EVIDENCE FOR AN ALKOXIDE INTERMEDIATE IN THE REACTION CATALYZED BY ALCOHOL OXIDASE

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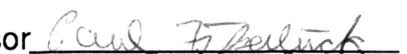
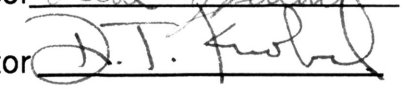
Fellows Advisor 

Honors Director 

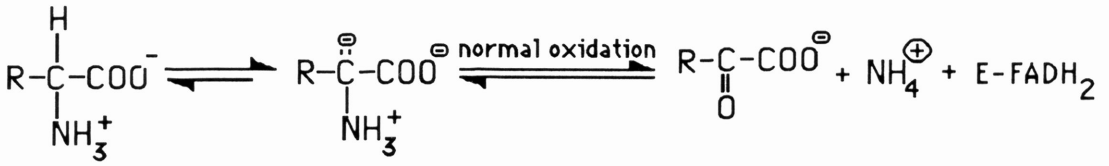
Alcohol oxidase from the yeast *Candida boidinii* is a flavin enzyme that catalyzes the oxidation of primary alcohols to their respective aldehydes using molecular oxygen.

$$R{-}CH_2{-}OH + O_2 \longrightarrow R{-}CHO + H_2O_2$$

The function of alcohol oxidase may involves in the metabolism of methanol as a carbon source. The enzyme is a octamer with subunits of 74,000 that are nonconvalently associated (Sahm and Wagner).

The enzyme contains one tightly bound FAD per subunit (Sahm*et al*). The native enzyme stabilizes a FAD semiquinone radical, which is catalytically inactive. As many as 30% of FAD of the enzyme is in the radical form (Geissler *et al*). Alcohol oxidase has a narrow substrate specificity.

Alcohol oxidase belongs to a large family of flavin dependent oxidases. Most members of this family catalyze reactions utilizing carbanion intermediate (Ghisla). A well-studied example is D-amino-acid oxidase. D-Amino-acid oxidase oxidizes D-amino acids and gives imino acids as primary products. Three lines of experimental evidence suggest that amino-acid oxidation may begin by enzyme-mediated abstraction of the hydrogen at C-2 as a proton and the formation of a carbanion (Walsh):



The primary indication for the carbanion intermediates is from the study on ring-substituted phenylglycines. The results indicate that substituents that stabilize carbanionic species increased V_{max} values, where as substituents that destabilize carbanions decreased V_{max}.

In contrast, there is evidence suggesting that alcohol oxidase follows a radical mechanism. Cyclopropanol irreversibly inactivates alcohol oxidase as a suicide inhibitor but not other oxidases. Alcohol oxidase is not inhibited by the substrates that form covalent flavin N(5) adducts, which are suicide inhibitors for several flavin oxidases that are known to form carbanion intermediates (Geissler *et al*). The experiments described in this thesis were designed to obtain evidence for or against this proposed radical mechanism.

Methods and Materials

Alcohol oxidase were obtained from Boehringer Mannheim. 8.34 mg of enzyme was dissolved in 3 ml of 20 mM pyrophosphate, pH of 8.9, to give the final concentration of 37.6 μM. Alcohols were obtained from standard chemical supply houses. α_2-$^2H_2$-Benzyl alcohol was a generous gift from Dr. Fitzpatrick. The standard conditions for the steady-state kinetics experiments were 20 mM sodium pyrophosphate buffer at 25°C. Aqueous alcohol solutions with 20 mM sodium pyrophosphate buffer were allowed to mixed with oxygen gas at 25°C. The enzyme was added to the alcohol-buffer aqueous mixture after oxygen and temperature equilibrations. The pH of the substrates for experiments was at 8.9 except for the pH profiles. Acetic acid was used to adjust the pH of the buffers. Assays monitored the rate of oxygen consumption using a Yellow Spring Instrument Model 5300 Biological Oxygen Monitor at the indicated pH. Under 1 atmosphere pressure at 25°C, the oxygen concentration used for the calculation is 26.6 μM.

