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Kinetic delay of cyclization/elimination-coupled enzyme assays: Analysis and solution

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ABSTRACT

A kinetic analysis of an enzyme assay employing a synthetic substrate that produces a detectable signal through a spontaneous organic cyclization/elimination reaction following the enzymatic reaction was conducted. The results from the calculation were used to predict the lag period and provide accurate measurements of the activity of alkaline phosphatase using the fluorogenic substrate (**1**).

Enzyme assays such as chromogenic assays and fluorogenic assays allow simple, sensitive, and rapid measurements of enzyme activities,[1,2] which are of great importance in diverse fields including diagnostics,[3] drug discovery,[4] cellular imaging,[5] and enzyme engineering and evolution.[6,7] Depending on the process of signal generation, enzyme assays are commonly classified into direct assays where substrates generate optical signals directly in response to enzymatic reactions, and coupled assays where an additional enzyme is usually employed to turn over the product of the initial enzymatic reaction to produce detectable signals. The kinetics of both types of enzyme assays have been well documented and widely applied in following activities of enzymes.[1,2,8,9] Recently, an increasing number of organic reaction (e.g., cyclization and elimination) coupled enzyme assays have been developed using chromogenic/fluorogenic substrates. As outlined in Scheme 1, these substrates function in two consecutive steps: (1) the enzymatic reaction of interest produces an unstable intermediate (I); and (2) the intermediate rapidly breaks down through a spontaneous cyclization reaction or a β-elimination reaction, releasing a chromogenic/fluorescent molecule (P). These assays can be viewed as a new type of direct enzyme assay since no additional enzyme/reagent is required to generate a detectable signal. Their kinetics, however, shares some characteristics with the coupled enzyme assay; the organic reaction is an additional step following the enzymatic reaction, which plays an important role when considering the kinetics of the whole process.[8,9] Compared to direct assays, the cyclization/elimination-coupled enzyme assays often show decreased background signal and improved substrate binding affinity.[2] More importantly, they provide a practical means for measuring the activities of enzymes that have been a challenge to evaluate by direct assays.[1,2] In addition, they avoid the complexities and potential errors involved in performing a coupled enzyme assay.[10] So far, this strategy has been successfully applied in numerous enzyme systems including catalytic antibodies,[11] proteases,[12,13] epoxide hydrolases,[14] phosphatases,[15–18] lipases and esterases,[18,19] β-lactamases,[20,21] tyrosinase,[22] coenzyme Q,[23] monoamine oxidases,[24,25] DT diaphorase,[26] ceramidases,[27] serum paraoxonases,[28] sphingosine-1-phosphate lyase,[29] Baeyer–Villiger monooxygenases,[30] hydroxylases,[30] lactonases,[30] and prostate specific antigen.[31]

Even though cyclization/elimination-coupled enzyme assays are widely used, to the best of our knowledge, there is no formal report about the kinetics of the assays, which can be used to determine the rate of the enzymatic reaction accurately. To facilitate future applications of these assays, herein, we carry out a detailed kinetics analysis of this system (Scheme 1) by viewing the cyclization/elimination reaction as a coupled step to the enzymatic reaction.[8,9] To use this assay to accurately determine the rate of the enzymatic reaction, it is important that one starts to record the data when the measured rate of the cyclization/elimination reaction (V_2) becomes equal to the rate of the enzymatic reaction (V_1) under experimental conditions—in other words, only if a steady state is reached (Scheme 1). Since the measurements are taken during the initial rate period when the concentration of the initial substrate is bigger than the Michaelis constant (K_m) and only a small fraction of the substrate is turned over by the enzyme (E), E is fully saturated by the substrate (S), therefore the rate equation

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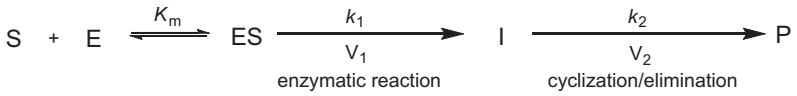


Scheme 1. Kinetic scheme for a cyclization/elimination-coupled enzyme assay.

