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A general method for the analysis of random bisubstrate enzyme mechanisms

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Abstract In the present communication, a general method for the kinetic analysis of random bisubstrate mechanisms is described. The method comprises a stepwise application of the following kinetic and ligand-binding experiments: determination of steady-state kinetic constants, product inhibition patterns, maximum rate relationships, application of alternate substrates, application of dead-end inhibitors, direct binding of substrates, kinetic isotope effects, and isotope exchange studies. This general method was applied to a practical example: a yeast alcohol dehydrogenase-catalyzed oxidation of 2-propanol by NAD^+ at pH 7.0, 25°C. It was found that this fully reversible reaction proceeds by a steady-state random Bi-Bi mechanism, whereby both dead-end complexes are formed.

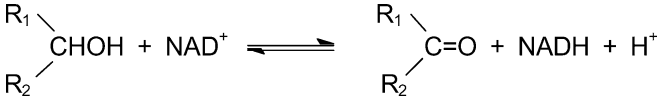
Keywords Yeast alcohol dehydrogenase · Kinetic mechanism of action

Introduction

Bisubstrate reactions are probably the most common type of reaction that occurs with enzyme-catalyzed processes in nature [1]. In bisubstrate reactions, both the ordered and the random-order addition of substrates were observed. However, it appeared to us that the latter type of reaction predominates in nature. The kinetic analysis of random-order bisubstrate reactions is rather difficult, because it can evoke several different kinetic mechanisms, such as rapid equilibrium random, steady-state random, or preferred-order bisubstrate mechanisms.

Recently, a textbook entitled "Comprehensive enzyme kinetics" was published, featuring a systematic analysis of enzyme kinetic mechanisms [2]. The principles of kinetic analysis outlined in that textbook have been applied in this work, in order to develop a general method for the analysis of random bisubstrate mechanisms. In order to support the general method described in this work with a practical example, the yeast alcohol dehydrogenase-catalyzed oxidation of alcohols by NAD^+ was chosen as a basis for analysis.

Yeast alcohol dehydrogenase (EC 1.1.1.1, constitutive, cytoplasmic) catalyzes the following reversible bisubstrate reaction (Scheme 1):



The experimental data reported on the steady-state kinetic and ligand-binding properties of this enzyme are exceptionally abundant, which considerably facilitates the kinetic analysis [3]. Specifically, the oxidation of 2-propanol to acetone by NAD^+ was chosen as a framework for kinetic analysis in this work.

Materials and methods

Materials

The kinetic measurements in this work, described by Leskovac and co-workers, were performed with yeast alcohol dehydrogenase preparations (lyophilized powder) from Boehringer Mannheim. The kinetic measurements, described by Dickinson and co-workers, were performed with a homemade preparation of yeast alcohol dehydrogenase [4].

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Methods

The concentration of the enzyme protein in solution was determined according to Hayes and Velick [5] and the concentration of enzyme active sites by the fluorescent method of Leskovac et al. [6]; and all enzyme concentrations in this work are given as the concentration of enzyme active sites. Initial velocity studies were performed and reaction progress curves were recorded using a double-beam spectrophotometer; and reaction rates were determined from the initial linear phase of reaction progress curves in 0.1 M sodium phosphate buffer, pH 7.0, at 25°C.

The initial rate data were collected at several different concentrations of a variable and a constant substrate. The initial rate data in the forward direction (oxidation of alcohols, obtained by varying the concentration levels of both substrates) were fitted to Eq. 1 with the Fortran program of Cleland [7]:

$$\frac{v_0}{E_0} = \frac{V_1 AB}{K_{iA}K_B + K_B A + K_A B + AB} \quad (1)$$

where v_0 is the initial rate (M s^{-1}), E_0 is the concentration of enzyme active sites (M), V_1 is the catalytic constant in the forward direction (s^{-1}), K_A and K_B are the Michaelis constants for NAD^+ and alcohols (M), K_{iA} is the inhibitory constant for NAD^+ (M), and A and B are the concentrations of NAD^+ and alcohols (M), respectively.

