Kinetics of Alcohol Dehydrogenase with competitive inhibition

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Abstract and Objectives

Alcohol Dehydogenase (ADH) allows the ablation of ethanol toxicity by oxidizing it to ethanal. The action of the enzyme follows Michaelis-Menten kinetics (1). In this paper, we present a standard curve for the reduction of NAD+ by yeast ADH and ethanol, as well as the corresponding kinetics with 2,2,2-triflouroethanol as a competitive inhibitor. We will determine the Michaelis-Menten constants for both reactions in order to characterize the degree of inhibition.

Introduction

A complete system involving ADH, NAD+, ethanol, and 2,2,2-triflouroethanol can be described by the state model in Figure 1. The binding of substrate and inhibitor are both reversible. Thus, it is easy to increase enzyme affinity for ethanol simply by increasing the concentration of ethanol. The intermediate complex (S:E) is tight binding of ethanol to a Zinc formation on ADH, permitting electron transfer to NADH. Using a Lineweaver-Burke linearization of the kinetics gives $\frac{1}{V} = \frac{\alpha K_M}{V_{\max}} \frac{1}{[EtOH]} + \frac{1}{V_{\max}}$ where $\alpha = 1 + \frac{[I]}{K_I}$. Thus, in the uninhibited case, $\alpha = 1$. Since triflouroethanol does not reduce NAD+, we can use the rate of production of NADH as an assay of enzyme affinity towards the substrate of inhibitor.

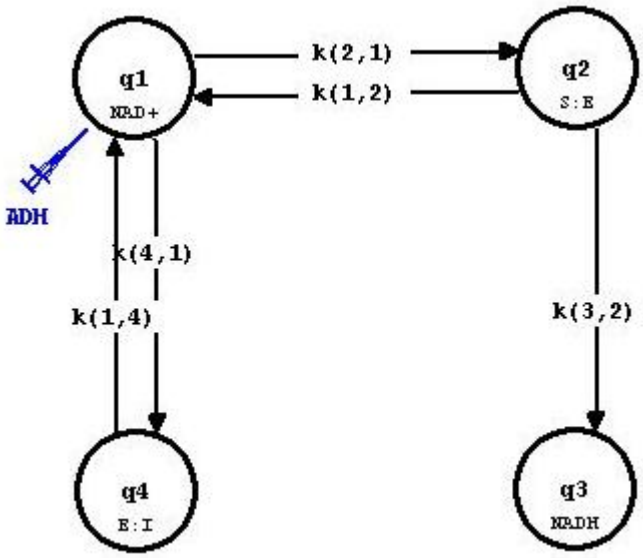

Figure 1 State-Model for the oxidation of ethanol to ethanal in the presence of a competitive inhibitor.

Methods

Standard Kinetics Eight cuvettes were loaded with volumes of 3M pure ethanol, pH 7.0 BSA and phosphate buffer shown in Table 1. A 1.5mM NAD+ solution was stored on ice to prevent decompositions and volumes were added prior to each experiment as shown. A UV-1700 PharmaSpec spectrophotometer was referenced the absorbance of cuvettes with NAD+ prior to adding ADH. Then, 0.06 mL of 0.02 mg/mL ADH was added and the reaction was allowed to occur while A_{340nm} was recorded every 15 s for 2 min in a light path of 1 cm. This was repeated with 3M methanol for the first two volumes in the table.

All in ml Cuvette #	1	2	3	4	5	6	7	8
Ethanol (3M)	0.3	0.24	0.18	0.12	0.09	0.06	0.03	0.015
NAD^+ (1.5 mM)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
pH 7.0 PB buffer (1mg/ml BSA)	1.69	1.75	1.81	1.87	1.90	1.93	1.96	1.975

Table 1 Volumes of substrate, NAD+ and buffer added to cuvettes with no inhibitor.

Inhibited Kinetics We repeated the above, but added 0.05 mL of 3M 2,2,2-triflouroethanol. Additionally, we used 0.01 mg/mL ADH for 0.06 and 0.015 mL of ethanol. The volumes added are shown in Table 2.

All in ml Cuvette #	1	2	3	4	5	6	7	8
Ethanol (3M)	0.3	0.24	0.18	0.12	0.09	0.06	0.03	0.015
NAD^+ (1.5 mM)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
3 M 2,2,2-trifluoroethanol	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
pH 7.0 PB buffer (1mg/ml BSA)	1.69	1.75	1.81	1.87	1.90	1.93	1.96	1.975

Table 2 Volumes of substrate, NAD+ and buffer added to cuvettes with inhibitor.