The multisubstrate mechanism experiments measured the steady state rate constants of several concentrations of benzyl alcohols under three oxygen concentrations, 1.27 μM (saturated), 0.266 μM and 0.127 μM. Aqueous benzyl alcohol solutions with 20 mM sodium pyrophosphate buffer at pH 8.9 were mixed with oxygen gas at 25°C. The alcohol-buffer mixtures were equilibrated with the oxygen gas (100%, 20% and 10%) for 10 minutes. Alcohol Oxidase was added to the alcohol-oxygen solution immediately after the equilibration period and the initial rate of catalysis was measured as the rate of oxygen consumption. The pH profiles were constructed by measuring the steady-state rates with benzyl alcohol as the substrate under the standard conditions at the indicated pH. The solvent isotope effects and the isotope fraction experiements were carried out under the standard conditions with pure protium oxide, pure

Results

Steady State Kinetics with Benzyl Alcohol The steady state kinetic mechanism of alcohol oxdiase with benzyl alcohol was examined before examining the effect of ring substitution upon the kinetic parameters. With a number of flavoprotein oxidases, the kinetics behave as if the mechanism were ping pong, in that the V/K values for the substrates are independent of the concentration of oxygen. If that were the case with alcohol oxidase, subsequent analyses would be greatly simplified. The experiment was done by measuring the initial rates varying both benzyl alcohol and oxygen concentrations. The data were fit to the equations for both ping pong and sequential mechanisms and gave a better fit to the former, as indicated by the parallel line pattern in a double reciprocal plot (Figure 1).

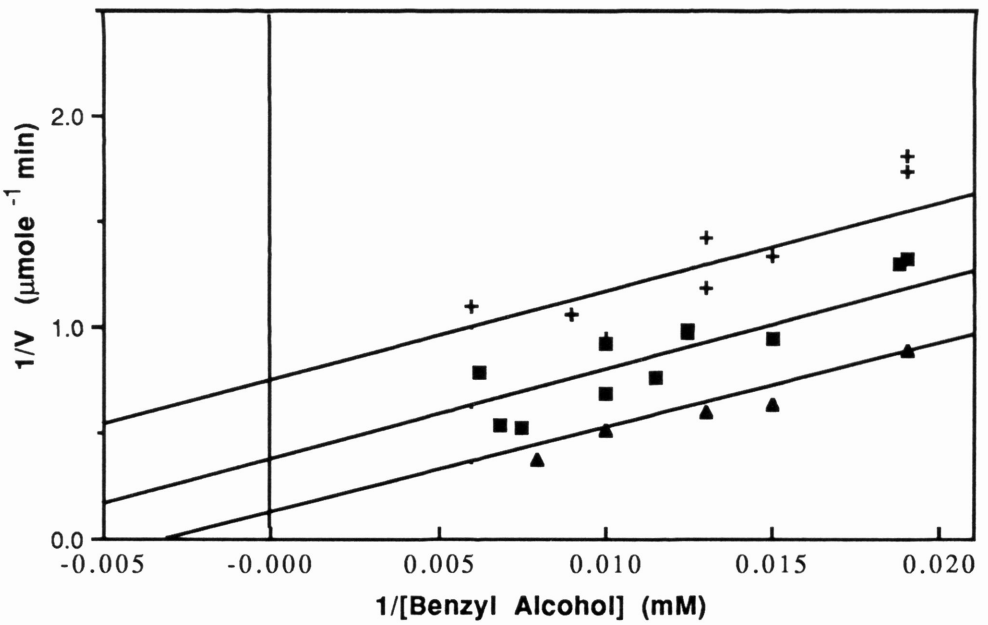


Figure 1: Steady State KInertics of Alcohol Oxidase with Benzyl Alcohol as Substrate Initial rates were determined at the indicated concentrations of benzyl alcohol and (+) 0.127 mM, (■)0.266 mM, and (▲)1.27 mM oxygen in 20 mM sodium pyrophosphate, pH 8.9, at 25 °C. The data were fit to $v = VAB/(AB+K_aB+K_bA)$.

Steady State Kinetics with para-Substituted Benzyl Alcohols The steady state kinetic parameters for para-substituted benzyl alcohols were measured by varying the concentrations of both oxygen and the alcohol (Table 1). In all cases apparent ping pong kinetics resulted. For some alcohols we were unable to determine V_{max} and K_m values due to low solubility in water. The V/K values for the alcohols showed a strong correlation with both σ and σ^* values of the substituents (Figure 2). Further, σ^* values of the alcohols correlate with pKa of the alcohols. The V/K values for the para-substituted benzyl alcohols are therefore plotted as a function of the pK_a of the alcohol in Figure 3.

Table 1: Kinetic Parameters of para-Substituted Benzyl Alcohols[a]

Substituent	V/K ($M^{-1}S^{-1}$)	σ	σ^{*b}	pKa[c]	WV ($Å^3$)[d]
H	106.32	0	0.75	15.25	7.22
Cl-	310.1	0.23	0.87	15.09	24.44
F-	132.9	0.06	0.81	15.17	10.29
CH_3-	38.98	-0.23	0.59	15.46	23.08
CH_3-O-	12.85	-0.27	0.60	15.45	30.64
NH_2-	7.088	-0.60	0.44	15.65	19.40
CF_3-	1.819	0.54	-	-	-
HO-	5.196	-0.7	-	-	-

[a]The (V/K) values were obtained as described for Figure 1.

