Flavin-dependent alcohol oxidase from yeast

Studies on the catalytic mechanism and inactivation during turnover

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The kinetic course of the reaction of methanol and deutero-methanol with FAD-dependent alcohol oxidase was investigated under single-turnover conditions [$k_{red} \approx 15000\ min^{-1}$ (^1H_3COH) and $\approx 4300\ min^{-1}$ (^2H_3COH)] and multiple-turnover conditions [$TN_{max} \approx 6000\ min^{-1}$ (^1H_3COH) and $\approx 3100\ min^{-1}$ (^2H_3COH)]. A kinetic scheme for the overall catalytic mechanism is proposed, which is characterized by (1) formation of a Michaelis complex between enzyme and substrate, (2) the reductive step involving partly rate-limiting scission of the substrate C-H bond, (3) reaction of the complex of reduced enzyme and aldehyde with dioxygen, and (4) a significant contribution of the dissociation rate of product from its complex with reoxidized enzyme to the overall rate.

Prolonged turnover of various alcohols, including methanol, results in progressive inactivation of the enzyme by two processes. In the absence of catalase the inactivation rate increases with time due to accumulation of hydrogen peroxide, which is a potent inactivator ($K_d \approx 1.6$ mM; $k_{inact} \approx 0.55\ min^{-1}$). In the presence of catalase inactivation during turnover is much slower, the process showing pseudo-first-order kinetics ($K_{inact} \approx 0.6$ mM; $k_{inact} \approx 0.005\ min^{-1}$ with methanol). The ratio k_{cat}/k_{inact} varies with different alcohols but is always $> 10^5$. Propargyl alcohol and methylenecyclopropyl alcohol cannot be considered as suicide substrates, as compared to analogous substrates of other flavin oxidases.

Alcohol oxidases (EC 1.1.3.13) from yeasts catalyze the oxidation of lower primary alcohols:

$$R\text{-}CH_2OH + O_2 \rightarrow R\text{-}CHO + H_2O_2.$$

The enzyme (subunit mass 70 – 80 kDa) are in general octameric with 1 FAD molecule/subunit as the only cofactor known [1]. Varying amounts of the anionic flavin radical $Fl^{\cdot-}$ are stabilized in these enzymes even in the presence of substrate and oxygen [2, 3]. Compared with the substrates of other flavin oxidases, such as the amino acid oxidases [4] or lactate oxidase [5], the C-H bond of the alcohol to be oxidized is much less polarized by the adjacent functional group. With the former enzymes substrate oxidation is initiated by formation of the α-carbanion, resulting from abstraction of H^+ from the C-H bond. Subsequently a transient covalent adduct is formed between the carbanion and N_5 of Fl_{ox} and this breaks down to yield E-Fl_{red} and the product (covalent catalysis; cf. [6]). This mechanism is difficult to envisage in the case of oxidation of alcoholic C-H bonds, since, due to the lack of stabilization of the negative charge, the alcohol carbanion would be an unlikely state from the standpoint of energetics [6, 7]. Thus, a mechanism for the flavin-catalyzed alcohol oxidation was proposed, which involves the transfer of two $1e^-$ entities from the substrate to Fl_{ox} without prior carbanion formation (radical mechanism) [7, 8].

Inactivation of alcohol oxidase by the suicide substrates cyclopropanone hydrate [9, 10] and cyclopropanol [3, 11] also indicates radical pathways. In either case inactivation was suggested to occur by formation of stable covalent adducts between a transient substrate/flavin radical pair. With propargyl alcohol, progressively increasing rates of inactivation during turnover have been reported [12]. This has been claimed to be due to the attack of the accumulating product propynal on an active-site residue of the enzyme and, hence, cannot be considered as a suicide inactivation. In contrast, with several other flavin-dependent oxidases, substrates with an acetylene function, or a methylenecyclopropyl function, act as efficient suicide substrates [13]. The actual inactivating species in these cases is probably the respective substrate α-carbanion which is unlikely to be formed in the reactions catalyzed by alcohol oxidase, and a suicide inactivation is therefore not to be expected.

In this study, the catalytic mechanism of alcohol oxidase from the yeast *Candida boidinii* is investigated by studying the reaction with methanol and deutero-methanol under single- and multiple-turnover conditions and an overall kinetic scheme is proposed. Furthermore, inactivation of the enzyme by turnover of various alcohols is investigated and discussed with respect to the potential underlying mechanism.

MATERIALS AND METHODS

Materials

Alcohol oxidase from *Candida boidinii* was isolated in collaboration with H. Schütte (Gesellschaft für Biotechnologische Forschung m.b.H., Stöckheim, FRG) es-

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Abbreviations. ABTS, 2,2′-azino-bis(3-ethyl-benzthiazolinsulfonic acid-6); EPR, electron paramagnetic resonance; Fl_{ox} (Fl_{red}), oxidized (reduced) flavin; $Fl^{\cdot-}$, anionic flavin radical; E-Fl_{ox} (E-Fl_{red}), oxidized (reduced) form of flavoenzyme; E-Fl_{red} · P, complex of reduced flavoenzyme and product; GSH, reduced glutathione; TN, turnover number; U, unit of activity of alcohol oxidase (1 μmol O_2 consumed × min^{-1}); SDS, sodium dodecyl sulfate.

