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IRREVERSIBLE INACTIVATION OF THE FLAVOENZYME ALCOHOL OXIDASE BY CYCLOPROPANONE

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SUMMARY: Incubation of the flavoenzyme alcohol oxidase from Candida boidinii with cyclopropanone hydrate in phosphate buffer at pH 7.5 and 20°C leads to a time dependent inactivation of the enzyme which cannot be reversed by removal of the cyclopropanone hydrate. The rate of inactivation is reduced in the presence of substrate, product, or glutathione, and the inactivation is prevented completely by reduction of the FAD coenzyme prior to addition of the inactivator. Inactivation of the enzyme is accompanied by a change in the absorption spectrum of the enzyme which indicates loss of the oxidized flavin chromophore. Denaturation of the inactivated enzyme under a variety of conditions does not cause reoxidation of the flavin which therefore must be covalently modified by cyclopropanone hydrate. A mechanism for the inactivation is proposed which involves radical intermediates and which suggests a similarity in reaction mechanism between alcohol oxidase and flavoprotein amine oxidases.

When grown with methanol as the sole source of carbon, the yeast Candida boidinii produces an alcohol oxidase (EC 1.1.3.13) which has flavin adenine dinucleotide (FAD) as the only cofactor (1). This enzyme oxidizes small primary alcohols to aldehydes while reducing O_2 to H_2O. It has been suggested that the mechanism of flavin-dependent alcohol oxidases should involve radical intermediates (or transition states) rather than the carbanion intermediates established for flavin-dependent α-hydroxy acid oxidases, which catalyze a formally similar reaction (2). Preliminary evidence for differences in mechanism between alcohol oxidase and α-hydroxy acid oxidases has been obtained (3), and support for a radical-like mechanism for the enzyme has been sought in the reactions of

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cyclopropyl compounds with the enzyme. Like the enzyme from Hanenula polymorpha (4), alcohol oxidase is irreversibly inactivated by reaction with cyclopropanol (5). It has now been found that cyclopropanone is also a suicide substrate for alcohol oxidase.

METHODS: Alcohol oxidase was purified to homogeneity from methanol grown C. boidinii by published procedures (1,6). Gel electrophoresis indicated a contamination of less than 3% based on stain density of the major and two minor bands. The activity of the enzyme was measured with a model 5331 oxygen electrode (Yellow Springs Instrument Co.) in a 1 ml volume of 50 mM sodium phosphate at pH 7.5 and 20°C which also contained 50 mM methanol. Cyclopropanone hydrate was prepared by hydrolysis of cyclopropanone ethyl hemiketal (7), which was prepared from ethyl 3-chloropropionate (8).

RESULTS: Incubation of alcohol oxidase (6 μM by FAD content) with 50 μM cyclopropanone hydrate in 50 mM sodium phosphate at pH 7.5 and 20°C leads to a pseudo-first-order loss of enzymatic activity which has a $t_{1/2}$ of 39 min. Other kinetic studies indicated that the inactivation exhibited saturation kinetics with an apparent K_I of 2.1 mM and a V_{Max}^{Inact} of 0.12 min^{-1} in air saturated buffer. The inactivation proceeds essentially to completion and cannot be reversed by 1000-fold dilution of the inactivated enzyme, by gel filtration through Sephadex G-25, or by dialysis against phosphate buffer at 4°C for 16 hr. Native alcohol oxidase is stable to all these procedures. The addition of 5 mM methanol, glutathione, or β-mercaptoacetic acid to the dialysis buffer had no effect on the recovery of activity. Preincubation of 50 μM cyclopropanone hydrate with 10 mM glutathione before addition of the enzyme caused a 40% decrease in the rate of inactivation. The presence of 12 mM methanol in the inactivation decreased the rate of inactivation 2.2-fold, and 20 mM acetaldehyde decreased it 2.8-fold. The cyclopropyl ring of cyclopropanone seems to be essential for inactivation, since neither acetone nor formaldehyde (which, like cyclopropanone, will be primarily hydrate) at 5 mM caused any inactivation of the enzyme after 3 hr. In addition, heating cyclopropanone hydrate to 90°C for 1 hr to convert it to propionic acid (9) destroys its ability to inactivate alcohol oxidase.

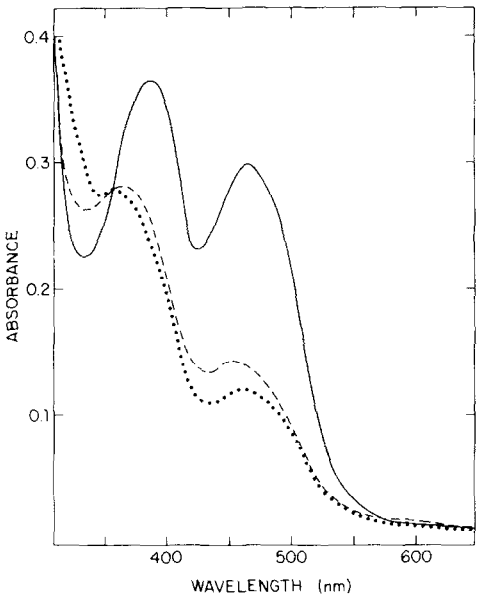


Figure 1. Visible absorption spectra of alcohol oxidase; solid line; native enzyme (2.5 mg/ml in sodium phosphate at pH 7.5); dashed line, enzyme reduced with methanol under anaerobic conditions; dotted line, enzyme inactivated with cyclopropanone hydrate (2% residual activity).

