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STUDIES WITH THE FLAVIN DEPENDENT ALCOHOL OXIDASE FROM YEAST: PROPERTIES AND CATALYTIC MECHANISM

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Introduction

Alcohol oxidase (E.C. 1.1.3.13) from Candida boidinii (octamer, M_r subunit 74000, 1 FAD/subunit) catalyzes the oxidation of primary alcohols: $RCH_2OH + O_2 \rightarrow RCHO + H_2O_2$. The native enzyme stabilizes a FAD radical, $Fl^{\cdot -}$, even in the presence of oxygen and substrate (1,2). The amount of $Fl^{\cdot -}$ (approx. 30% of FAD of our enzyme) is increased 2.5 fold by hydroxylamine with concomitant quantitative inactivation of the enzyme (1). $Fl^{\cdot -}$ is considered to be catalytically inactive. Otherwise little is known on the catalytic mechanism: Cyclopropanol (2) and cyclopropanone (3,4) irreversibly inactivate the enzyme probably via radical pathways. In contrast to several other flavin oxidases (5) α-alkynoic substrates are not suicide inhibitors of alcohol oxidase: Upon turnover of propargyl alcohol inactivation is observed but is claimed to be due to reaction of the accumulating propynal with an active side residue (6).

Results and Discussion

The catalytic activity of alcohol oxidase is increased 1.7 fold in the presence of the redox dye radical $ABTS^{\cdot +}$ (fig. 1) which is produced via the peroxidase (POD) catalyzed oxidation of 2,2'-azino-bis-(3-ethylbenzthiazoline-sulfonic acid-6) ($ABTS_{red}$) by H_2O_2 (7). $ABTS^{\cdot +}$ might react with $Fl^{\cdot -}$ to give $ABTS_{red}$ and Fl_{ox}. The electron spectrum of the activated enzyme also indicates the loss of $Fl^{\cdot -}$ upon $ABTS^{\cdot +}$-treatment (fig. 1). This sup-

ports the view of $Fl^{\cdot -}$ being catalytically inactive in alcohol oxidase. According to steady-state kinetic experiments (data not shown) the catalytic mechanism includes a ternary complex of alcohol oxidase with alcohol and oxygen. The K_M-values are 1.4 mM (8mM) for methanol ($[^2H_3]$-methanol) and 0.6 mM for oxygen. Steady-state and transient kinetics are accounted for by an overall kinetic scheme (scheme 1) as proposed for lactate oxidase (8). It is assumed that $k_{-2} \ll k_2$. As K_D (i.e. k_{-1}/k_1) is not very different from K_M(ROH) $k_2 \ll k_{-1}$ is implied (eq. III). The values for k_5 are calculated from eq. I. The observed rate constant k_{ox} for $E\text{-}Fl_{red} + O_2 \rightarrow E\text{-}Fl_{ox} + H_2O_2$ probably corresponds to k_6: Enzyme monitored turnover indicates a rate of approx. $7 \times 10^6\ M^{-1}min^{-1}$ for k_4(9), which governs the re-oxidation rate in catalysis (ternary complex mechanism).

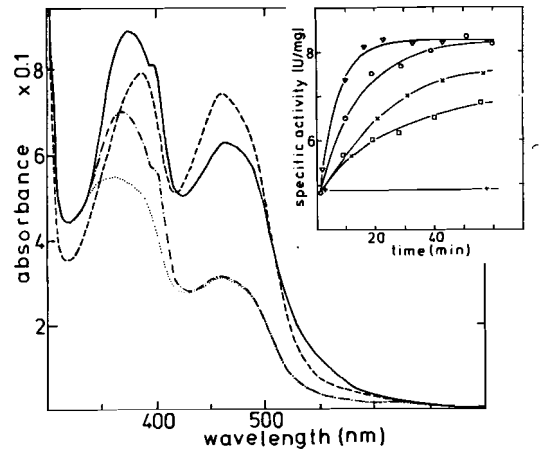
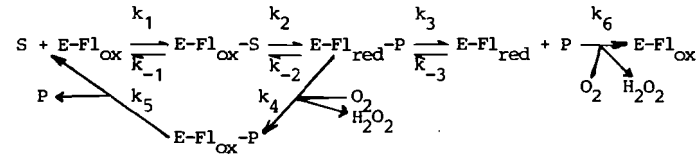


Fig. 1: Electron spectra of native and activated alcohol oxidase enzyme, 90µM, incubated for 19 h, 0°C, with $ABTS^{\cdot +}$, 2mM. After incubation the dye was removed by Sephadex G-25 gel filtration, 0.1M phosphate, pH 7.5. Activity (1 U = 1 µmole O_2/min) was assayed with an oxygen electrode, 0.1M methanol, air-saturated buffer, 25°C ($O_2 \sim 0.25$mM).
—— native enzyme, 90µM (4.9 U/mg); -·- native enzyme reduced with 0.1M methanol; --- enzyme after incubation and gel filtration (6.8 U/mg); ··· activated enzyme with 0.1M methanol.
Insert: Activation of alcohol oxidase by $ABTS^{\cdot +}$: 5µM enzyme, 1mM $ABTS_{red}$, 25µg POD in 1 ml; prior to the addition of enzyme $ABTS^{\cdot +}$ was produced with H_2O_2 (1 H_2O_2 yields 2 $ABTS^{\cdot +}$). (■ 1.1µM H_2O_2; (x) 2.1µM ; (o) 5.3µM; (▽) 21µM; (+) no H_2O_2 added.



