A Kinetic Study of Yeast Alcohol Dehydrogenase

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Kinetic investigations are powerful techniques to probe the mechanisms of enzymes, but many biochemistry laboratory courses focus solely on single-substrate enzymes that display well-behaved Michaelis–Menton kinetics. Although this type of treatment may be wholly satisfactory in a general junior- or senior-level course, work intended for advanced undergraduate or graduate students should incorporate a system that is more challenging. When enzymes with two substrates (e.g., tyrosinase) are studied, one of the substrates is often treated as "invisible" (e.g., oxygen, in the case of tyrosinase). Such treatment has obvious pedagogical problems.

A Two-Substrate Enzyme System

A system that is more challenging than the single-substrate case and has only "visible" substrates is the yeast alcohol dehydrogenase (ADH) system in which the enzyme has two substrates—ethanol and NAD^+—both of which can be easily varied. Yeast ADH catalyzes the oxidation of ethanol by NAD^+, producing NADH and acetaldehyde.

$$CH_3CH_2OH + NAD^+ \rightleftarrows CH_3CHO + NADH + H^+ \quad (1)$$

The variation of ethanol concentration displays the expected Michaelis–Menton kinetics, whereas variation of NAD^+ concentration shows substrate inhibition. ADH has been studied in great depth and is considered a "classic" enzyme because many early enzyme studies were carried out with it (*1*, *2*).

ADH is a zinc-containing, NAD-dependent dehydrogenase. Two forms of the enzyme have been commonly studied, one isolated from liver, and the other from yeast. The liver enzyme is a dimer, and the yeast enzyme a tetramer. The crystal structure of the liver enzyme has been solved (*3*) as well as an enzyme·NAD^+·4-bromobenzyl alcohol complex (*4*).

In the oxidation of ethanol, the enzyme displays an ordered Bi–Bi mechanism. The ternary complex does not accumulate, giving rise to a classic Theorell–Chance mechanism. The rate-limiting step is the release of NADH from the E·NADH complex at low ionic strength and saturating substrate concentrations (*5*). Although there are many possible studies for this enzyme, I have used it primarily as a tool to introduce students to substrate inhibition of enzymes.

Experimental

The solutions listed in the table should be prepared for this experiment. All solutions are prepared using 20 mM Tris–Cl^- buffer, pH 8.8. It is very important that the water used to prepare the buffer be pure—free of both organic and inorganic contaminants. The amounts shown in the table assume that students will work alone and do not allow for waste.

Solutions to be Prepared

40 U/mL Yeast Alcohol Dehydrogenase (ADH)	3 mL/student
0.1% (V/V) Ethanol	40 mL/student
0.010 M NAD^+	17 mL/student

The rate of reaction can be determined by following NADH formation. Measurement of the NADH formation is quite easily accomplished by monitoring the absorbance of the reaction mixture at 340 nm. All volumes have been set to give a total of 6 mL, a convenient volume for Spectronic 20 cuvettes. It can be modified as needed. The concentration of ADH used here, 40 U/mL, is well-suited for situations in which the students have access to a stripchart recorder or other device to record absorbance versus time measurements. If students are simply reading a meter at a set time interval, the concentration of enzyme should be lowered to allow more accurate, less harried readings.

The ADH solution must be kept on ice to prevent loss of activity, and the other solutions should be kept at room temperature. Although the alcohol solution is stable over time, the ADH solution will lose activity overnight. The NAD^+ solution may be rendered useless by the formation of inhibitors if stored overnight.

Small changes in room temperature will not drastically affect the rate of reaction, and relatively few measurements are made in this experiment. Thus, thermostating of samples is not necessary if the room is reasonably temperature-stable. The measurements should nevertheless be carried out as expeditiously as possible in the absence of drafts, direct sunlight, or temperature changes.

Effect of Ethanol Concentration

The first study is the effect of ethanol concentration on the reaction rate. This gives students the results that they expect and builds confidence in their technique for the unexpected results in the second study. During this study the NAD^+ concentration will be kept constant at 1.67 mM (1.00 mL), and the concentration of ethanol will vary between 1.45 and 13.9 mM (0.50, 1.00, 2.00, 3.00, 4.00, and 4.80 mL of the 0.1% (V/V) ethanol stock solution).

Buffer is added to the NAD^+/ethanol mix to give a total

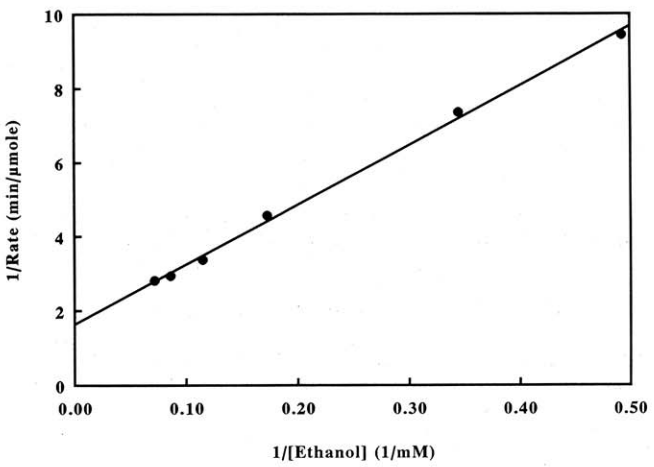

Figure 1. Double-reciprocal plot showing the dependence on ethanol concentration of the ADH-catalyzed oxidation of ethanol by NAD^+.

