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IRREVERSIBLE INACTIVATION OF THE FLAVOENZYME ALCOHOL OXIDASE WITH ACETYLENIC ALCOHOLS

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SUMMARY: Alcohol oxidase is irreversibly inactivated by the acetylenic substrates propargyl alcohol and 1,4-butynediol. In both cases, inactivation results from affinity labeling of amino acid residues in the active site of the enzyme by propynal and 4-hydroxy-2-butynal, the electrophilic products of the oxidation of these alcohols. The inactivation by 4-hydroxy-2-butynal occurs with the aldehyde bound in the active site of the enzyme in the orientation which is opposite to that in which it had been produced. Alcohol oxidase is inactivated by acetylenic substrates in a fundamentally different manner than are the formally similar flavin-dependent α-hydroxy acid oxidases.

The development of mechanism based, irreversible enzyme inactivators (suicide substrates or k_{cat} inhibitors) has shown considerable promise in the development of new kinds of drugs and in the elucidation of enzyme reaction mechanisms (1-4). These inactivators are enzyme substrates which can be converted by at least a part of the normal catalytic mechanism of the target enzyme into reactive species which become covalently attached to the active site of the enzyme before dissociation occurs. Flavin dependent enzymes which catalyze the oxidation of α-hydroxy acids to α-keto acids have been found to be particularly susceptible to suicide inactivation by substrates with an acetylene function adjacent to the site of oxidation (5,6). These inactivations occur by covalent attachment of the γ-carbon of the β,γ-acetylenic α-hydroxy acid substrate to the flavin coenzyme rather than to an amino acid of the protein. The mechanism for these inactivations has not been established, but it has been suggested to involve rearrangement of the carbanion known to be generated at the α-carbon of the substrate during normal catalysis (5,6).

Because of the absence of an adjacent carbonyl group to stabilize negative charge, the oxidation of primary alcohols to aldehydes by flavoenzymes would

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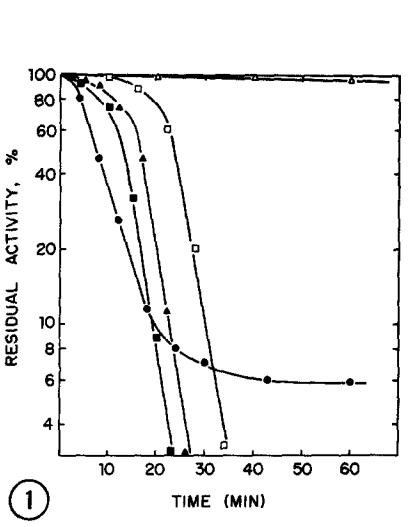
not be expected to proceed through carbanion intermediates. Based on model system studies, Bruice has suggested that radical-like intermediates should be involved (7). For comparison with enzymes catalyzing the formally similar oxidation of α-hydroxy acids, the reaction of alcohol oxidase (E.C. 1.1.3.12) from the yeast Candida boidinii with acetylenic substrates was explored. This enzyme has FAD as cofactor and oxidizes a variety of small primary alcohols to aldehydes with the reduction of oxygen to H_2O_2 (8).

METHODS: Alcohol oxidase was purchased from Boehringer Mannheim and further purified by chromatography on Sephadex G-200 or was isolated and purified from C. boidinii grown on methanol (8). The enzyme was assayed with a Clark-type oxygen electrode (Yellow Springs Instrument Co.) in 50 mM sodium phosphate, pH 7.5 at 23°C containing 50 mM methanol. Propargyl alcohol and 1,4-butynediol were purchased from Aldrich and purified by distillation before use. 4-Hydroxy-2-butynal was prepared by the hydrolysis of 4,4-diethoxy-2-butyn-1-ol (9) in 0.25 M sulfuric acid at room temperature and was monitored by the increase of the aldehyde signial in the NMR. After 90 min benzene was added, and the solution was azeotroped at reduced pressure. The original volume was restored with water, and the procedure was repeated until no further increase in the aldehyde signal in the NMR was observed. The aldehyde, which could not be extracted into organic solvents without decomposition, was isolated by chromatography on Merck F-254 cellulose TLC plates developed with 0.1 M phosphate buffer, pH 4.2 (R_f = 0.7). The structure was confirmed by mass spectrometry and the NMR of the 2,4-dinitrophenylhydrazine derivative (10).