In the fully reversible Bi-Bi mechanism, the rate equation in the reverse direction is analogous to Eq. 2:

$$\frac{v_0}{E_0} = \frac{V_2 PQ}{K_{iQ}K_P + K_P Q + K_Q P + PQ} \quad (2)$$

In the reverse direction, reduction of aldehydes or ketones, V_2 is the catalytic constant (s^{-1}), K_Q and K_P are the Michaelis constants for NADH and aldehydes or ketones (M), K_{iQ} is the inhibitory constant for NADH (M), and Q and P are the concentrations of NADH and aldehydes or ketones (M), respectively [2]. Statistical evaluation of initial rate and ligand-binding data are described in detail in the corresponding source references [4, 8–11].

Results

The general method for the kinetic analysis of random bisubstrate mechanisms, as described in this work, comprises the stepwise application of the initial rate and ligand-binding experiments listed in Table 1. The sequence of experiments in Table 1 is found to be the best approach for the practical application of this method.

This general method provides an analytical basis for the study of all random bisubstrate reactions that occur in nature. As we stated in the opening section, in order to illustrate the method with a practical example, the oxidation of 2-propanol to acetone by NAD^+ was chosen as a framework for analysis, since the experimental data in the literature reported for the steady-state kinetic and ligand-binding properties of this reaction are abundant. In doing so, we give the reason for performing the particular analysis, the expected results, the actual observations, and the implication or conclusion from the observed results.

Steady-state kinetic constants with various substrates

The acquisition of steady-state kinetic constants for a particular enzyme reaction is always the first step in analysis; and this analysis provides the first insight into the nature of enzyme-catalyzed reaction. Table 2 shows the steady-state kinetic constants for various substrates of yeast alcohol dehydrogenase.

Product inhibition patterns

Product inhibition is often the best means for distinguishing between different mechanisms, since different mechanisms usually afford very different product inhibition patterns [2]. Figure 1 shows the product inhibition patterns for yeast alcohol dehydrogenase-catalyzed oxidation of 2-propanol by NAD^+.

Figure 1 shows only two product inhibition patterns for the forward reaction, the oxidation of 2-propanol with NAD^+. In order to save space, the remaining

Table 1 A general method for a stepwise analysis of random bisubstrate mechanisms applied to yeast enzyme alcohol dehydrogenase-catalyzed oxidation of 2-propanol with NAD^+, at pH 7.0, 25°C

Number	Method	Forward reaction	Reverse reaction	Source of experimental data
1	Steady-state kinetic constants	+[a]	+	[4, 8]
2	Product inhibition patterns, primary and secondary double-reciprocal plots	+	+	[8]
3	Maximum rate relationships	+	+	[8]
4	Alternative substrates	+	+	[3, 4, 8, 11]
5	Effects of dead-end inhibitors	+	–	[8–10]
6	Direct binding of substrates	+	+	[8, 15, 16]
7	Kinetic isotope effects	+	–	[8]
8	Isotope exchange studies	–	–	–
9	Nonhyperbolic secondary plots	+	–	[8]
10	Direct estimation of rate constants	+	+	[19]

[a]Data for the acetone/2-propanol reaction were collected and analyzed in this work

Table 2 Kinetic constants of yeast alcohol dehydrogenase-catalyzed reactions with various pairs of substrates, measured at pH 7.0, 25°C. The symbol ≈ indicates extrapolated values

Constant	Ethanol/ acetaldehyde[a]	Propanol/ propionaldehyde[a]	Butanol/ butyraldehyde[a]	2-Propanol/ acetone[b]	2-Butanol/ 2-butanone[b]
K_A(μM)	109	150	250	597	376
K_B(mM)	21.7	29.2	32	117	35
K_{iA}(μM)	325	235	156	378	398
$V_1(s^{-1})$	454	66.7	25	7	0.86
K_Q(μM)	96	≈96	97	43	38
K_P(mM)	0.93	–	27.6	477	285
K_{iQ}(μM)	12.5	≈14	15.4	17.5	15.2
$V_2(s^{-1})$	3,846	≈3,650	3,448	9	0.7
K_A/V_1(μM s^{-1})	0.24	0.24	10	86	435
K_B/V_1(μM s^{-1})	48	438	1,280	25.3	40.8
$K_{IA}V_1/K_AV_2$	0.352	–	0.0045	0.492	1.30
K_Q/V_2(μM s^{-1})	0.025	≈0.0265	0.028	4.8	5.6
K_P/V_2(μM s^{-1})	0.24	–	8.0	54,160	423,105
$K_{IQ}V_2/K_QV_1$	1.10	≈1.14	1.28	0.52	0.315