[b]These are σ * values for substituents at the para-position of the aromatic ring.

[c]The pKa values were calculated from σ^* values using the data of Takahash *et al*.

[d]WV is the van der Waals volume of the substitute using the data of Motoc.

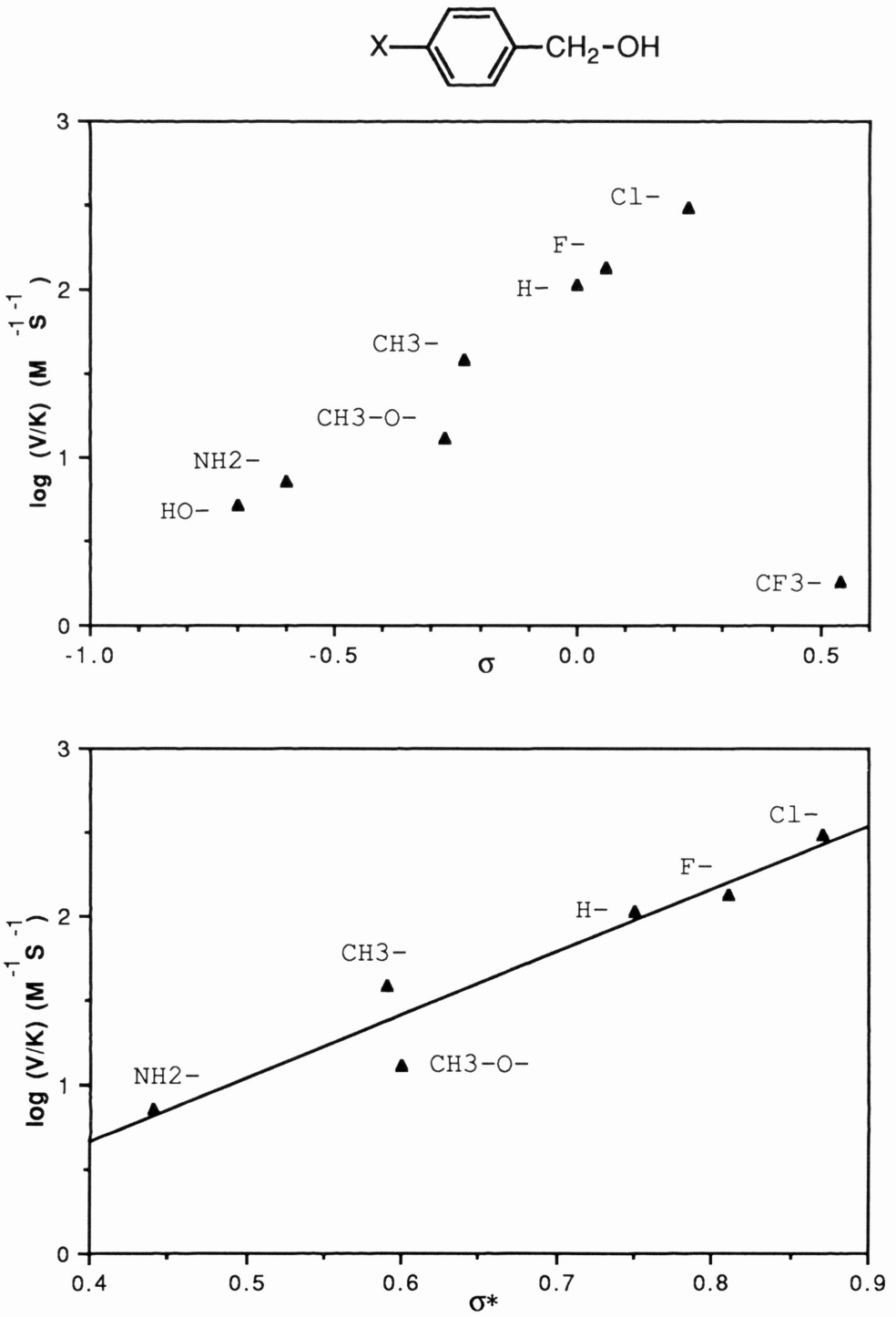


Figure 2: V/K Values of Substituted Benzyl Alcohols as a function of σ and σ*

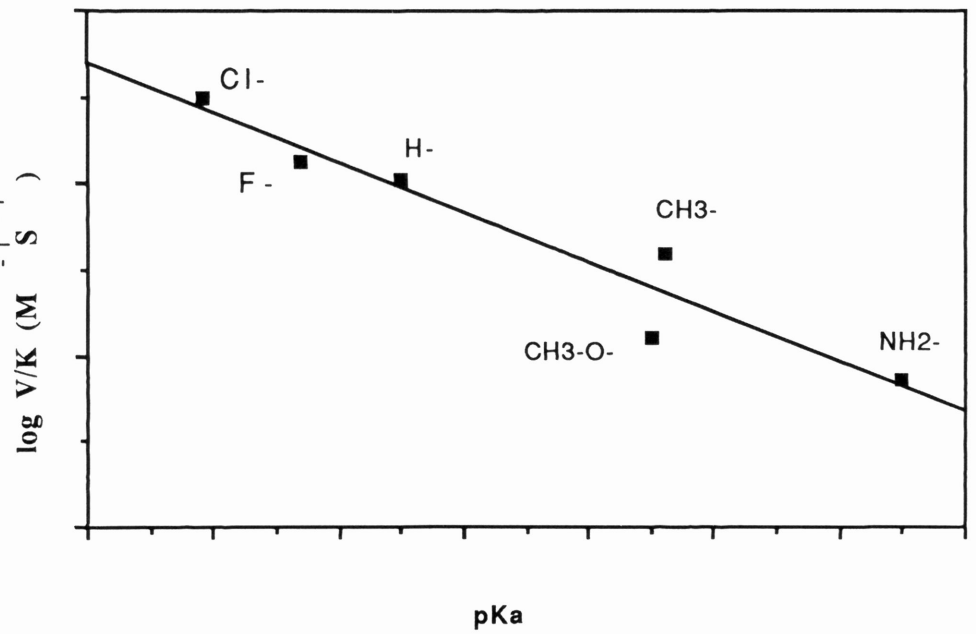


Figure 3: Correlation of V/K Values for Benzyl Alcohols with pKa Values.

pH-rate Profiles for Alcohol Oxidase The effect of pH on the apparent V_{max} value and on the V/K value for benzyl alcohol was determined. As shown in FIgure 4, both V and (V/K) profiles show the effect of a base with a pK value of 8.1 which must be deprotonated for catalysis.

Kinetic Isotope Effects with Benzyl Alcohol Replacement of the benzyl hydrogens in benzyl alcohol with deuterium had no significant effect on the V/K value for the alcohol (Table 3), indicating that the benzylic carbon-hydrogen bond is not cleaved in the rate-limiting step. In constrast, running the reaction in D_2O results in a solvent isotope effect of 2.3. The number of protons involved in the rate-limiting step of the catalysis was determined by measuring the solvent isotope effects in mixtures of D_2O and H_2O. The data show a linear correlation between the isotope effect and the mole fraction of D_2O, indicating that there is single exchangeable proton in flight in the rate-limiting step with benzyl alcohol. Isotope effects of deuterated alcohols indicate which covalent bond is involved in the rate-limiting step.