$$v_{Max} = \frac{k_2 \times k_5}{k_2 + k_5} \quad \text{(eq. I)}$$

$$K_M(O_2) = \frac{k_5(k_2 + k_{-2})}{k_4(k_2 + k_5)} \quad \text{(eq. II)}$$

$$K_M(ROH) = \frac{k_5(k_{-1} + k_2)}{k_1(k_2 + k_5)} \quad \text{(eq. III)}$$

k_{-1}/k_1 = 2 (8) mM
v_{max} = 4670 (3100) min^{-1}
k_5 = 12400 (11100) min^{-1}
k_2 = 7500 (4300) min^{-1}
k_{ox} = $3.6 \times 10^6\ M^{-1}min^{-1}$
k_4 ~ $7 \times 10^6\ M^{-1}min^{-1}$

Scheme 1: Kinetic model of the turnover of methanol and $[^2H_3]$-methanol by alcohol oxidase (values in parentheses refer to deuterated methanol)

The enzyme is irreversibly inactivated by H_2O_2 in a process exhibiting saturation kinetics with K_I = 1.6 mM and k_{inact} = 0.55 min^{-1} (9). During methanol turnover the rate of inactivation increases as H_2O_2 accumulates. Methanol delays the inactivation as shown by decreasing rates of inactivation with increasing concentrations of methanol (fig. 2A). Even when H_2O_2 is eliminated by catalase prolonged alcohol turnover slowly inactivates the enzyme in a process showing (pseudo)first order saturation kinetics with all alcohols used (fig. 2B). With methanol k_{inact} = 0.005 min^{-1}, "K_I" = 0.6 mM, which is identical to K_{app} of methanol turnover under the same conditions. The characteristic ratio k_{cat}/k_{inact} varies for different alcohols but is always greater than 10^5:

$\frac{k_{cat}}{k_{inact}} \times 10^5$	3.00	2.80	2.66	2.18	2.11	1.70	1.57	1.43
R- (ROH)	CH_3-	C_2H_5-	C_3H_7-	C_4H_9-	CH_2=$\Delta$$CH_2$-	$C_6H_5CH_2$-	$(CH_3)_2CH$-	HC≡CCH_2-

In the case of other flavin oxidases substrates with the methylenecyclopropyl or the acetylene function often act as efficient suicide substrates with $k_{cat}/k_{inact} < 100$ (5). With propargyl alcohol an inactivation mecha-

nism via free propynal (6) is excluded by the (pseudo)first order kinetics of inactivation when catalase is present.

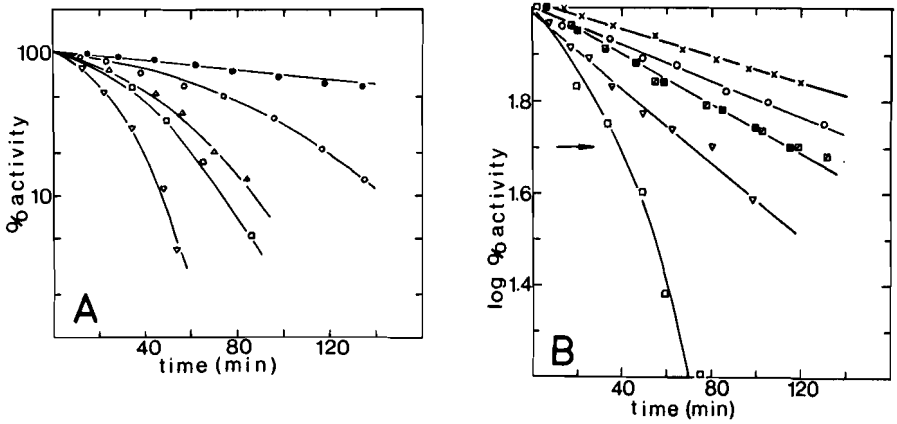


Fig. 2A: Inactivation by turnover of methanol
33nM enzyme and (●) 50mM methanol, 1.1 U/ml catalase; (o) 50mM methanol ; (Δ) 10mM methanol; (□) 5mM methanol; (▽) 1mM methanol.
Fig. 2B: Inactivation by turnover of various alcohols with catalase present 17nM enzyme, 1.1 U/ml catalase, except (x) with 67nM enzyme; substrates are 0.1M (x) isopropanol, (o) butanol, (▨) methylenecyclopropyl alcohol, (■) methylenecyclopropyl alcohol, 1mM semicarbazide, (□) methylenecyclopropyl alcohol without catalase, (▽) propargyl alcohol.

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