Enzymes. Alcohol oxidase (EC 1.1.3.13); catalase (EC 1.11.1.6).

sentially as described [1]. A typical preparation had a specific activity of 4.6 ± 0.5 U/mg protein (from three different preparations). Liver catalase (EC 1.11.1.6) was from Boehringer (Mannheim, FRG), $^{2}H_3COH$ was from Merck (Darmstadt, FRG). Methylenecyclopropyl alcohol, C_5H_8O, was synthesized according to [14] and contained 4% butadiene-1-ol and 1% spiro-2,2-pentyl-carbinol, as determined by gas chromatography and NMR [15]. Propargyl alcohol was vacuum-distilled prior to use. Pure gases (nitrogen and oxygen) and nitrogen/oxygen mixtures were from Linde (Spaichingen, FRG). All other chemicals were from the best available commercial sources and used without further purification.

Methods

All experiments were performed at 25°C in 0.1 M phosphate buffer, pH 7.5. Concentrations of alcohol oxidase refer to the FAD concentration of the enzyme solutions. Standard activity tests were performed with 0.1 M methanol in air-saturated buffer (0.25 mM oxygen) using an oxygen electrode (Yellow Springs Instruments Co., model 53, Yellow Springs, Ohio, USA). Oxygen concentrations in the assay solutions were varied by flushing the test buffer with nitrogen or oxygen. Enzyme activities are also given as turnover numbers, defined as either mol O_2 consumed (mol total FAD)$^{-1}$ × min^{-1} (TN*) or as mol O_2 consumed (mol active FAD)$^{-1}$ min^{-1} (TN). This distinction was made because in alcohol oxidase only part (30 – 35%) of the FAD is catalytically active. The fraction of active FAD is calculated from the change of absorbance at 460 nm upon reduction of the enzyme with 0.1 M methanol using ε_{460} of $Fl_{ox} \approx 10000$ M^{-1} cm^{-1} and assuming negligible absorbance of Fl_{red} at this wavelength [16]. Optical absorbance spectra were recorded with a Cary 118 spectrophotometer. For anaerobic experiments Thunberg-type cells were used. Rapid kinetic measurements were carried out with a stopped-flow spectrophotometer as described elsewhere [17]. To generate reduced enzyme in order to investigate its reoxidation by oxygen the enzyme was stored for two days under the exclusion of air at room temperature. This procedure is known to cause autoreduction of the enzyme [2]; $t_{1/2}$ of reduction was 4 h.

Anaerobiosis of enzyme and substrate solutions was achieved by flushing the solutions at 0°C for 1 min with pure nitrogen followed by 5 s of evacuation, this procedure being repeated 10 times.

RESULTS

Properties of alcohol oxidase and the reaction with alcohols

From the optical absorbance spectrum of the enzyme as isolated (Fig. 1, spectrum A) the ratio A_{370}/A_{460} is determined as 1.5. This value differs significantly from ratios determined for other flavin oxidases ($A_{370}/A_{460} \approx 1$). Denaturation of the enzyme with trichloroacetic acid, subsequent neutralization and removal of the protein by centrifugation liberates oxidized FAD and leads to a substantial increase of the absorbance in the region 420 – 460 nm (Fig. 1, spectrum E). This suggests that our preparations also contain flavin in a redox state other than Fl_{ox}. In fact a shoulder at 395 nm indicates the presence of anionic flavin radical ($Fl^{\cdot -}$), which is quantified by EPR spectroscopy and makes up approximately 30 – 35% of the flavin determined by the trichloroacetic acid procedure [2].

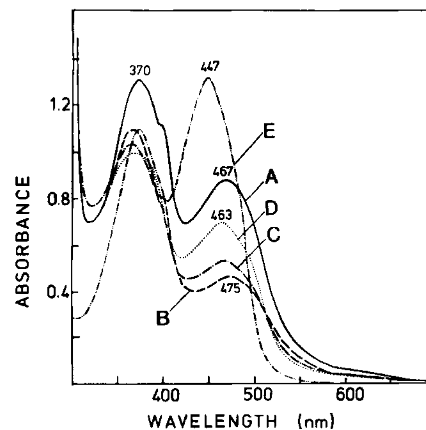


Fig. 1. *Absorption spectra of alcohol oxidase under various conditions.* The enzyme was 0.12 mM in 0.1 M phosphate buffer, pH 7.5, 25°C. (A) Native enzyme; (B) 5 min after addition of 0.1 M methanol; (C) 60 min after addition of methanol; (D) 140 min after addition of methanol; (E) after aerobic denaturation with 2.5% trichloroacetic acid, neutralisation and centrifugation, spectrum corrected for dilution. During the incubation with methanol the enzyme solution was gently stirred at 25°C to enhance the oxygen uptake of the system