If a solution of alcohol oxidase is made anaerobic by flushing with N_2 (10), and the enzyme is reduced by a small molar excess of methanol or of dithionite, treatment of the enzyme solution with 0.2 mM cyclopropanone hydrate for 3 hr causes no loss of activity. If the soltuion is then made aerobic by stirring in air, inactivation begins and is complete in about 10 min. Inactivation is paralleled by a change in the visible absorption spectrum of the FAD coenzyme (Figure 1) to one similar to the spectrum of the reduced enzyme (11). The difference spectrum between native and cyclopropanone hydrate-inactivated enzymes (Figure 2) clearly shows the loss of the oxidized flavin chromophore. Denaturation of the modified enzyme with ethanol, trichloroacetic acid, urea, or guanidinium hydrochloride in the presence of oxygen followed by removal of insoluble protein does not lead to return of the modified flavins to the oxidized form. The resulting difference spectrum is stable for 6 hr in all cases, so modification of the oxidized flavin is not reversed by O_2 or even by O_2 in acid.

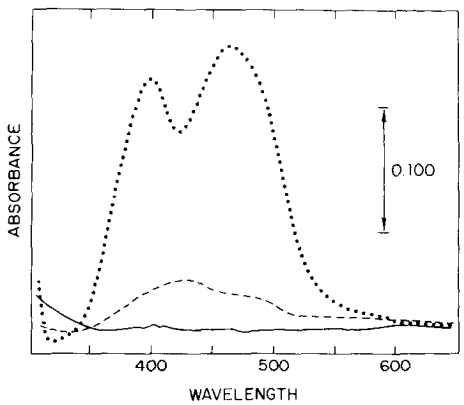
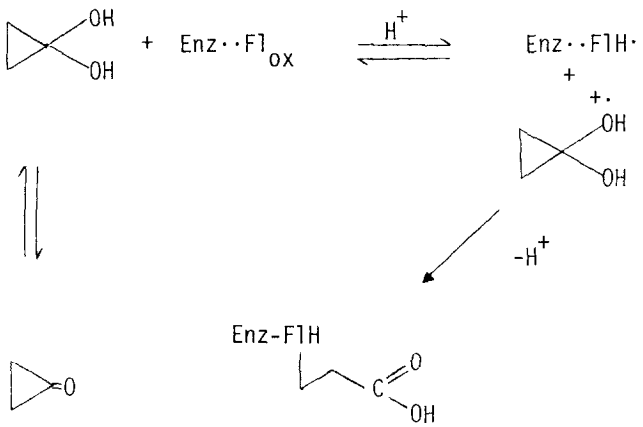


Figure 2. Difference absorption spectra of alcohol oxidase. The solid line is native enzyme (2.5 mg/ml) in both sample and reference cuvette, the dotted line is native enzyme in sample cuvette and cyclopropanone hydrate-inactivated enzyme in the reference cuvette, and the dashed line is methanol reduced enzyme in the sample cuvette and cyclopropanone hydrate-inactivated enzyme in the reference cuvette.

DISCUSSION: Cyclopropanone (as the hydrate in aqueous solution) has been reported to be a potent but reversible inhibitor of several enzymes, including especially aldehyde dehydrogenase, which have catalytically essential sulfhydryl residues (7,12). The inhibition was found to result from formation of an unusually stable thiohemiacetal between cyclopropanone and a sulfhydryl group on the enzyme. Essentially complete recovery of activity resulted from dilution of the inhibited enzyme. In contrast, cyclopropanone hydrate irreversibly inactivates alcohol oxidase by a process which has the characteristics of a suicide inactivation (13). The inactivation is pseudo-first order, shows saturation kinetic behavior with increasing substrate concentration, and exhibits substrate protection. The inactivation occurs only when the enzyme is in the oxidized form and, surprisingly, causes an apparent reduction of the flavin chromophore. Oxidized flavin is not recovered on denaturation of the inactivated enzyme or even on acid treatment. This evidence strongly suggests that the FAD coenzyme has suffered a stable, covalent modification by the hydrate rather than just being sequestered



Scheme I: Possible Mechanism for Inactivation of Alcohol Oxidase by Cyclopropanone

from oxygen by reaction of the hydrate with the apoprotein. The structure of the flavin-cyclopropanone hydrate adduct has not yet been determined. Clearly it must be known before the mechanism of inactivation can be established.

Silverman and Hoffman have recently reported that N-(1-methyl) cyclopropylbenzylamine is a mechanism-based irreversible inactivator of the flavoprotein monoamine oxidase (14). Their results are similar to the present work in that they also observed inactivation of a flavoenzyme with a cyclopropyl substrate which cannot undergo normal oxidation by loss of a hydrogen and 2 electrons. They rationalized their results in terms of an amine cation radical. A similar mechanism is proposed to explain the inactivation of alcohol oxidase by cyclopropanone hydrate (Scheme I). For alcohol oxidase, a covalent modification of the coenzyme occurs, and the most chemically reasonably mechanism involves ring opening of the hydrate, which is especially susceptible to ring opening reactions via radical pathways (15, 16). Electron transfer from cyclopropanone hydrate to flavin to give flavin semiquinone and a β-propionate radical, which could couple to give a stable modified flavin, seems more likely than collapse of the initial radicals to give the cyclopropanone hydrate attached to the flavin through an oxygen atom, for this latter adduct would be expected to break up after denaturation of the enzyme in aerobic,

acid solution to give oxidized flavin. Regardless of the exact mechanism of inactivation or of the structure of the modified flavin, it is clear that cyclopropanone can inactivate oxidase-type enzymes in ways more complex than formation of thiohemiketals with essential sulfhydryl groups.

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