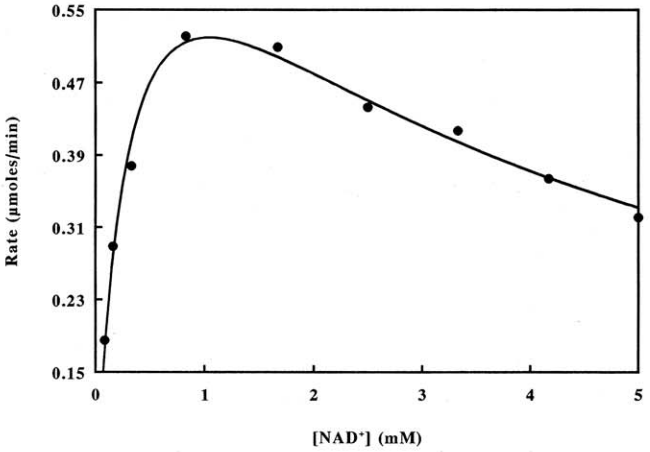

Figure 2. The dependence of the ADH-catalyzed oxidation of ethanol on NAD^+ concentration.

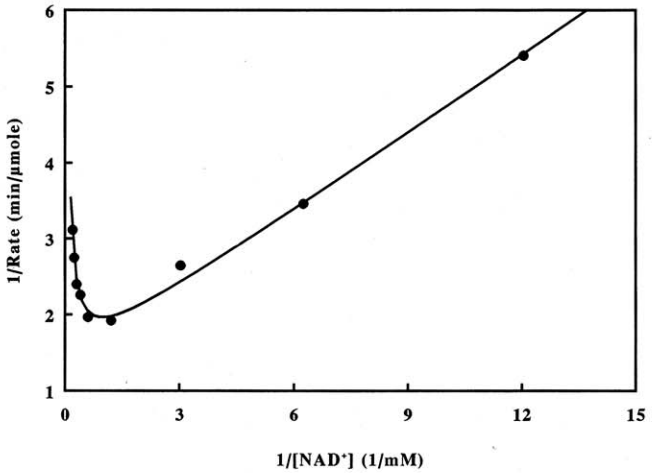

Figure 3. Double-reciprocal plot showing the classical hook shape indicating substrate inhibition by NAD^+.

volume of 5.8 mL. Add 0.2 mL of enzyme solution, and quickly but gently mix the solution. Then monitor the absorbance until the total absorbance change is at least 0.2 or monitor for 5 min, whichever is shorter. The increase in absorbance at 340 nm with time represents the production of NADH. This may be converted to the initial rate using the extinction coefficient for NADH, ϵ_{340} = 6,220 M^{-1} cm^{-1}, and the total volume of the assay.

These rates are plotted versus substrate concentration in a Michaelis–Menton plot or as the double-reciprocal Lineweaver–Burk plot to give the apparent V_{max} and K_M. Figure 1 is a graphical plot of student data showing the expected linear double-reciprocal plot.

Effect of NAD^+ Concentration

The second study is the variation of NAD^+ concentration from 0.083 to 5 mM (0.05, 0.10, 0.20, 0.5, 1.0, 1.5, 2, 2.5, 3.0 mL of the 0.010 M NAD^+ stock solution) at an ethanol concentration of 7.25 mM (2.5 mL of the 0.1% (V/V) ethanol stock solution). It will again be necessary for the students to calculate the initial velocity of each reaction before plotting the reaction rate versus NAD^+ concentration. Figure 2 shows the Michaelis–Menton plot of this data, showing that high concentrations of NAD^+ slow the rate of reaction. Figure 3 shows the hooked double-reciprocal plot characteristic of substrate inhibition.

With No Substrate Inhibition

When substrate inhibition is not displayed, as when ethanol concentration is varied, the Michaelis–Menton plots (eq 2) will be curved, whereas the double-reciprocal plot (eq 3) will be linear.

$$V = V_{max}\frac{[S]}{[S] + K_M} \tag{2}$$

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}}\frac{1}{[S]} \tag{3}$$

With Substrate Inhibition

When substrate inhibition is displayed, as when NAD^+ concentration is varied, both the Michaelis–Menton plots (eq 4) and the double-reciprocal plot (eq 5) will be curved.

$$V = \frac{V_{max}}{1 + \frac{K_M}{[S]} + \frac{[S]}{K_{si}}} \tag{4}$$

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}}\frac{1}{[S]} + \frac{V_{max}}{K_{si}}\frac{1}{1/[S]} \tag{5}$$

Data Analysis

The curved nature of these plots makes graphical data analysis difficult beyond the simple shape of the curve. It is also somewhat less than satisfying to the student. In my teaching labs I have used the EZ-FIT program, written and distributed by Frank W. Perrella (Medical Products Department, E.I. Du Pont de Nemours) to allow students to analyze their data. This program was written specifically to analyze enzyme kinetic data and gives nice graphical output. Another choice for nonlinear fitting is the BASIC routine of Duggleby (*6*), which can be easily modified to fit any equation. Either of these two choices will also allow the instructor to introduce the students to computer methods of data reduction.

Conclusion

The study of more-complex enzyme kinetics enriches the student's biochemistry laboratory experience, and the study of ADH is an excellent means to obtain that goal. The study of this enzyme system in the teaching lab may be expanded even further by the use of other alcohols as inhibitors or alternate substrates.

Literature Cited

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