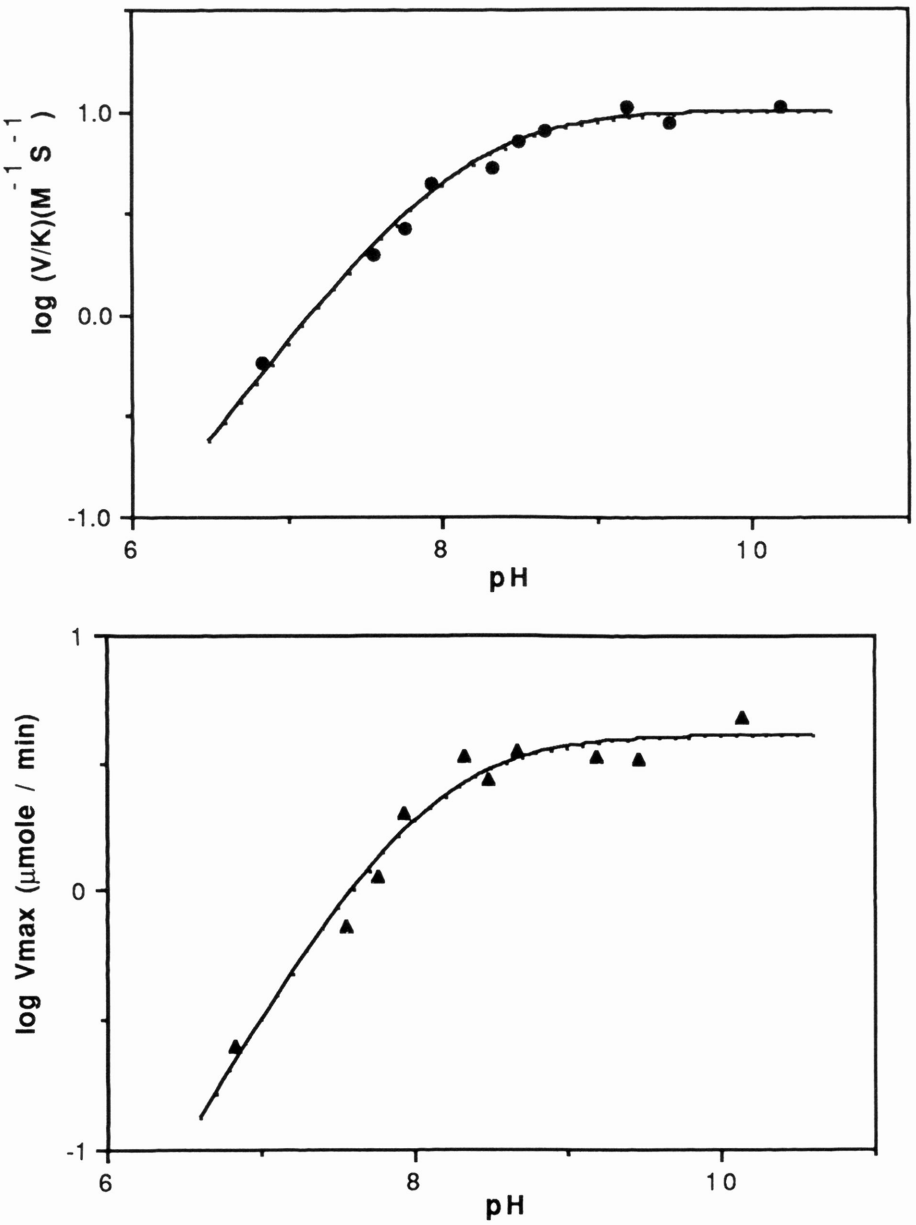


Figure 4: pH-Rate Profiles for Alcohol Oxidase with Benzyl Alcohol as Substrate.

The experiments were carried out at 25°C. The line is a fit to log v = log(C/(1 + H/Ka) where H is the proton concentration. For (A), C = 10 and pKa = 8.11 ± 0.02; for (B), C = 4.1 and pKa = 8.05 ± 0.04

Table 2: Steady State Kinetic Constants of ß-Substituted Ethanols[a]

alcohol	Km [mM]	V/K ($M^{-1}S^{-1}$]	pKa	WV[b] ($Å^3$)
Ethanol	3.4	1200	15.9	23.8
FCH_2CH_2OH	9.9	310	14.5	28.27
ICH_2CH_2OH	53	58.6	14.6	57.12
Cl_2CHCH_2OH	29	2.7	12.9	55.97
CF_3CH_2OH	80.5	2.9	12.3	34.55

[a]The Km and (V/K) values are obtained as described in Figure 1.

[b]The van der Waals volumes of the constituents at the β carbon. The values are from the same source of Table 1.