[a] Calculated from the data of Dickinson and Monger [4]
[b] Calculated from the data of Trivić and Leskovac [8]

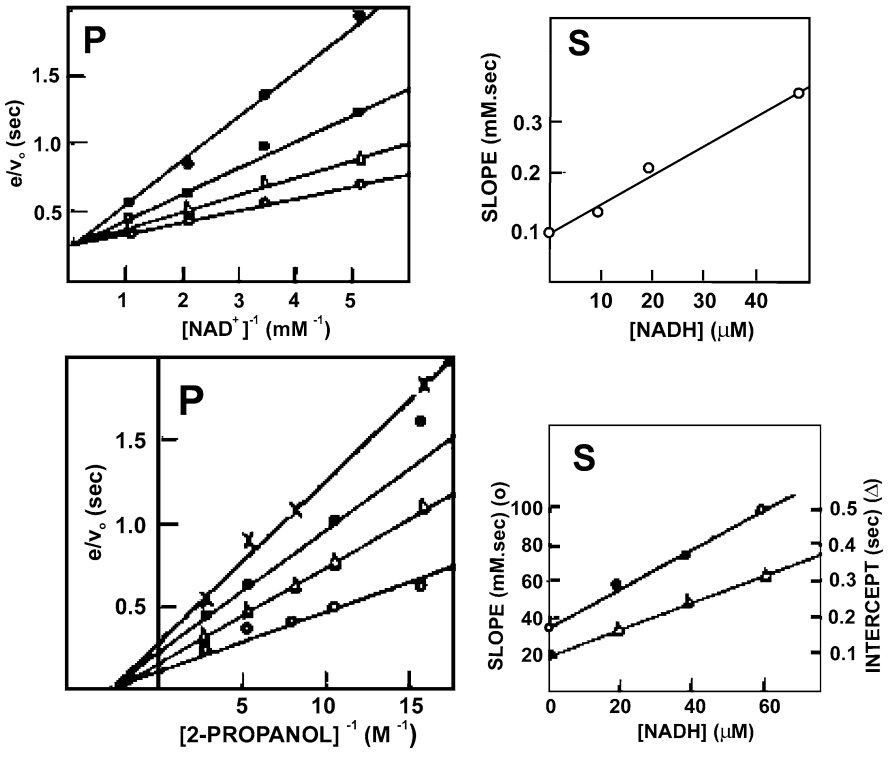


Fig. 1 Product inhibition patterns in yeast alcohol dehydrogenase-catalyzed reactions at pH 7.0, 25°C, in primary (*P*) and secondary plots (*S*). *Top* Oxidation of 2-propanol (243.3 mM) by increasing concentrations of NAD^+ (0.195, 0.292, 0.484, 0.965 mM) in the presence of increasing concentrations of NADH (0, 9.8, 19.5, 48.1 μM). *Bottom* Reduction of NAD^+ (0.81 mM) with increasing concentrations of 2-propanol (63.7, 95.3, 126.8, 189.2, 373 mM) in the presence of increasing concentrations of NADH (0, 18.8, 37.2, 59.2 μM)

patterns are shown in Table 3, which lists qualitatively the complete product inhibition patterns for the redox pair 2-propanol/acetone, both in the forward and reverse directions.