The spectra obtained upon addition of methanol (Fig. 1, spectrum B) show that there is only partial bleaching of the absorbance at 460 nm (approximately 50%). Hence, part of the cofactor does not react with methanol and is catalytically inactive. The fraction of the reducible Fl_{ox}, i.e. catalytically active FAD, was estimated as about 35% of the total FAD content (cf. Materials and Methods). The fraction of flavin present as stable radical ($Fl^{\cdot -}$, 30 – 35%) is not reduced by substrate [2, 3]. The structure and oxidation state of the residual catalytically noncompetent flavin (30 – 35%) are not known with certainty. A clue to its possible structure is the considerable absorbance both in the 370-nm and the 460-nm region which must be accounted for after subtraction of the estimated contributions of catalytically active Fl_{ox} and of $Fl^{\cdot -}$ to spectrum A (Fig. 1) and of Fl_{red} and of $Fl^{\cdot -}$ to spectrum B. A simple explanation would be the presence of Fl_{ox} bound to a modified active site being not reducible by the substrate. The spectra of the enzyme samples shown in Fig. 1 (spectra A – D) can be interpreted quantitatively assuming the presence of about 30% of catalytically inactive cofactor in addition to about 35% of functional Fl_{ox} and about 35% of $Fl^{\cdot -}$. The specific activity of approximately 4.5 U/mg determined for three different preparations varied within a range of ±10%. The activity of the enzyme could be increased up to 1.7-fold by reaction with the redox dye ABTS (Geissler, J. and Kroneck, P. M. H., unpublished results). This increase was correlated with the formation of oxidized flavin and a decrease in $Fl^{\cdot -}$ content as measured by EPR. Upon prolonged turnover in the absence of catalase, a gradual increase of the absorbance in the 465-nm region is observed (spectra C, D), which cannot be reversed by additional substrate; i.e. part of the enzyme is irreversibly converted to a form with a higher absorption coefficient in the 465-nm region than Fl_{red}, possibly non-functional Fl_{ox}. In agreement with this assumption, the enzymatic activity decreases to 80% (spectrum C) and 25% (spectrum D) of the original value. It is possible that the inactive flavin components contribute to some of the slow, catalytically nonrelevant reactions described later in the stopped-flow experiments. In the absence of oxygen (not shown) no increase in absorbance is observed after 24 h of

incubation with 0.1 M methanol; the enzyme remains in the reduced state (corresponding to spectrum B) and the enzymatic activity remains constant.

The same absorption spectrum (curve B), indicative of reduced enzyme, is also obtained and persists for weeks, when the enzyme solution is made anaerobic in the absence of added substrate [2]. The reduction process in this case is slow ($t_{1/2} \approx 4$ h at room temperature). This peculiar reaction is not understood at present; it might be related to the presence of tightly bound H_2O_2 as suggested earlier [2]. The process does not require light and can be repeated in cycles. It represents a convenient method for the preparation of reduced enzyme. Anaerobic reduction by dithionite proved inadequate since loss of up to 50% of the total activity of alcohol oxidase was observed.

Steady-state turnover experiments with methanol and deutero-methanol

Steady-state kinetic parameters were determined for the oxidation of ^1H_3COH and ^2H_3COH with oxygen catalyzed by alcohol oxidase (Fig. 2A and B; Table 1). Upon variation of alcohol and oxygen concentrations a pattern of converging lines in the Lineweaver-Burk plot results with both alcohols, as shown in Fig. 2A for ^1H_3COH. This finding indicates the formation of a ternary complex during catalysis [18], i.e. oxygen reacts with a binary complex which, in analogy to other flavin oxidases, is reasonably assumed to be E-Fl_{red}-$CH_2{=}O$. The K_m and TN_{max} values (Table 1) for the two alcohols and oxygen are obtained from the secondary plots of data such as those of Fig. 2A (shown in Fig. 2B for ^2H_3COH).

A similar converging line pattern was reported with alcohol oxidase from *Hansenula* [19]. K_m values for methanol and oxygen at saturating oxygen and methanol concentrations of the *Candida* enzyme were 2.6 mM and 0.8 mM, compared to 1.4 mM and 0.4 mM of the *Hansenula* enzyme. The $K_m(O_2)$ values of both enzymes thus are far above physiological oxygen concentrations.

The similarity of the kinetic parameters and of the line pattern in the Lineweaver-Burk plot indicate a very similar catalytic mechanism for the alcohol oxidases from the two yeasts. On the other hand, the specific activity of the *Hansenula* enzyme is approximately 10 times higher (50 U/mg protein) at 0.25 mM oxygen [19] compared to that of the *Candida* enzyme. A direct comparison of the activities of the two enzymes is not very meaningful since the values depend on the fraction of active enzyme which is not known in the case of the *Hansenula* enzyme used in the cited study.

The magnitude of the isotope effect $^1H/^2H$ on turnover is dependent on the alcohol concentration, and K_m(R-OH) is affected by isotopic substitution [20]. At saturating (deutero)methanol concentrations (0.1 M) the limiting $^1H/^2H$ value on turnover is 1.9 (Table 1). Thus, it is apparent that the reduction of the enzyme by the substrate is at least partially rate-limiting under steady-state turnover conditions.

Substrate turnover was also monitored by the method of Gibson [21] (enzyme concentrations ≈ 0.01 mM) according to which absorbance changes are registered upon mixing the enzyme with alcohol and oxygen in a stopped-flow spectrometer (Fig. 3). The TN_{max} values obtained by systematic variation of methanol and deutero-methanol concentrations were essentially the same as those obtained by monitoring the oxygen concentration, although the scattering of the data points was larger due to uncertainties in the evaluation of the curves at the end of the reaction (Fig. 3). The