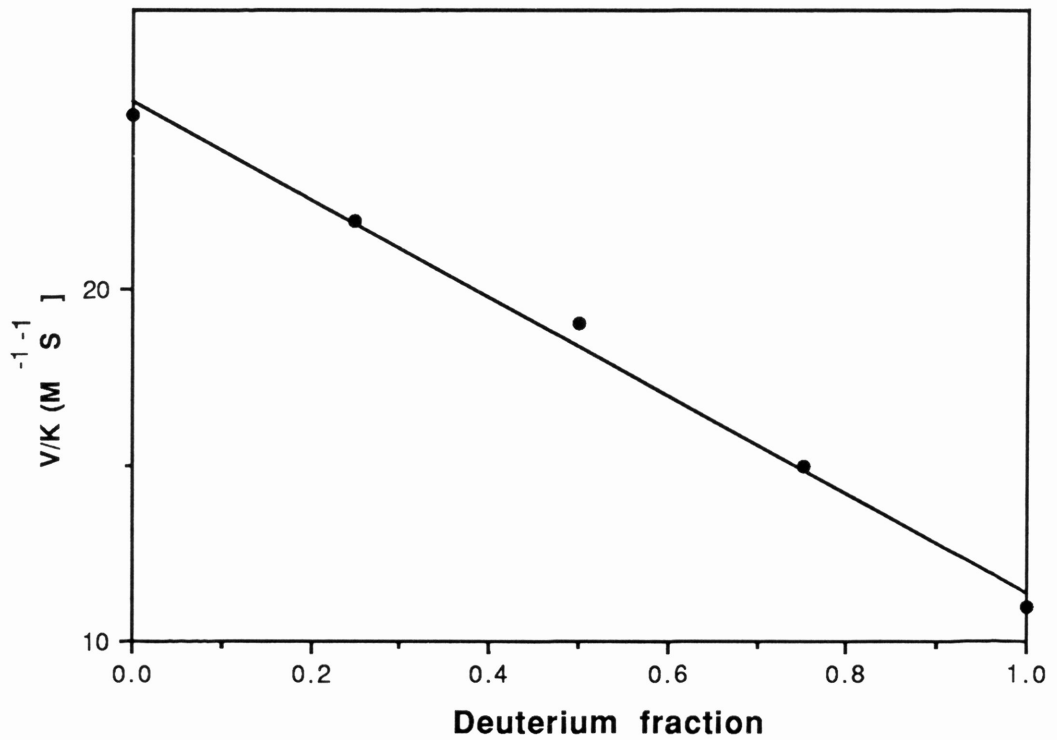


Figure 5. Proton Inventory for Alcohol Oxidase with Benzyl Alcohol as Substrate.

Steady State Kinetic Study on β-Substituted Ethanols Apparent V_{max} nad V/K values were determined with a series of ß-substituted ethanols. The results

showed a weak correlation between (V/K) values and the pK_a of the alcohol. In contrast to data for para-substituted benzyl alcohols, the rates of catalysis for this family of substrates increased with the pK_a value of the alcohol.

Primary deuterium kinetic isotope effects were determined with ethanol and trifluoroethanol. With 2,2,2-trifluoroethanol (pK_a 12.3), the primary isotope effect is 7.1. However, the primary isotope effect for ethanol is only 1.3 (Tables 3 and 4).

Table 3: Isotope effects on Benzyl Alcohol (pKa 15.25)

Primary isotope effect	$(V/K)_H/(V/K)_D$[a]	$^D(\frac{V}{K})$[b]
C_6H_5-CH_2OH / C_6H_5-CD_2OH	10/9.5	1.1

Solvent isotope effect	$(V/K)_H/(V/K)_D$[c]	$^D(\frac{V}{K})$
$C_6H_5CH_2O$-H / $C_6H_5CH_2O$-D	25/11	2.3

[a]The (V/K) values were obtained as in Table 1.

[b]The $^D(\frac{V}{K})$ value is the ratio of $(V/K)_H$ vs. $(V/K)_D$, the isotope effect is fit to the equation of v = Vm *S/(Km*(1 + Fi*Evk) + S*(1 + Fi * Ev)

[c]The $(V/K)_H$ is from the experiments in 100% protium oxide and $(V/K)_D$ is in 100% deuterium oxide.

Table 4: Isotope Effects with Ethanol and 2,2,2-Trifluoroethanol as Substrates

Primary isotope effect	$(V/K)_H$ / $(V/K)_D$[a]	$^D(\frac{V}{K})$[b]	pKa
CH_3-CH_2OH / CD_3-CD_2OH	697 / 550	1.3	15.9
CF_3-CH_2OH / CF_3-CD_2OH	5.763 / 0.811	7.1	12.3

Solvent Isotope effect	$(V/K)_H$ / $(V/K)_D$[c]	$^D(\frac{V}{K})$
CH_3CH_2O-H / CH_3CH_2O-D	1146 / 362	3.1

a), b), and c) are as for Table 3.

Discussion

The use of different substrates demonstrates the different rate-limiting steps of the catalysis by alcohol oxidase. Alcohols of different characteristics were used in this study. Methanol is the best substrate, with Km of 1.1 mM and V/K of 2900 $M^{-1}S^{-1}$. The V/K rate constants decrease drastically as the length of the carbon chains grow longer. Our results are consistent with a model that alkoxide is the important intermediate before the electron transfer that eliminates the α hydrogen and forms the aldehyde product.

Substituted benzyl alcohols were chosen for a detailed study on the effects of electron donating substituents. The drawback of using para-substituted benzyl alcohols is often their low solubilities in water. Kinetic constants such as V_{max} and K_m values can not be determined accurately unless the concentration of the substrate is high enough. In several cases, only V/K values could be determined as the slope in a direct plot of rate vs. substrate concentration.

The steady-state kinetic study on para-substituted benzyl alcohols showed that the initial rate constant (V/K) correlates with the Hammett constant (σ) and the Taft constant (σ^*) of the substitutes. Whether the mechanism involves radical or carbanion formation can be inferred from the slope of the log (V/K)s vs. the σ values plot. For enzymes that involve carbanion formation, the slope of this plot generally greater than 2. If a radical mechanism is followed, the slope is usually between -1 to +1. The plot of log (V/K) vs. σ values for para-substituted benzyl alcohols is 2.1. However, this result is not sufficient to establish that a carbanion is involved the mechanism of alcohol oxidase.