If the combination of reactants in bisubstrate reactions is random, all product inhibition patterns are competitive, except between the molecules that can form dead-end complexes with the enzyme; and these are

Table 3 Product inhibition patterns for the redox pair 2-propanol/acetone, measured at pH 7.0, 25°C [8]. A, B, P, Q, and E, respectively denote NAD^+, 2-propanol, acetone, NADH, and enzyme

Experiment	Substrate		Product inhibitor	Pattern		Dead-end complex
	Variable	Fixed		Primary plot	Secondary plot	
1	A	[B] = 2.1 K_B	Q	Competitive	Linear	–
2	B	[A] = 1.4 K_A	Q	Noncompetitive	Linear	EBQ
3	A	[B] = 0.9 K_B	P	Noncompetitive	Linear	EAP
4	B	[A] = 0.8 K_A	P	Competitive	Parabolic	(PEP)
5	Q	[P] = 1.3 K_P	A	Competitive	Linear	–
6	P	[Q] = 2.2 K_Q	A	Noncompetitive	Linear	EAP
7	Q	[P] = 0.5 K_P	B	Noncompetitive	Linear	EBQ
8	P	[Q] = 2.9 K_Q	B	Competitive	Linear	–

noncompetitive. The two reactants lacking the piece transferred during the reaction will always form a dead-end complex with the enzyme, while the two reactants possessing this piece may or may not form such a complex [2, 12]. Thus, in Table 3, we have two competitive and two noncompetitive patterns in each direction, showing that both dead-end complexes, EAP and EBQ, are formed.

Maximum rate relationships

The maximum rate relationships were defined by Dalziel [13] as:

$$1 \leq K_{iA}V_1/K_AV_2 \quad \text{and} \quad 1 \leq K_{iQ}V_2/K_QV_1 \tag{3}$$

For a simple ordered Bi-Bi mechanism, the above ratios are always greater than unity; and values close to unity indicate a Theorell–Chance mechanism [2]. Thus, when these criteria are applied to the redox pair 2-propanol/acetone, it appears that the ordered mechanism is excluded in both directions (Table 2).

Alternative substrates

Table 2 shows the comparison of kinetic constants for the redox pair 2-propanol/acetone with kinetic constants for the redox pairs of several alternative substrates. A difference between the ordered and random-order addition of substrates is the comparative values of the kinetic constants K_{iA} and K_A/V_1 in the forward direction and the kinetic constants K_{iQ} and K_Q/V_2 in the reverse direction. A constant value of K_A/V_1 or K_Q/V_2 indicates an ordered addition of substrates, while the opposite is true for the random addition of substrates [14].

In Table 2, in the acetone/2-propanol reaction, the value of K_A/V_1 changes considerably with substrate. Also, the value of K_Q/V_2 changes considerably in going from acetaldehyde to acetone or 2-butanone as a substrate. Thus, both cases are compatible with the random addition of substrates. In contrast, the values of K_{iA} and K_{iQ} are nearly constant for all substrates in Table 2, which is not incompatible with the random addition of substrates [14]. Thus, the above analysis by both criteria clearly indicates that, in both directions, the addition of substrates is random and not ordered [2].

Effects of dead-end inhibitors

A further distinction between the ordered and random addition of substrates can be made by using dead-end inhibitors. In the yeast alcohol dehydrogenase-catalyzed reaction, pyrazole is a dead-end inhibitor competitive with respect to 2-propanol. Pyrazole acts as a competitive inhibitor for 2-propanol in the presence of subsaturating amounts of NAD^+; and it acts as a noncompetitive inhibitor for NAD^+ in the presence of subsaturating amounts of 2-propanol [8, 9]. This kinetic criterion also suggests that, in the acetone/2-propanol reaction, the addition of substrates on the alcohol side is random, because the ordered addition would afford an uncompetitive pattern with NAD^+ and a competitive pattern with 2-propanol [2].

Direct binding of substrates

In a preferred-order mechanism, K_{iA} and K_{iQ} represent the dissociation constants of NAD^+ and NADH from their respective binary complexes with enzyme and are independent of the structure of substrates [13]. This expectation is met with K_{iA} and K_{iQ}, especially with regard to difficulties in obtaining K_{iQ} (Table 1).

An independent estimation of the dissociation constants of NAD^+ and NADH from their binary complexes with enzyme indicates that they are practically identical with K_{iA} and K_{iQ} at neutral pH [15, 16].