RESULTS AND DISCUSSION: Propargyl alcohol is a good substrate for alcohol oxidase (K_m = 10 mM and V_{max} = 30 μmol/min/mg at pH 7.5 and 23°C compared with K_m = 3.0 mM and V_{max} = 3.3 μmol/min/mg for methanol in air saturated buffer), but one which leads to a time dependent loss of enzymatic activity (Fig. 1). At inactivator concentrations below 3 mM, the inactivation was incomplete and dependent on the initial concentration of propargyl alcohol. Complete loss of activity was observed at higher concentrations of propargyl alcohol. The inactivation was not reversed by Sephadex chromatography or prolonged dialysis of the inactivated enzyme in 50 mM sodium phosphate at pH 7.5 containing 0.1 μM FAD and 1 mM glutathione (conditions to which native enzyme is stable), so the inactivation is irreversible. Under anaerobic conditions, incubation of alcohol oxidase with propargyl alcohol for up to 3h caused no loss of activity, indicating the inactivation is linked to enzymatic catalysis. However, the time lag before the maximum rate of inactivation is reached suggests the loss of



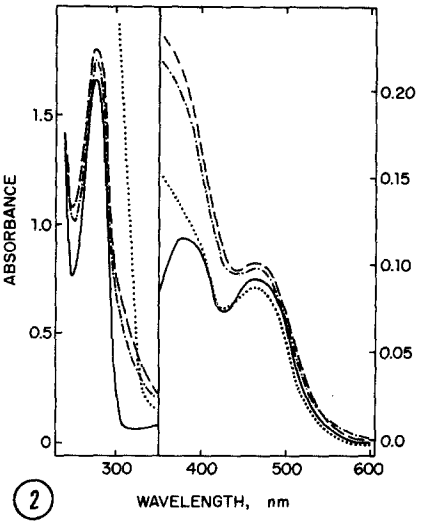


Figure 1. Inactivation of 0.5 μM alcohol oxidase in 50 mM sodium phosphate at pH 7.5 and 23°C with various concentrations of propargyl alcohol: (●), 1.8 mM; (■), 4.3 mM; (▲), 8.2 mM; and (□), 16.4 mM. Inactivation under identical conditions with 4.3 mM propargyl alcohol in the presence of 25 mM glutathione is shown by (▲). Rates were measured in 50 mM phosphate buffer, pH 7.5 and 23°C, containing 50 mM methanol with a Clark-type oxygen electrode.

Figure 2. Absorption spectra of alcohol oxidase in 50 mM phosphate buffer at pH 7.5: (——), native enzyme; (·····), enzyme inactivated with propargyl alcohol (the offscale peak has λ_{max} = 290 and A_{max} = 3.4); (- - -), enzyme inactivated with 1,4-butynediol; (—·—·), enzyme inactivated with 4-hydroxy-2-butynal. After inactivation to 5% residual activity, the enzyme samples were dialyzed, centrifuged, and adjusted to an enzyme concentration of 0.75 mg/ml.

activity is not a suicide inactivation but arises from reaction of the enzyme with the product propynal after it has been released from the enzyme and has accumulated in solution. As anticipated, the presence of glutathione, which should trap propynal released into solution via a Michael addition (5), almost completely prevented the inactivation. The near identity of the maximum rates of inactivation at propargyl alcohol (and thus eventually at propynal) concentrations above 3 mM indicates saturation kinetics for the inactivation. Since increasing concentrations of propargyl alcohol extend the time lag before the maximum rate of inactivation is reached, the substrate protects the enzyme from inactivation by the product, which implies the inactivation occurs at the active site of the enzyme. Inactivation resulted in a large increase in the absorbance of the enzyme in the 275-325 nm region, but the 450 nm absorption

characteristic of the bound FAD coenzyme was not significantly changed (Fig. 2). Significant absorption changes in the 400-500 nm region due to covalent attachment of the inactivator to the flavin coenzyme occur in the irreversible inactivation of hydroxy acid oxidases with the acetylenic suicide substrate 2-hydroxy-3-butynoate (5,6,). The spectral changes caused by irreversible inactivation of alcohol oxidase are more similar to those seen in the inactivations of flavoenzymes in which amino acid residues on the protein are alkylated by electrophilic products produced by the enzyme (5,11). Thus propynal acts as an active-site-directed affinity label which alkylates an amino acid residue in alcohol oxidase.