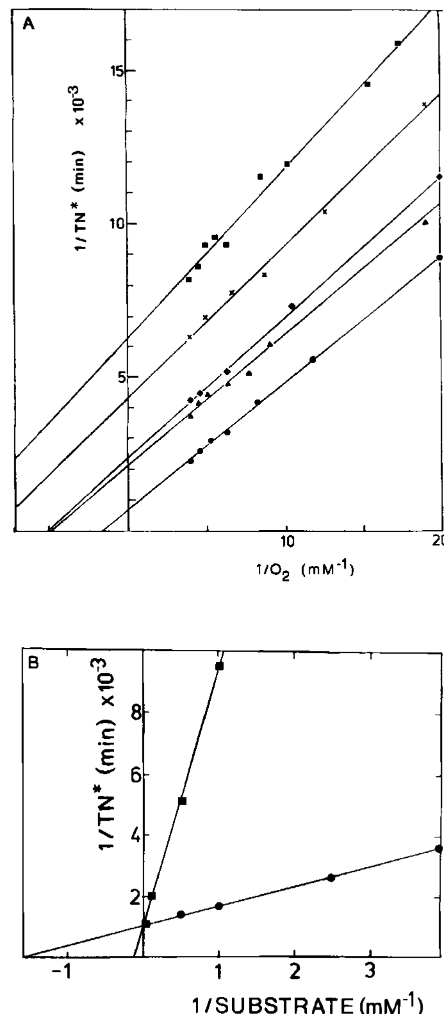


Fig. 2. *(A) Effect of the oxygen concentration on the rate of methanol turnover by alcohol oxidase. (B) Evaluation of TN_{max} and K_m values for (■) deutero-methanol and (●) oxygen.* (A) Methanol concentrations: (●) 10 mM; (▲) 1 mM; (◆) 0.7 mM; (×) 0.35 mM and (■) 0.2 mM). The TN* values were determined by monitoring the oxygen concentration and are calculated on the basis of the total FAD concentration of the enzyme. (B) Data obtained from primary plots such as shown in A by plotting the ordinate intercept values ($1/TN_{max}$*) vs concentration of the limiting substrate

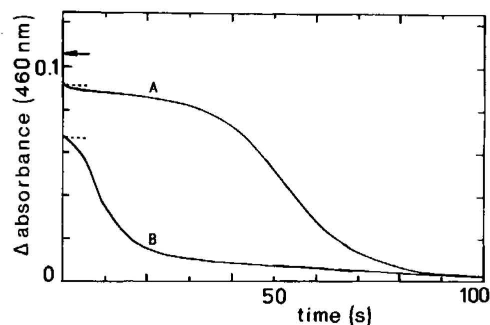


Fig. 3. *Enzyme-monitored turnover of (A) deutero-methanol and (B) methanol.* 19 μM alcohol oxidase was allowed to react with 0.4 mM alcohol; air-saturated solutions (0.25 mM O_2) were mixed in the stopped-flow spectrophotometer and absorbance changes at 460 nm were recorded. Dotted lines indicate the absorbance of the mixtures at the beginning of the steady-state phase

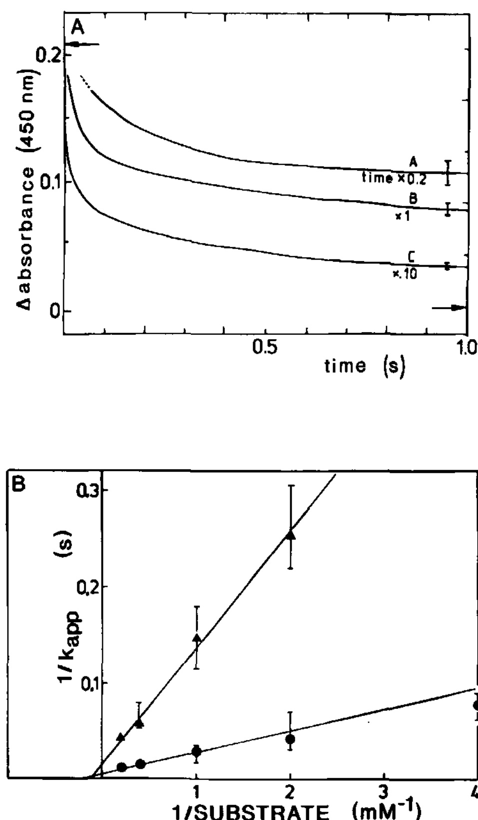


Fig. 4. *(A) Time course of the anaerobic reaction of alcohol oxidase with methanol. (B) Rates of the anaerobic reaction of alcohol oxidase with (●) methanol and (▲) deutero-methanol.* (A) The trace of the absorbance changes at 450 nm of a mixture of 34 μM enzyme and 0.5 mM methanol in the absence of oxygen is given as an example. The left arrow indicates the absorbance of oxidized minus reduced enzyme; the right arrow indicates the endpoint of the reaction, 3 min after mixing the components in the stopped-flow cell; observation intervals (curve A) 0.2 s; (curve B) 1 s and (curve C) 10 s. Noise is indicated by bars. Approximately 40% of the absorbance changes are attributable to the fast reaction phase, which follows (pseudo)first-order kinetics over about 4 half-lives