Deuterium kinetic isotope effects

The primary deuterium isotope effects of yeast alcohol dehydrogenase-catalyzed oxidation of 2-propanol to acetone, at pH 7.0, are: $V_1{}^D = 2.20 \pm 0.20$, $^D(V_1/K_A) = 3.23 \pm 0.30$, and $^D(V_1/K_B) = 2.54 \pm 0.15$ [8]. The value of $^D(V_1/K_A)$ is significantly larger than $^D(V_1/K_B)$, which indicates that, on the alcohol side, NAD^+ adds before the alcohol. In a sequential mechanism, an isotope effect equal or close to one on one of the two substrate V/K values suggests a steady-state ordered mechanism; and this is obviously not the case in this reaction. Finite but unequal isotope effects on the two substrate V/K values suggest a steady-state random kinetic mechanism, which is obviously the case in the forward direction [2].

Isotope exchange studies

Isotope exchange studies not only tell the subtle differences between the ordered and random addition of substrates, but can also tell the differences between the rapid equilibrium and steady-state random addition of substrates [2]. However, isotope exchange studies with the redox pair 2-propanol/acetone were not reported in the literature.

Nonhyperbolic secondary plots

Generally, the nonhyperbolic secondary plots present complexities that complicate the kinetic analysis. Table 3 shows that, in the acetone/2-propanol reaction, all the secondary plots were linear, except the plot of $1/v_0$ vs

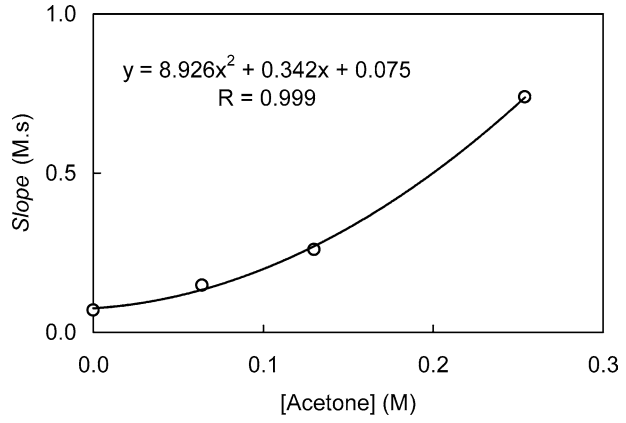
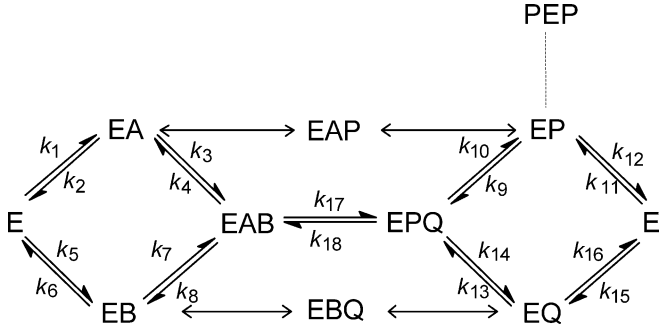


Fig. 2 A nonhyperbolic secondary plot for the oxidation of increasing concentrations of 2-propanol by NAD^+ (0.49 mM) in the presence of increasing concentrations of acetone (0, 65.2, 129.9, 253.4 mM). The primary double-reciprocal plot was competitive; and the figure represents the re-plot of the slope function from the primary plot

1/[B] in the presence of constant [A] and increasing [P]; and this plot was parabolic (Fig. 2).

$$v_0 = \frac{V_1 V_2 \left(\mathrm{AB} - \frac{\mathrm{PQ}}{K_{\mathrm{eq}}}\right)}{V_2 K_{\mathrm{iA}} K_{\mathrm{B}} + V_2 K_{\mathrm{B}} \mathrm{A} + V_2 K_{\mathrm{A}} \mathrm{B} + V_2 \mathrm{AB} + \frac{V_1 K_{\mathrm{P}}}{K_{\mathrm{eq}}} \mathrm{Q} + \frac{V_1 K_{\mathrm{Q}}}{K_{\mathrm{eq}}} \mathrm{P} + \frac{V_1}{K_{\mathrm{eq}}} \mathrm{PQ}} \quad (4)$$

Generally, parabolic secondary plots are caused by the combination of at least two molecules of the inhibitor with some form of the enzyme [2, 12, 17, 18]. Thus, a nonlinear parabolic plot in Fig. 2 indicates that two molecules of an inhibitor (acetone) are bound to some form of the enzyme. We assume that this form is an EP complex and consequently a PEP complex is formed (Scheme 2).