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Initial Reaction Rates The initial rates were determined by a linear fit of the discrete differential of absorbance against time. The linear fit was a good approximation since R^2 correlations were high (see results). Velocity of absorbance was converted to velocity of NADH by using Beer's law.

$$\frac{d[NADH]}{dt} = \frac{1}{\varepsilon b}\frac{dA}{dt} \qquad (1)$$

We use the molar extinction of NADH at 340 nm as $\varepsilon = 6200\, L/mol \cdot cm$. The *initial* amount of ethanol and NAD+ was calculated by simple stoichiometric conversion of M to mol by using the volume of the cuvette as $v = 2.94\, mL$. Plots of initial rate against concentration of ethanol were made, using the computed amount of substrate and the linear fit slope. This data was linearized by the Lineweaver-Burk method, for which the Michaelis-Menten constants were determined. We assumed that the concentration of NAD+ was constant over the short period of the reaction. This effectively made the concentration of the enzyme infinite, so that competition between ethanol and the inhibitor could be observed.

Reaction with Inhibitor The above was repeated with the data from 2,2,2-triflouroethanol. The inhibition factor, α, was determined by comparison of K_M with the uninhibited case. As expected, there was no change in $V_{\max}$, but such a change would give us the inhibition factor for any non-competitive inhibition.

Non-initial Rates

Results

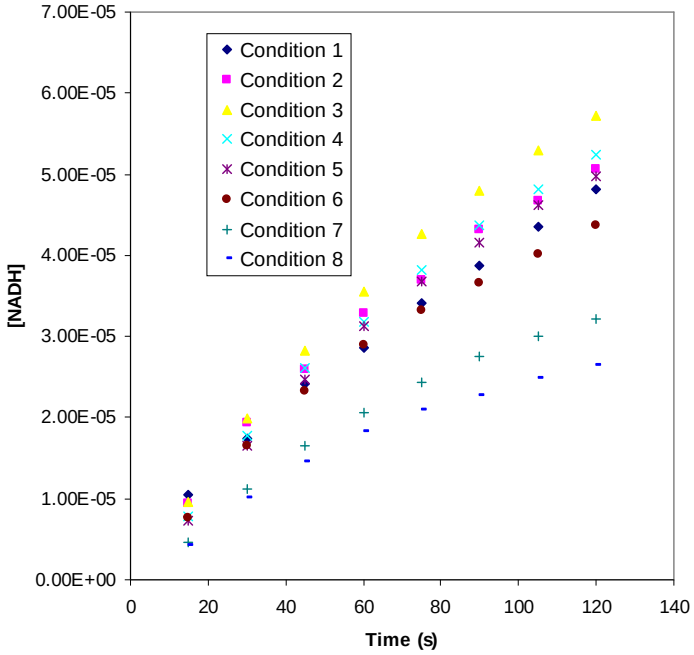

Figure 2 Production on NADH over time without inhibitor.

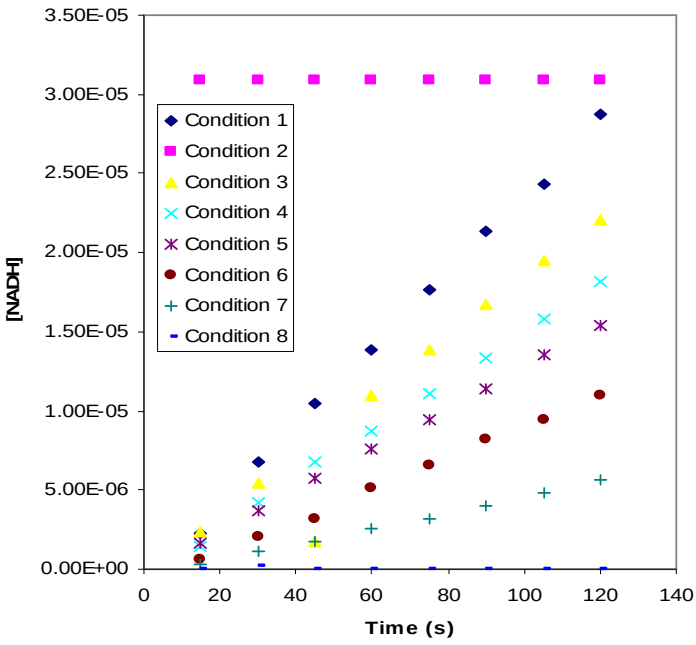

Figure 3 Production of NADH over time with inhibitor. The data for condition 2 was omitted in linearization since there was some error when adding substrate.