Table 1
Relationship between t^* and $t_{1/2}$ for different assay accuracies

$[I]/[I]_{ss}$·100% (%)	t^*
99	$6.65 \times t_{1/2}$
95	$4.32 \times t_{1/2}$
90	$3.32 \times t_{1/2}$

of the enzymatic reaction is $V_1 = k_1[ES] \approx k_1[E_0]$ (E_0 is the total concentration of the enzyme used in the assay). The product of this reaction, I is depleted irreversibly from the enzymatic reaction through a first-order intramolecular cyclization/elimination reaction with a rate constant of k_2.

It is important to establish whether the steady state (i.e., $V_2 = V_1$) is likely to occur within a reasonable period of time. Moreover, a quantitative prediction of the lag period before the steady state is reached would serve as an indispensable guideline for when to begin collection of data points. Based on the discussion above, the rate equation for the enzymatic reaction in Scheme 1 can be shown as

$$\frac{d[I]}{dt} = k_1[E] - k_2[I] \quad (1)$$

Integrate both sides of Eq. 1,

$$[I] = \frac{k_1[E]}{k_2}(1 - e^{-k_2 t}) \quad (2)$$

At the steady state ($t \to \infty$)

$$\frac{d[I]}{dt} = k_1[E] - k_2[I]_{ss} = 0 \quad (3)$$

Thus

$$[I]_{ss} = \frac{k_1[E]}{k_2} \quad (4)$$

Take the natural log of both sides of Eq. 2 and rewrite the equation as

$$\ln\left[1 - \frac{k_2}{k_1[E]}[I]\right] = -k_2 t \quad (5)$$

Plug Eq. 4 into Eq. 5,

$$\ln\left[1 - [I]/[I]_{ss}\right] = -k_2 t \quad (6)$$

Therefore, the time required to reach a certain [I] can be calculated by

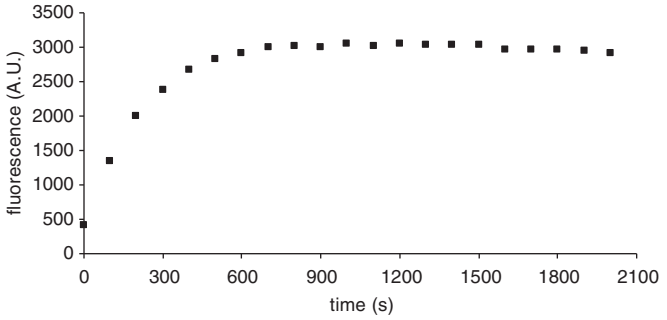


Figure 1. Time courses of cyclization reactions of **2** in 10 mM Tris buffer at pH 8.0. The initial concentration of **2** for the measurement was 100 μM. The increase in fluorescence intensity (I, λ_{em} = 460 nm) was recorded.

$$t^* = -\frac{\ln(1 - [I]/[I]_{ss})}{k_2} \quad (7)$$

Since the conversion of I to P is a first-order reaction, the rate constant is defined as

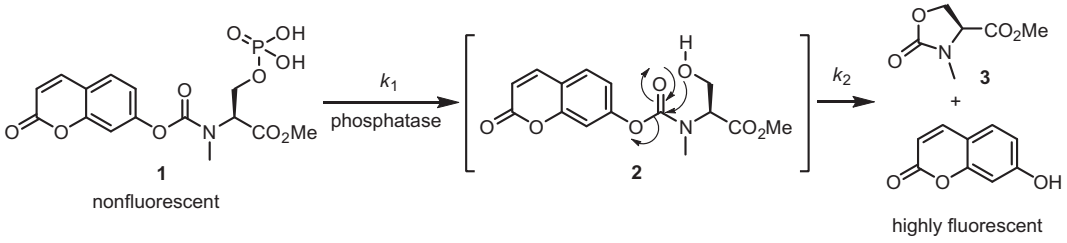
$$k_2 = \frac{\ln 2}{t_{1/2}} \quad (8)$$

Thus

$$t^* = -\frac{\ln(1 - [I]/[I]_{ss})}{\ln 2} \cdot t_{1/2} \quad (9)$$

As shown in Eq. 9, the time required (t^*) to reach a certain percentage of the steady state of the coupled enzyme system ($[I]/[I]_{ss}$) is directly and only dependent on the half life ($t_{1/2}$) of the coupled cyclization/elimination reaction that follows the enzymatic reaction of interest. Table 1 lists simple calculations of t^* values that can be used to obtain a desired assay accuracy. For a precise kinetic study, the value of $[I]/[I]_{ss}$ should be >99%, so one needs to pre-incubate the assay mixture for a period of $6.65 \times t_{1/2}$ before collecting the first data point. In more qualitative measurements, the corresponding value of $[I]/[I]_{ss}$ may be lowered to 95% or 90%. To achieve these accuracies, one can start recording the data of the corresponding assay after $4.32 \times t_{1/2}$ and $3.32 \times t_{1/2}$, respectively.