Direct estimation of rate constants

The numerical values of some of the rate constants in Scheme 2 were estimated by kinetic methods [19] and are presented in Table 4; and the implications of these values for the kinetics mechanism are put forward in the Discussion.

Table 4 Rate constants in Scheme 2 for the redox pair ethanol/acetaldehyde, measured at pH 7.0, 25 °C [19]

Forward direction	S^{-1}	Reverse direction	S^{-1}
k_2	2,100	k_{12}	388
k_{17}	4,000	k_{18}	35,000

Discussion

The experimental data outlined in the Results indicate that the reversible oxidation of 2-propanol by NAD^+ proceeds by the mechanism shown in Scheme 2. Overwhelming kinetic evidence indicates that the addition of substrates is random in both directions, including the product inhibition patterns, maximum rate relationships, alternative substrates, and the direct binding of substrates. The effects of dead-end inhibitors and deuterium isotope effects also indicate the random binding from the alcohol side of the reaction.

The general rate equation for the rapid equilibrium random Bi-Bi system, with both dead-end complexes included, is [2]:

From Eq. 4, it follows that if the combination of reactants is random, all product inhibition patterns are competitive, except between the molecules that can form dead-end complexes with the enzyme; and these are noncompetitive. All kinetic constants are equal to the dissociation constants of various forms of enzyme complex.

In Table 3, we have two competitive and two noncompetitive patterns in each direction, showing that both dead-end complexes, EAP and EBQ, are formed. Rigorously, Eq. 4 applies only to rapid equilibrium mechanisms, which in the pure form are rare. However, simulation studies show that random mechanisms, unless very unusual values are assumed for the rate constants, resemble rapid equilibrium mechanisms in their initial velocity and product inhibition patterns, even though the rate-limiting step is not solely the interconversion of two central complexes [12]. Therefore, the initial velocity and product inhibition patterns for the rapid equilibrium and steady-state random mechanisms are very similar. The initial velocity patterns in a steady-state random mechanism may seem linear, depending on the values of the off-rate constants for the substrates from their complexes with enzyme, relative to k_{cat}. These patterns can be fitted to Eq. 4, but this fit gives incorrect values for the dissociation constants [18].

Thus, the real problem in random mechanisms is to distinguish between the *rapid equilibrium* and the

steady-state condition. In our particular case, from the alcohol side of the reaction, the kinetic mechanism is steady-state random, as evidenced by the deuterium kinetic isotope effects.

Further evidence for a steady-state mechanism comes from the direct estimation of the magnitude of rate constants. For a mechanism in Scheme 2 to become a rapid equilibrium random, only the off-rate constants of A and B from their binary complexes have to be much faster then k_{cat}; and similarly, in the reverse direction, only the off-rate constants of P and Q from their binary complexes have to be much faster then k_{cat}. A direct measurement of rate constants for the redox pair ethanol/acetaldehyde was reported, which indicates the following numerical values (Table 4).

The values of rate constants k_{17} and k_{18} in Scheme 2 for the redox pair 2-propanol/acetone are not known. However, it is obvious from the above analysis that, for this redox pair, $k_2 \leq k_{17}$. Consequently, it is very unlikely that $k_{12} \gg k_{18}$. The latter inequality strongly suggests that the mechanism is also steady-state random in the reverse direction.

The above analysis indicates that a difference between a random and an ordered addition in either direction is easily obtained by a variety of kinetic methods. A distinction between the rapid equilibrium and steady-state condition is much more difficult to obtain; and kinetic isotope effects are usually necessary to establish this. In doubtful cases, the isotope exchange methods can afford definitive conclusions [2].

A direct estimation of all or most rate constants in the mechanism is the most powerful method to determine the mechanism. However, this approach falls outside the scope of this work, as it is often technically extremely demanding.

The nonlinear parabolic secondary plots indicate the binding of at least two molecules of substrate to some form of the enzyme (Fig. 2, Scheme 2). A kinetic analysis in such cases may become very demanding. Such cases also fall outside the scope of this communication and will be treated in detail in a forthcoming communication dealing with this topic.

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