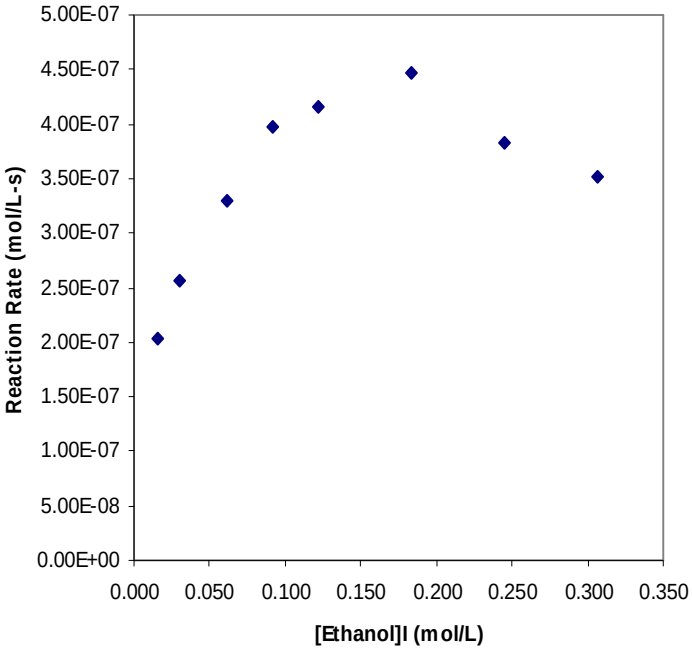

Figure 4 Michaelis-Menten curve for reaction velocity as a function of substrate without inhibitor.

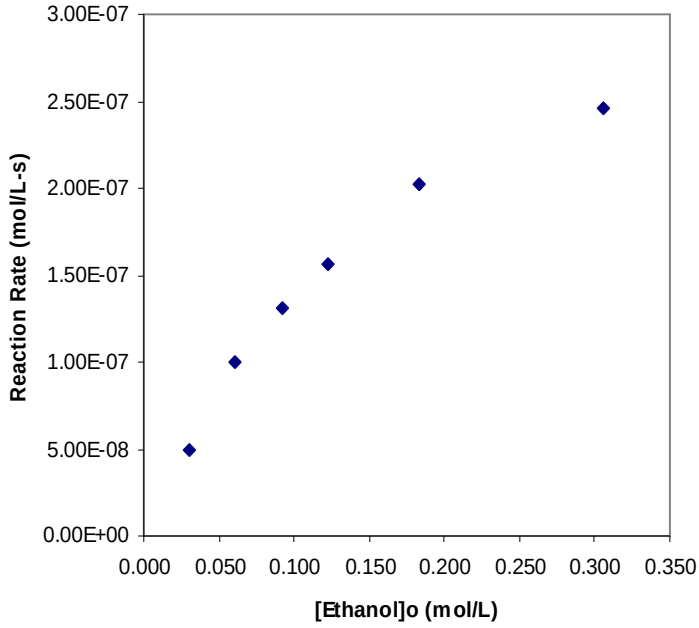

Figure 5 Michaelis-Menten curve for reaction velocity as a function of substrate with inhibitor.

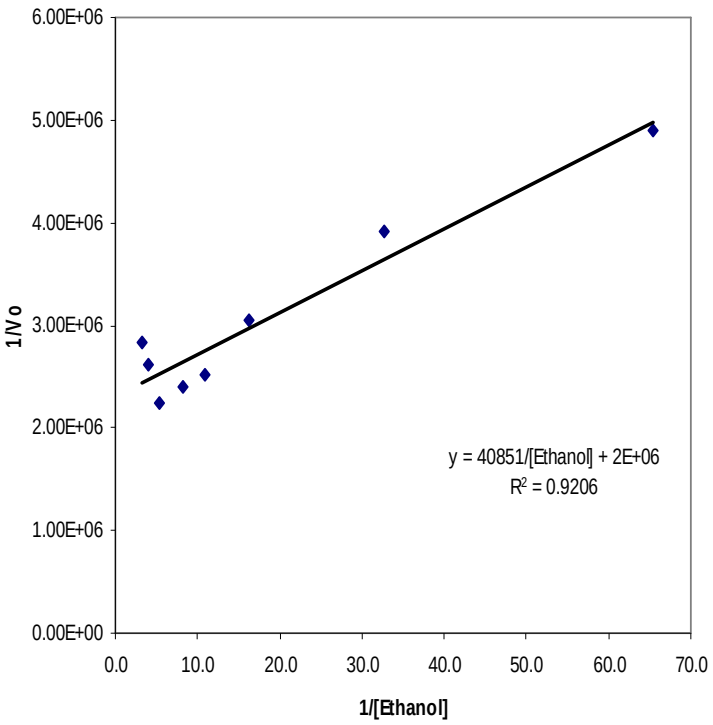

Figure 5 Lineweaver-Burke linearization without inhibitor.