There is no kinetic primary isotope effect observed with α_2-$^2H_2$-benzyl alcohol. Therefore,carbon-hydrogen bond cleavage is not the rate-limiting step of the catalysis for benzyl alcohols and is not represented in the V/K values.

Therefore, the log (V/K) vs. σ plot provides no information for the involvement of a radical or carbanion intermediate.

The Taft constant is directly related to the pK_a of the respected benzyl alcohols by Equation 1 (Takahashi *et al*).

$$pKa = 16.23 - 1.316\ \sigma^* \qquad (1)$$

The pK_a values of para-substituted benzyl alcohols were calculated using this equation. As shown in Figure 3, there is a good correlation between the pK_a of the alcohol and the V/K value. This suggests that formation of an alkoxide is the rate-limiting step.

This conclusion is further supported by the isotope effect data. The deuterium solvent isotope effects is 2.3 for benzyl alcohol, consistent with alkoxide formation in the rate-limiting step. The proton inventory study shows that the solvent isotope effect for benzyl alcohol correlates linearly with the deuterium fraction in the solvent. Theoretical models for different mechanisms can be calculated from the isotope effect . The data fit best to a single proton model, which indicates that only a single exchangeable proton is involved in the rate-limiting step of catalysis. Therefore, the formation of alkoxide intermediate is an important step for the alcohol oxidase catalysis. The base on enzyme with an apparent pK_a value of 8.1 probably assists the formation of alkoxides from alcohols.

There are two possible paths for the alkoxides affecting the initial rates of catalysis. The first path is that the catalysis proceeds only when the enzyme binds to an alkoxide ion. With the other path, the formation of the alkoxide occurs after the substrate binds to the enzyme. The first path is limited by the diffusion rate of alkoxide, about $10^7\ M^{-1}s^{-1}$.

One way to distinguish which path the enzyme uses is to adjust the initial rate constant V/K of an alcohol for the actual alkoxide concentration. The

corrected V/K values for methanol (6.82 x 10^9 $M^{-1}S^{-1}$ at pH 8.9) and for benzyl alcohol (2.04 x 10^8 $M^{-1}S^{-1}$ at pH 7.9) exceed the diffusion limit. Since about 30% of alcohol oxidase is in its inactive form, the actual initial rates could be even higher. Thus, alkoxide formation must occur on the enzyme surface.

The pH study using benzyl alcohol (pK_a 15.25) as substrate shows that the enzyme catalyzes best at higher pH range. As pH of the medium increases, both Vmax and V/K increase until reaching a maximum rate. A pK_a value of 8.1 was observed in both profiles. The pK_a for the substrate is known; therefore this kinetic pK_a can only be due to a functional group on enzyme. The catalytic reaction requires this group, a base, to be deprotonated for the enzyme to be active. However, this base can not be identified with kinetic methods.

In summary, the results from para-substituted benzyl alcohols study indicate that (1) alkoxides are formed when the alcohol binds to the enzyme; (2) there is a base on enzyme with pK of 8.1 value which presumable removes the alcoholic proton; (3) a single exchangeable proton is in flight in the rate-limiting step in catalysis. A model that is consistent with these findings involves the following steps: (a) the alcohol binds to the active site of alcohol oxidase; (b) the base (pK_a of 8.1) on enzyme pulls off the acidic hydrogen from the alcohol, forming the alkoxide ion, and (c) the enzyme and FAD cofactor proceed to remove the α-hydrogen from the alkoxide, then forms the aldehyde product.

When the substrates are para-substituted alcohols, step (b) of the model is rate-limiting. In order to study the events in step (c), substrates with lower pK_a values were examined.

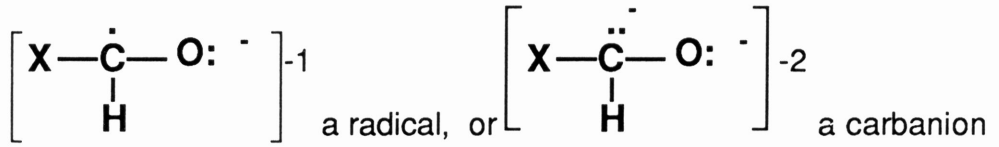
Substituted ethanols were used to study the carbon-hydrogen bond cleavage event. This group of alcohols has pK_a values between 15.9 (ethanol) to 12.3 (2,2,2-trifluoroethanol). The V/K values, however, show a different trend

from the data of para-substituted benzyl alcohols. Statical analysis of the data indicate that V/K values have only a minute correlation to the steric parameter. The effects of the substituents on the strength of α carbon-hydrogen bond will be the subject of further investigation.