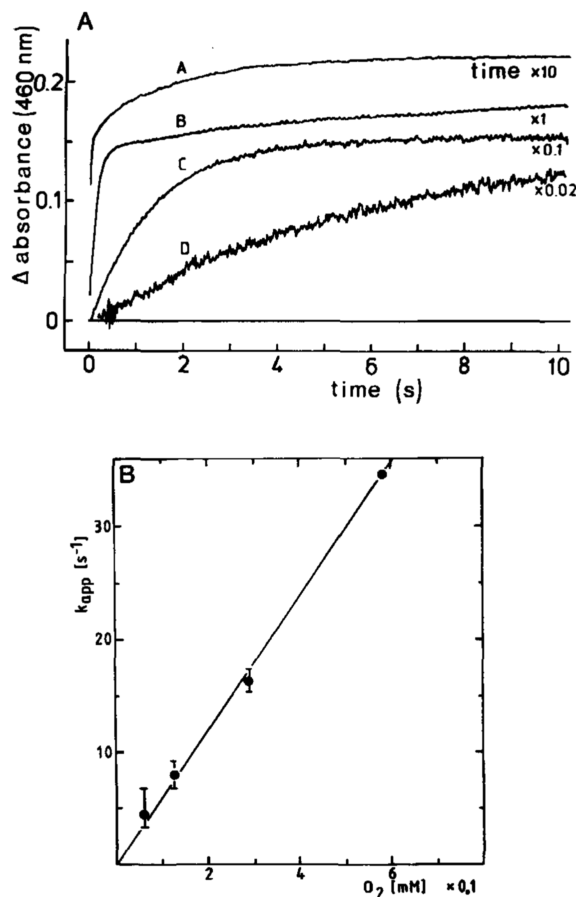


Fig. 5. *(A) Time course of the reaction of reduced alcohol oxidase with oxygen. (B) Rates of the fast phase of the reaction of reduced alcohol oxidase with oxygen.* (A) The trace of the absorbance change at 460 nm of a mixture of 30 μM enzyme, which had been reduced by anaerobiosis (cf. Materials and Methods) and 0.13 mM oxygen is given as an example; observation intervals (curve A) 100 s, (curve B) 10 s, (curve C) 1 s and (curve D) 0.2 s. Approximately 75% of the absorbance change occurs in the fast reaction phase, which follows (pseudo)first-order kinetics

isotope effect on TN_{max} estimated by this method was about 1.5. Experiments as illustrated in Fig. 3 also allow the estimation of the ratio of reduced to oxidized enzyme at steady state, which depends on the relative rates of the reductive and oxidative steps. The ratios of the rates of reduction and reoxidation (k_{red}/k_{ox}) were obtained from the absorbance at 460 nm, taking into account the absorbance before mixing (oxidized enzyme) and after completion of the reaction (reduced enzyme) (Fig. 3), and by extrapolation to the onset of steady-state conditions. With 0.4 mM alcohol and 0.25 mM oxygen these ratios were 0.56 for methanol and 0.15 for deutero-methanol.

Kinetic course of the anaerobic reaction of alcohol oxidase with methanol and deutero-methanol

The spectral course of the anaerobic reaction of the enzyme with substrate is shown in Fig. 4A. With $^{1}H_3COH$ and $^{2}H_3COH$, it is described by three reaction phases each following (pseudo)first-order kinetics. The intermediate phase ($k \approx 100\ min^{-1}$) and the slow phase ($k \approx 10\ min^{-1}$) do not exhibit any isotope effect using $^{1}H_3COH$ and $^{2}H_3COH$. Their extent is approximately 30% each of the total absorbance changes observed at 460 nm. Both phases are much too slow to account for catalytic rates of methanol and deutero-methanol turnover. In contrast, the rapid phase of reduction (Table 1), which comprises approximately 40% of the absorbance changes at 460 nm, shows a substantial isotope effect of 3.5 at saturating alcohol concentrations. The rates of the rapid reaction phase show saturation kinetics (Fig. 4B) which indicates the formation of a Michaelis complex prior to the reductive step. The estimated rates and constants are summarized in Table 1. The activity of the enzyme remained essentially unchanged after the anaerobic stopped-flow measurements at 25 °C.

Kinetic course of the reaction of reduced alcohol oxidase with oxygen

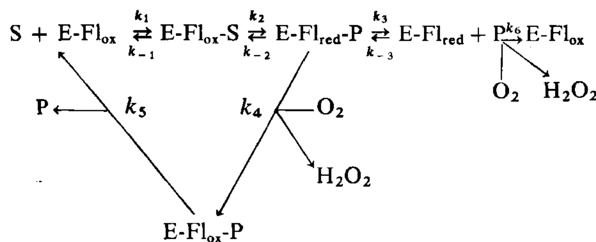
The course of the oxidation reaction (Fig. 5A) is described by two reaction phases with (pseudo)first-order kinetics. The second phase ($k \approx 3 \times 10^4\ M^{-1}\ min^{-1}$) comprises only 20% of the total absorbance changes observed and is too slow to be of catalytical significance. With the rapid phase of reoxidation, a plot of the apparent (pseudo)first-order rate constants k_{app} vs the concentration of oxygen yields a second-order rate

Table 1. *Kinetic parameters of methanol and deutero-methanol turnover catalyzed by alcohol oxidase, of the anaerobic reaction of alcohol oxidase with methanol (deutero-methanol) and of the reaction of reduced alcohol oxidase with oxygen*
Turnover rates were determined with an oxygen electrode under standard conditions. Enzyme-monitored turnover was registered with the stopped-flow spectrophotometer. The values for k_2 and k_6 (Scheme 1) are the rate constants determined by stopped-flow spectrophotometry for the anaerobic reaction of the oxidized enzyme with methanol (deutero-methanol) and the reaction of the reduced enzyme with oxygen