1,4-Butynediol is also a substrate (K_m = 36 mM, V_{max} = 1.9 μmol/min/mg) and an inactivator of alcohol oxidase which is similar in most respects to propargyl alcohol. Thus, inactivation by 1,4-butynediol is irreversible, has a time lag before the maximum rate of inactivation is reached (Fig. 3), does not occur under anaerobic conditions, is prevented by the presence of glutathione, and shows saturation kinetics with respect to increasing concentrations of 1,4-butynediol. The absorption spectrum of the inactivated enzyme shows the 450 nm region due to the FAD cofactor is not significantly altered, but there is increased absorbance in the 300-400 nm region (Fig. 2). The spectrum of alcohol oxidase inactivated with 1,4-butynediol is different from that which results from inactivation with propargyl alcohol. Inactivation is probably due to the enzymatic production of 4-hydroxy-2-butynal, a potent electrophile which could function as an affinity label for the enzyme. 4-Hydroxy-2-butynal was found to be a substrate (K_m = 0.44 mM and V_{max} = 0.25 μmol/min/mg) and an inactivator of the enzyme (Fig. 3). Although low concentrations of the aldehyde caused only partial loss of activity, complete loss of activity in a pseudo-first-order process was found with higher concentrations. The inactivation was irreversible and showed saturation kinetics with a K_I of 0.42 mM for the aldehyde. The close correspondence of the K_m for 4-hydroxy-2-butynal as a substrate and the K_I as an irreversible inactivator implies the inactivation

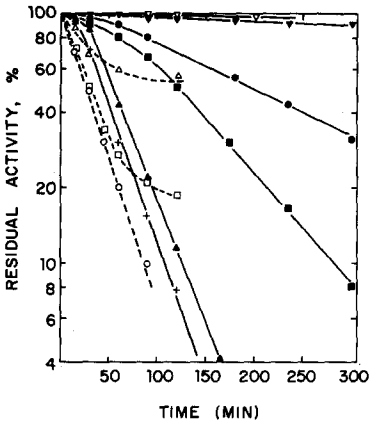
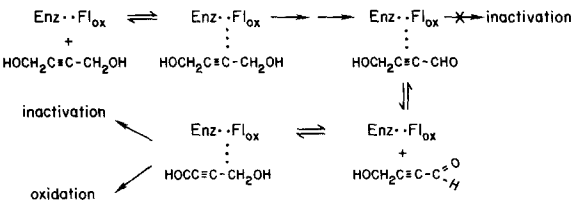


Figure 3. Inactivation of 0.4 μM alcohol oxidase in 50 mM sodium phosphate at pH 7.5 and 23°C by acetylenic inactivators. Closed figures: 1,4-butynediol: (●), 0.22 mM; (■), 0.83 mM; (▲), 1.67 mM; and (+), 3.3 mM. Inactivation by 1.67 mM 1,4-butynediol in the presence of 20 mM glutathione: (▼). Open figures; 4-hydroxy-2-butynal: (○), 0.23 mM; (□), 0.15 mM; (△), 0.07 mM. Inactivation by 0.23 mM 4-hydroxy-2-butynal which had been preincubated 10 min with 10 mM glutathione: (▽).

occurs at the active site with the aldehyde bound in the same manner for oxidation and for inactivation. There was no time lag for this inactivator and the rate of inactivation under anaerobic conditions was within a factor of 2 of the aerobic rate of inactivation. Thus, the inactivation of alcohol oxidase by 4-hydroxy-2-butynal occurs without further oxidation of the inactivator. The absorbance spectrum of alcohol oxidase inactivated with 4-hydroxy-2-butynal is essentially identical to that found on inactivation by 1,4-butynediol, which is strong circumstantial evidence that an identical inactivated enzyme is produced in both cases. From all the evidence, the unique mechanism for the inactivation of alcohol oxidase by 1,4-butynediol shown in scheme I is proposed. The key feature is that initial oxidation of the diol gives 4-hydroxy-2-butynal, which does not inactivate the enzyme but dissociates into solution. It then returns to bind to the enzyme in the opposite orientation, e.g. with the alcohol moiety in the position at the active site where oxidation can occur. Thus 4-hydroxy-2-butynal functions as an affinity label based on the substrate alcohol rather than on the product aldehyde.

The inactivation of alcohol oxidase by acetylenic substrates occurs by



SCHEME I

addition of electrophilic products of oxidation to amino acid residues of the enzyme. In contrast to all reported cases of inactivation of α-hydroxy acid oxidases with 2-hydroxy-4-butynoate, no addition of the inactivator to the FAD coenzyme is observed. These results support the contention that the mechanism of oxidation of alcohols by alcohol oxidase differs from that for the oxidation of α-hydroxy acid oxidases by flavin-dependent hydroxy acid oxidases, although they give no indication as to the mechanism of the alcohol oxidase.

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