containing two very similar or identical subunits, shows negatively cooperative (anticooperative) interactions between NADH binding sites in the presence, but not in the absence, of 0.1 *M* L-malate. This behavior is observed consistently whether the technique used employs protein fluorescence quenching, NADH fluorescence enhancement, or ultrafiltration dialysis. Fluorescence titration shows that L-malate is also anticooperatively bound in the presence of saturating concentrations of NADH. The data are consistent with an "induced asymmetry" model in which conformational change accompanies the formation of the ternary complex. Two of the three chromatographically resolvable forms of the enzyme have been tested and found to have anticooperative behavior.

Although the biological significance of heteropolymeric enzyme structure is now better understood in terms of regulatory function, the significance of homopolymeric enzyme function is still in question. The near ubiquitous occurrence of multiple catalytic subunits in heteropolymeric as well as homopolymeric enzymes suggests a strong evolutionary selection for as yet poorly understood functional advantages. The importance of protein conformational alteration has been established as a significant feature in heteropolymeric proteins, suggesting that conformational mobility also may be important in homopolymeric enzyme function. One consequence of conformational change related to catalytic mechanism might be negatively cooperative (anticooperative) interactions between ligand binding sites on enzyme multimers. The prediction that negatively cooperative interactions might occur (Koshland et al., 1966) has been followed by experimental reports (Malhotra and Bernhard, 1968, 1973; Levitzki and Koshland, 1969, 1972; Simpson and Vallee, 1970; Long et al., 1970; Lazdunski et al., 1971; Levitzki et al., 1971; Dalziel and Egan, 1972) of this sort of binding behavior.

We report here studies aimed at exposing possible anticooperative interactions in pig heart supernatant malate dehydrogenase. These studies were prompted by the fact that such behavior would be predicted in mitochondrial malate dehydrogenase by the previously published studies of Harada and Wolfe (1968) described in a reciprocating compulsory order kinetic mechanism which assumed functional interdependence between enzyme subunits. Previous attempts to discover anticooperative binding of ligands by this and the mitochondrial enzyme (Holbrook and Wolfe, 1972) were apparently unsuccessful because of subtle dependence of the phenomenon on the structure of the substrate analog used in binding studies.

Materials and Methods

Reagents. Pig heart cytoplasmic malate dehydrogenase (s-MDH)[1] was prepared by a modification (Eberhardt, 1972) of the method of Gerding and Wolfe (1969). The enzyme had a specific activity of 100 IU/mg of protein using $A_{280}(1\%) = 9.0$ (Gerding and Wolfe, 1969). All binding data reported here were taken with the use of the second s-MDH peak eluting from hydroxylapatite column chromatography in the final purification step with one exception which will be considered below. The crystalline enzyme was

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[1] Abbreviation used is: s-MDH, supernatant or cytoplasmic malate dehydrogenase.