Parameter	Value for		Ratio ($^1H/^2H$)	Conditions
	^1H_3COH	^2H_3COH		
K_m^{app}	0.6 mM	2.7 mM	0.2	turnover with 0.25 mM oxygen (oxygen electrode)
TN_{max}^{app}	1500 min^{-1}	910 min^{-1}	1.7	
K_m(R-OH)	2.5 mM	8.0 mM	0.3	turnover with alcohol and oxygen (oxygen electrode)
$K_m(O_2)$	0.8 mM	0.6 mM	1.3	
TN_{max}	6000 min^{-1}	3100 min^{-1}	1.9	
Reaction:	$E\text{-}Fl_{ox} + CH_3OH \rightarrow E\text{-}Fl_{red} + CH_2O$			
k_2	15000 min^{-1}	4300 min^{-1}	3.5	stopped-flow (single turnover)
K_d	5 mM	8 mM	0.6	
Reaction:	$E\text{-}Fl_{red} + O_2 \rightarrow E\text{-}Fl_{ox} + H_2O_2$			
k_{ox}	$3.6 \times 10^6\ M^{-1}\ min^{-1}$			stopped-flow (single turnover)
k_4	$6 \times 10^6\ M^{-1}\ min^{-1}$			stopped-flow (enzyme-monitored multiple turnover)
k_5	10000 min^{-1}	11100 min^{-1}		estimated from Eqn (1) (Scheme 1)

constant $k_{ox} \approx 3.6 \times 10^6\ M^{-1}\ min^{-1}$. The plot also indicates that the formation of a Michaelis complex between oxygen and the reduced enzyme does not play a major role in the reoxidation reaction, which is essentially irreversible. These findings are in agreement with results reported for the reaction between oxygen and other flavin oxidases [22, 23].

An apparent discrepancy emerges by comparing the ratio of the rate constants obtained from stopped-flow measurements (Figs 4 and 5) with the ratio of reduction and reoxidation rates estimated under similar experimental conditions from the enzyme-monitored multiple-turnover experiments in Fig. 3. Thus, with 0.4 mM alcohol and 0.25 mM oxygen, $k_{red}(^1H)/k_{ox}$ is $1000\ min^{-1}/900\ min^{-1}$ (= 1.1) which compares with a ratio of 0.56 estimated from enzyme-monitored turnover. Similarly $k_{red}(^2H)/k_{ox}$ is $195\ min^{-1}/900\ min^{-1}$ (= 0.22) and compares with 0.15 estimated from enzyme-monitored turnover. Since the turnover rates with 0.25 mM oxygen and saturating alcohol concentrations are 1500 min^{-1} and 910 min^{-1} with methanol and deutero-methanol, respectively (Table 1), the $k_{ox} \approx 900\ min^{-1}$ clearly cannot be adequate for turnover. From the rates of reduction (k_{red}, determined by stopped-flow spectroscopy, Table 1) and the ratios of k_{red}/k_{ox} (enzyme-monitored turnover), the rates of reoxidation (k_4) can be estimated as 1750 min^{-1} for ^1H_3COH and 1300 min^{-1} for ^2H_3COH. The difference between these two rates might be due to a small isotope effect (≈ 1.3). However, the uncertainties in the estimation of these rates do not allow an unambiguous interpretation. Such an isotope effect could result from breaking the N(5)-^{1}H bond of the reduced flavin cofactor [as compared to N(5)-^{2}H] during reoxidation. It would require that the hydrogen attached to the N(5) position of the flavin nucleus originates from the α-position of the substrate and that it does not undergo a fast exchange with hydrogen from solvent molecules. The second assumption is rather unlikely. Since no direct information is available on this crucial point, the assumption is made that this isotope effect does not prejudice the estimation of k_4. Accordingly, the rate constant of the reoxidation during catalytic turnover, k_4, is about 1500 min^{-1} (average of 1750 and 1300). Thus, k_4 must be approximately 1.7 times the value of k_{ox} determined for the oxidation reaction in the absence of substrate or product ($3.6 \times 10^6\ M^{-1}\ min^{-1}$; Fig. 5B), i.e. approximately $6 \times 10^6\ M^{-1}\ min^{-1}$. These values can account for the measured rates of turnover.



$$TN_{max} = \frac{k_2 \times k_5}{k_2 + k_5} \quad (1)$$

$$K_m(O_2) = \frac{k_5(k_2 + k_{-2})}{k_4(k_2 + k_5)} \quad (2)$$

$$K_m(ROH) = \frac{k_5(k_{-1} + k_2)}{k_1(k_2 + k_5)} \quad (3)$$

Scheme 1. *Minimal kinetic model for the turnover of methanol and deutero-methanol by alcohol oxidase.* For scheme and equations cf. [22]

Clearly, the catalytically relevant reoxidation rate (k_4) should be measured in the presence of $^1H_2C{=}O$ and $^2H_2C{=}O$. However, this is not possible since the hydrate of the product formaldehyde is a substrate of the enzyme itself [24]. A faster reaction of $E\text{-}Fl_{red}\text{-}CH_2{=}O$ with oxygen compared to uncomplexed $E\text{-}Fl_{red}$ has been documented for the flavin-dependent L-lactate oxidase [22] and monoamine oxidase [25]. An overall kinetic scheme is proposed for the substrate turnover catalyzed by alcohol oxidase (Scheme 1), with the assumptions that $k_2 \gg k_{-2}$, and $k_1, k_{-1} > k_2$. In this scheme the value of k_5 was estimated from Eqn (1) while the deduction that $k_3 < k_4$ results from the data discussed above.