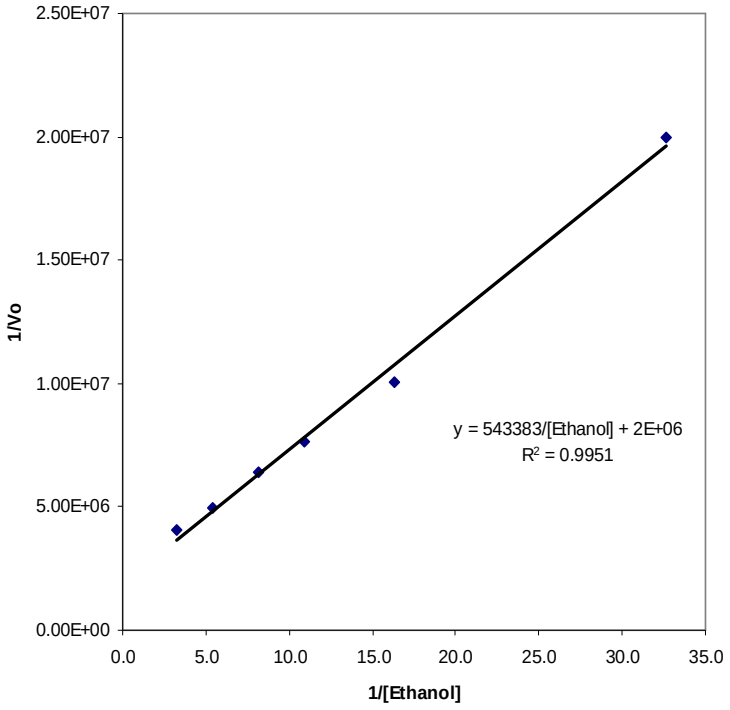

Figure 6 Lineweaver-Burke linearization with inhibitor.

The initial concentrations of ethanol and NAD+ are shown below in mol/L.

	1	2	3	4	5	6	7	8
[Ethanol]i	3.06E-01	2.45E-01	1.84E-01	1.22E-01	9.18E-02	6.12E-02	3.06E-02	1.53E-02
[NAD+]i	4.59E-04	4.59E-04	4.59E-04	4.59E-04	4.59E-04	4.59E-04	4.59E-04	4.59E-04

From the uninhibited kinetics, we have $\frac{1}{V_{\max}} = 2\times10^{6}\, s\cdot L/mol$ and $\frac{K_M}{V_{\max}} = 40851 s$. This gives $V_{\max} = 5\times10^{-7}\, mol/L\cdot s$ and $K_M = 0.02\, mol/L$. From the inhibited kinetics, we observe the same $V_{\max}$, indicating non-competitive behavior. We have $\frac{K_M'}{V_{\max}} = 543383 s$, thus $K_M' = 0.27\, mol/L$. Thus $K_M' = 13.5 K_M$, or 1250% inhibition. In other words, it takes a 12.5 factor increase in substrate in the inhibited reaction to achieve the same half-max velocity in the uninhibited reaction. Since $\alpha = 13.5$, we can determine the binding affinity of inhibitor relative to ethanol: $K_I = \frac{[I]}{\alpha - 1} = \frac{v_I [I]_0 V}{\alpha - 1} = 4.08\, mol/L$.

Appendix

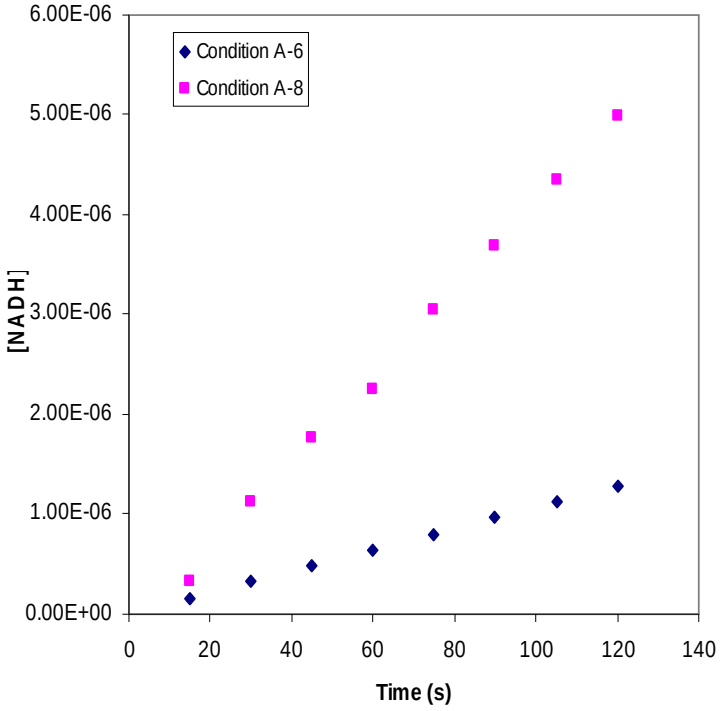

Figure 7 Production of NADH over time with inhibitor and 0.01 mg/mL ADH.

References

1. A study of the kinetics and mechanism of yeast alcohol dehydrogenase with a variety of substrates FM Dickinson, GP Monger. Biochem. J. (1973) 131, 261-270