Ethanol shows only small primary isotope effect of 1.3, but the primary isotope effect for trifluoroethanol is 7.1. Since trifluoroethanol has a low pK_a (12.3), the formation of alkoxide is very swift. Thereafter, the carbon-hydrogen bond cleavage at the α carbon becomes the slow step of the catalysis.

The carbon-hydrogen bond cleavage is the prelude to the formation of aldehyde product. There are two possible mechanisms based on the intermediate to describe how electrons are transfer to FAD cofactor. One forms the radical; the other, a carbanion. Most flavin oxidases follow the carbanion mechanism (Ghisla). There are no direct methods to distinguish the unstable intermediate after C-H bond cleavage in the catalysis of alcohol oxidase. However, the alkoxide precursor favors the radical mechanism over the formation of a carbanion.

The stability of the intermediate determines which mechanism is kinetic favorable. The intermediates involved in the two possible mechanisms after the formation of alkoxides are:

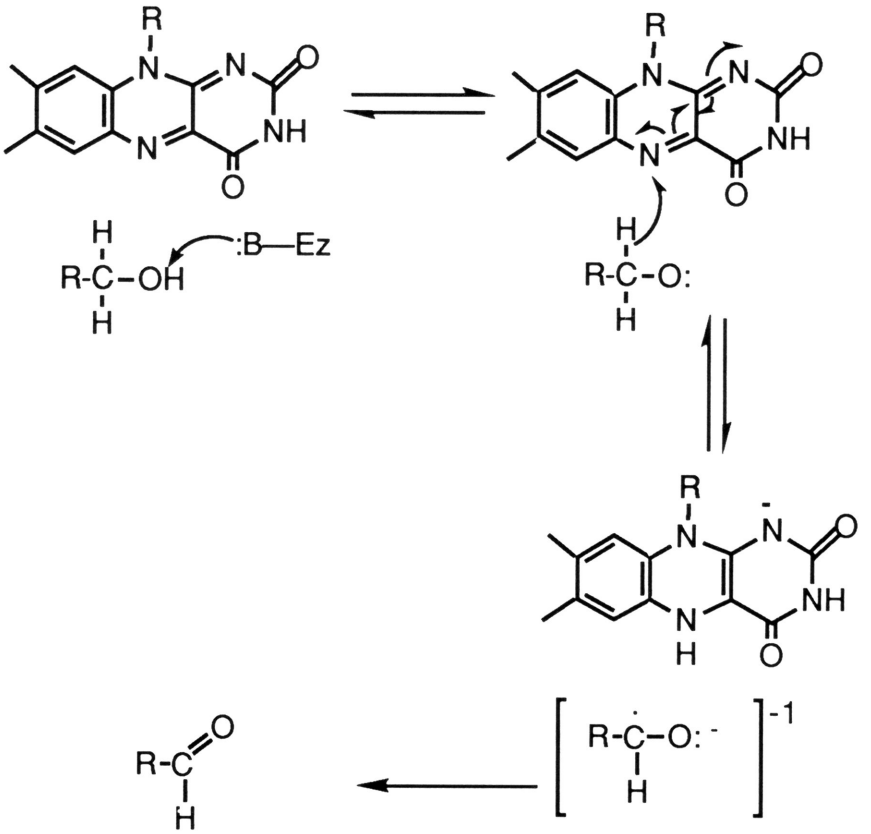


The electron density on the intermediate will be the determining factor of the stability. On a carbanion of the alkoxide, there are two adjacent negative charges. The a hydrogen is removed as a proton. The stability of this intermediate can be improved with a strong electon-withdrawing group on the

alcohols. However, a majority of the substrates used in this experiment lacks such ability to stabilize the carbanion intermediate.

In the radical mechanism, the a hydrogen is removed as a hydride. A radical forms at the a carbon of the alkoxide. The total charge of the radical remains as -1. This radical alkoxide has less energy than the carbanion form, is more stable. The radical also does not need a extended structure on the alkoxide to be stablized. Therefore, the radical mechanism describes the primary alcohol oxidation by alcohol oxidase better.

In summary, the course of the catalysis by alcohol oxidase is showed in Scheme 1. At first, the substrate enters enzyme's active site. The alcohol then loses the acidic proton to a base on enzyme and forms an alkoxide. An α-proton on the alkoxide is eliminated through electron transfer as a hydride. The radical intermediate then loses another electron to FAD, and forms the aldehyde product.



Scheme 1: Proposed Mechanism for Alcohol Oxidase

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