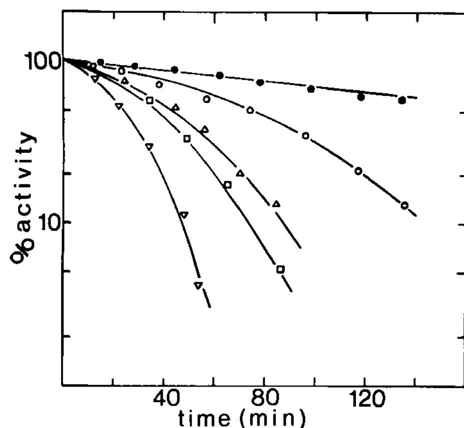


Fig. 6. *Inactivation of alcohol oxidase during turnover of methanol.* Decrease of observed rates of turnover of methanol by 33 nM enzyme in air-saturated buffer; methanol concentrations were (▽) 1 mM, (□) 5 mM, (△) 10 mM, (○) 50 mM, (●) 50 mM and 1.1 U/ml catalase

Inactivation of alcohol oxidase during turnover of substrate

Inactivation of the enzyme by hydrogen peroxide. The enzyme is irreversibly inactivated by H_2O_2 in a process following (pseudo)first-order saturation kinetics, with $K_d \approx 1.6$ mM and $k_{inact} \approx 0.55$ min^{-1} (data not shown). Qualitatively inactivation by H_2O_2 has also been shown with alcohol oxidases from *Pichia pastoris* [26] and *Kloeckera* sp. 2001 [27]. The *Pichia* enzyme can be reactivated by incubation with 1 mM GSH for 60 min at 25 °C [26], whereas the *Candida* enzyme remains inactive under these conditions (data not shown).

Inactivation during methanol turnover. Inactivation of the enzyme is observed also during turnover of methanol (Fig. 6). The rate of inactivation at a given alcohol concentration increases as hydrogen peroxide accumulates. The substrate partially protects the enzyme from inactivation, since at higher methanol concentrations lower inactivation rates are observed.

Inactivation during alcohol turnover in the presence of catalase. With catalase present, however, the typical increasing rate of inactivation at a fixed alcohol concentration is not observed (Fig. 7A, B). Since inactivation still proceeds at a significant rate, it is concluded that, in addition to inactivation by hydrogen peroxide, another inactivation process must occur during turnover. In the presence of catalase only this latter mechanism is operative, the course of inactivation exhibiting (pseudo)first-order saturation kinetics: with methanol $k_{inact} \approx 0.005$ min^{-1} and 'K_{inact}' ≈ 0.6 mM; this latter value is identical to K_m^{app} for turnover under the same experimental conditions (0.25 mM O_2). With k_{cat} (TN_{max}^{app} in Table 1) ≈ 1500 min^{-1}, the partitioning between catalysis and inactivation, k_{cat}/k_{inact}, is 3×10^5. (Pseudo)first-order kinetics of inactivation are also observed during turnover of all other alcohols tested (Table 2), in particular with propargyl alcohol and with methylenecyclopropyl alcohol.

Substrates with an acetylenic substituent or the methylenecyclopropyl function have been shown to be potent suicide inhibitors of several flavoenzymes [13]. Alcohol oxidase, however, is only very slowly inactivated by propargyl alcohol and methylenecyclopropyl alcohol: the partitioning between turnover and inactivation, k_{cat}/k_{inact}, is similar to that of all other alcohols measured and is greater than 10^5 (Table 2). With increasing chain length of the saturated alcohols k_{cat}/k_{inact} decreases, i.e. inactivation is more efficient. Similarly, the secondary alcohol isopropanol is a more efficient inactivator than its primary isomer. Since these differences are relatively small, it is likely that in all cases the same mechanism of inactivation is operative.

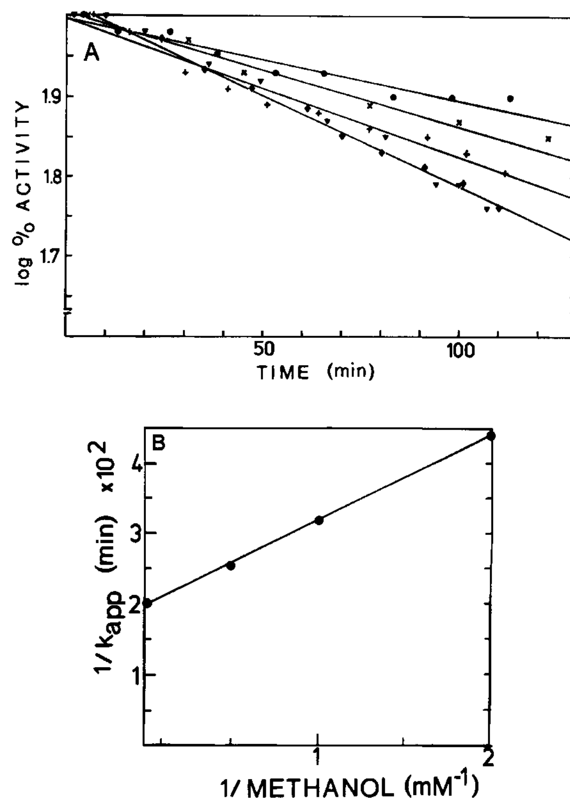


Fig. 7. *(A) Inactivation of alcohol oxidase by turnover of methanol in the presence of catalase. (B) Evaluation of 'K_{inact}' and k_{inact} of the inactivation process.* (A) 17 nM enzyme, 1.1 U/ml catalase (except ▼ with 11 U/ml) and methanol (●) 0.5 mM; (×) 1 mM; (+) 2 mM; (▼, ◆) 100 mM in air-saturated buffer. With the low enzyme concentrations used, the alcohol concentrations remained essentially constant during the observation time. (B) Apparent (pseudo)first-order rate constants k_{app} are calculated from A