Next, we applied this kinetic analysis to the measurement of the activity of alkaline phosphatase (AP) using fluorogenic substrate (**1**).[32] As outlined in Scheme 2, when treated with AP, the O–P bond in the nonfluorescent substrate (**1**) is hydrolyzed generate the unstable intermediate (**2**). This intermediate undergoes a rapid cyclization reaction to produce **3** while releasing the strongly fluorescent molecule 7-hydroxycoumarin.



Scheme 2. Function of fluorogenic substrate (**1**) of AP.

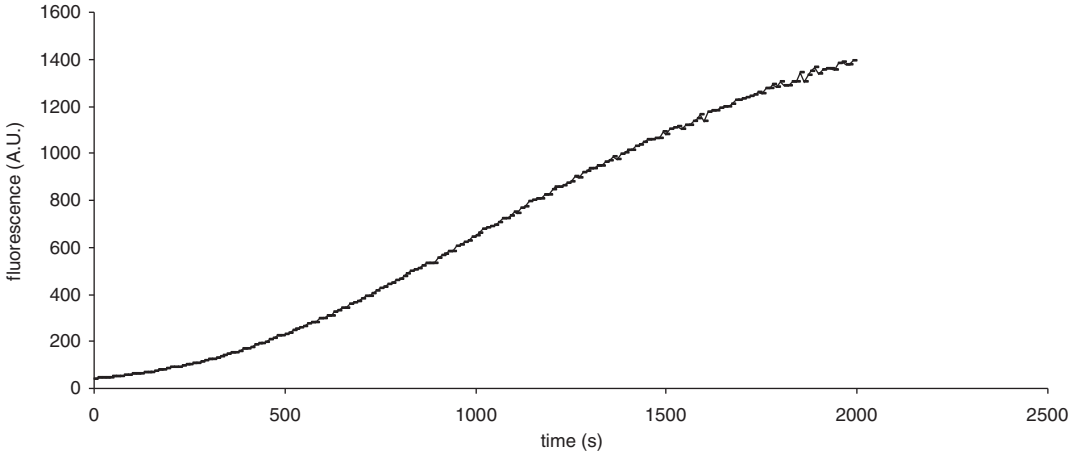


Figure 2. The activity of AP (Sigma P6774, from bovine intestinal mucosa) was measured in 96-well microtiter plates with **1** as the substrate. The enzymatic reaction mixture (50 μL) consisted of 10 mM Tris buffer (pH 8.0) and 1 mM $MgCl_2$. Substrates were added as solutions in DMSO. The overall concentration of DMSO was 10% for each measurement. The enzymatic reaction was run at 37 °C and initiated by the addition of substrates. For fluorogenic substrate **1**, the fluorescence emission generated from the reaction was measured using a Galaxy microplate reader (FLUOStar, BMG Labtechnologies) with an excitation filter of 340 nm and an emission filter of 460 nm.

Under the same conditions that were used for the AP activity assay (10 mM Tris buffer, pH 8.0), the half life ($t_{1/2}$) of the cyclization reaction of **2** was determined to be 100 s (Fig. 1). The corresponding AP assay using **1** as the fluorogenic substrate was conducted (Fig. 2). The lag period of the assay before the steady state was reached lasted from 0 to 600 s. At the same time, the predicted lag period for 99% accuracy based on Table 1 is 665 s (calculated by 6.65 × 100 s). This prediction matches the experimental data well.

In summary, we have studied the kinetics of a cyclization/elimination-coupled enzyme assay, and established a simple relationship between the lag period of the assay and the half life of the coupled organic reaction. This result may be applied to the development of other novel assays for enzymes using indirect colorimetric substrates.

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