Table 2. *Relative rates of turnover and inactivation of alcohol oxidase by turnover of various alcohols in the presence of catalase*
Experiments were performed as described for methanol (Fig. 7), except that with isopropanol the assay was 67 nM in alcohol oxidase

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

DISCUSSION

Flavin-dependent alcohol oxidases are a class of flavin enzymes characterized by a peculiar property: the absorbance

spectrum of the enzyme as isolated is significantly different from that of other flavin oxidases (Fig. 1) and also varies depending upon the source [1, 3, 28]. The enzyme used in this work is homogeneous as analyzed by SDS gel electrophoresis, but is heterogenous in its chromophore content: it contains approximately one third of catalytically active oxidized FAD, the residual two thirds consisting of approximately equal amounts of flavin radical [2] and, probably, inactive oxidized flavin. For these reasons, estimations of rate constants of the enzyme will yield values that are too low when related to the total flavin content. The fraction of active enzyme is best estimated by experiments such as those of Fig. 1, in which the decrease of absorbance observed upon addition of substrate is assumed to reflect conversion of oxidized to reduced flavin; no change in radical content is observed during this process [2, 3].

The isotope effect of 1.9 observed on turnover (Table 1) is approximately half of that observed for the reductive half reaction, the rate-limiting step of which is represented by k_2 (Scheme 1). Rupture of the C-H bond (step k_2) is thus only partially rate-limiting and another step in the catalytic cycle (either k_4 or k_5, Scheme 1) must be of similar magnitude. The results from the kinetic studies are indicative of a system in which k_{-2} (Scheme 1) has a finite value [29] and is compatible with the formation of a ternary complex between the reduced enzyme, the product aldehyde and oxygen as shown in Scheme 1. Another important rate in the minimal catalytic cycle of this scheme is represented by k_5. A reasonable value can be obtained from Eqn (1) by using the experimentally determined values of k_2 and TN_{max}, as was done in several similar cases with other flavin enzymes [22, 23]. Clearly, our data cannot differentiate between k_5 representing the dissociation of either aldehyde or H_2O_2 from oxidized enzyme. However, with several flavin oxidases the dissociation of product (not H_2O_2), has been shown to be (partially) rate-limiting [22, 30]. A simple test of the validity of the kinetic Scheme 1 can be obtained by comparing the values of K_m(R-OH) and $K_m(O_2)$ calculated from Eqns (2) and (3) with the experimentally determined values (Table 1): $K_m(^1H_3COH) = 2.6$ mM and $K_m(^2H_3COH) = 6.3$ mM are in good agreement with experimental values 2.5 mM and 8 mM respectively, and $K_m(O_2)$ 1 mM (^1H_3COH) and 0.5 mM (^2H_3COH) with 0.8 mM and 0.6 mM, respectively. In view of the problems inherent in the experimental determination of these parameters (presence of inactive radical etc.), this may be considered satisfactory.

During the reaction between Fl_{ox} and alcohol no intermediates can be observed in the stopped-flow spectrophotometer. This suggests that intermediates such as radical species or covalent adducts, if they occur, must have a very short life time (< 5 ms). Similarly no intermediates could be detected during the reaction of reduced enzyme with O_2. Of particular interest is the inactivation occurring during substrate turnover. Inactivation of flavin-dependent alcohol oxidase has been reported to occur upon incubation with propargyl alcohol [12] and has been proposed to result from reaction of the accumulating propynal with an active-site residue. In this work [12], however, the strong inactivating effect of the second product, H_2O_2, was neglected. Since we were not able to reproduce the rapid inactivation using this substrate analog in the presence of catalase and since rapid inactivation occurs upon formation of H_2O_2, the interpretation by Cromartie [12] is probably erroneous. Methylenecyclopropyl alcohol, an alcohol carrying the same potential suicide function as hypoglycine (α-amino-β-2-methylenecyclopropylpropionic acid) [31], behaves similar as methanol. This suggests that the oxidation of the carbinol function is not initiated by proton abstraction as is the case with other flavin oxidases and dehydrogenases [6].

The inactivation by H_2O_2 probably involves oxidation of an essential function at the active site of the enzyme [32]. This idea is supported by the reversal of inactivation with reagents such as GSH in the case of the *Pichia* enzyme [26]. The slow inactivation accompanying substrate turnover in the presence of catalase cannot be correlated to a specific chemical or spectroscopic event. Unfortunately the high numbers of turnover required for obtaining substantial inactivation lead to formation of turbidity and thus exclude direct spectroscopic monitoring. With regard to the mechanism of alcohol oxidation, our observations are compatible with recent results reported by Abeles and coworkers, which involve the inactivation of the enzyme by cyclopropyl alcohol [11]. These authors suggest that flavin-dependent alcohol oxidases function a radical-type mechanism such as also proposed for the flavin-dependent monoamine oxidase [33].

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