

**BIOC 463A
2008-2009**

Kinetic Data for Inactivation of Alkaline Phosphatase (AP) by EDTA.

Introduction

In this experiment, we look at the kinetics of inactivation of alkaline phosphatase (AP) by the metal chelator EDTA, and we will suggest a mechanism for inactivation of the enzyme that is consistent with the kinetic data. Based on the AP literature, we know that exposure of AP to metal chelators such as EDTA leads to inactivation of the enzyme, presumably due to extraction of one or more of the Zn metals at the active site. As a starting point we may suggest the following simple bimolecular collisional inactivation reaction in to understand of the mechanism of inactivation:



Where k represents the second-order rate constant for the inactivation reaction. The rate equation (or law) for this mechanism is:

$$\text{(Eq. 2) } \text{rate (v)} = k [\text{EDTA}][\text{AP}_{\text{active}}]$$

By studying this reaction we hope to determine two things. First, is Eq. 1 an accurate description of the mechanism of inactivation. Second, we want to determine the value for the rate constant, k , or any other rate constants that may be associated with this reaction.

Determination of the rate constant requires that we understand how this constant is calculated from the rate law given in Eq. 2. The more laborious way to determine the rate constant is to start with **approximately equal** concentrations of EDTA and $\text{AP}_{\text{active}}$ and then to be able to determine how the initial concentrations of EDTA, $\text{AP}_{\text{active}}$, and $\text{AP}_{\text{inactive}}$ vary with time. Since the rate of the reaction can be measured by change in product per unit time, $d[\text{P}]/dt$, and assuming a 1:1 relationship between the amount of product formed and the amount of EDTA or $\text{AP}_{\text{active}}$ lost then we can rewrite Eq. 2:

$$\text{(Eq. 3) } d[\text{AP}_{\text{inactive}}]/dt = d(x)/dt = k [\text{edta}_o - x][\text{ap}_o - x]$$

Where edta_o and ap_o are the initial or starting concentrations and x is the concentration of product formed at any time, t . The rate constant, k , is determined by first integrating Eq. 3, the solution for which is given by:

$$\text{(Eq. 4) } \frac{1}{(\text{a}_o - \text{b}_o)} \ln \frac{\text{b}_o(\text{a}_o - x)}{\text{a}_o(\text{b}_o - x)} = kt$$

Once all of the values have been calculated, the rate constant, k , can be determined by plotting the left-hand side of the equation as a function of time, t ,

and the slope of the straight line (if one is obtained) is the second-order rate constant. Fortunately, there are easier ways of determining this rate constant.

The value for k can also be determined by performing the reaction under **pseudo-first order conditions** that are obtained by having the concentration of one reagent in excess of the other reagent. Assuming that $[\text{EDTA}] \gg [\text{AP}_{\text{active}}]$, then it should be obvious that the $[\text{EDTA}]$ does not vary significantly during the course of the reaction (i.e. $[\text{EDTA}]$ is also a constant). The rate law (Eq. 2) can therefore be simplified to:

$$\text{(Eq. 5) rate} = k[\text{EDTA}] [\text{AP}_{\text{active}}] = k_{\text{obs}} [\text{AP}_{\text{active}}]$$

Eq. 5 states that the rate will only be a function of the rate constant k_{obs} and the concentration of active AP and since this rate equation only takes into account the concentration of one reagent, $\text{AP}_{\text{active}}$, it is considered to be a “first-order” reaction. However, because $k_{\text{obs}} = k[\text{EDTA}]$ we say k_{obs} is a **pseudo-first order** rate constant, hence the reaction is more stringently a pseudo-first order reaction. The reason for making this simplification is that graphical analysis of kinetic data is much easier for (pseudo-) first-order reactions than it is for true second-order or bimolecular reactions, especially when plots of k_{obs} vs. $[\text{EDTA}]$ are non-linear (see below).

It can be shown (please refer to any physical (bio)chemistry or kinetics textbook), that for a (pseudo) first-order reaction, the decay in $[\text{AP}_{\text{active}}]$ should occur by a simple exponential process:

$$\text{(Eq. 6) } [\text{AP}_{\text{active}}] \text{ at any time, } t, = ([\text{AP}_{\text{active}}]_0)(e^{-k(\text{obs})t})$$

where $[\text{AP}_{\text{active}}]_0$ is the initial concentration of active AP. Thus, a plot of $[\text{AP}_{\text{active}}]$ (or $[\text{product}]$) vs. time (referred to as a kinetic trace or action spectrum) can be fit with a single exponential function and the rate constant is derived from the fit. Alternatively, using the plot of $[\text{AP}_{\text{active}}]$ or $[\text{product}]$ vs. time, it is possible to determine the first half-life of the reaction, $t_{1/2}$, which corresponds to the time required for $[\text{AP}_{\text{active}}] = [\text{AP}_{\text{active}}]_0/2$. For an exponential process, $t_{1/2}$ is related to the rate constant by:

$$\text{(Eq. 7) } k_{\text{obs}} = \ln 2 / t_{1/2} = 0.693 / t_{1/2}$$

Again, the value for k_{obs} that is determined from this type of graphical analysis corresponds to: $k_{\text{obs}} = k[\text{EDTA}]$.

By studying the rate of inactivation of AP over a range of EDTA concentrations, we can determine a k_{obs} value at each EDTA concentration. A plot of k_{obs} vs. $[\text{EDTA}]$ should give a straight line with a slope equal to the second-order rate constant, k , *if the reaction is a simple bimolecular collisional process*.

Although we cannot directly monitor the reaction between EDTA and AP by any direct spectral method, we can use the initial velocity of the catalytic hydrolysis of PNPP in order to determine the $[\text{AP}_{\text{active}}]$ during the period of

incubation with EDTA. This is accomplished by measuring v_o for the catalytic reaction of AP when $[PNPP] \gg K_m$, since under these conditions:

$$\text{(Eq. 8) } v_o = V_{\max} = k_{\text{cat}}[\text{AP}_{\text{active}}]$$

Therefore, under these conditions, v_o is directly proportional to $[\text{AP}_{\text{active}}]$.

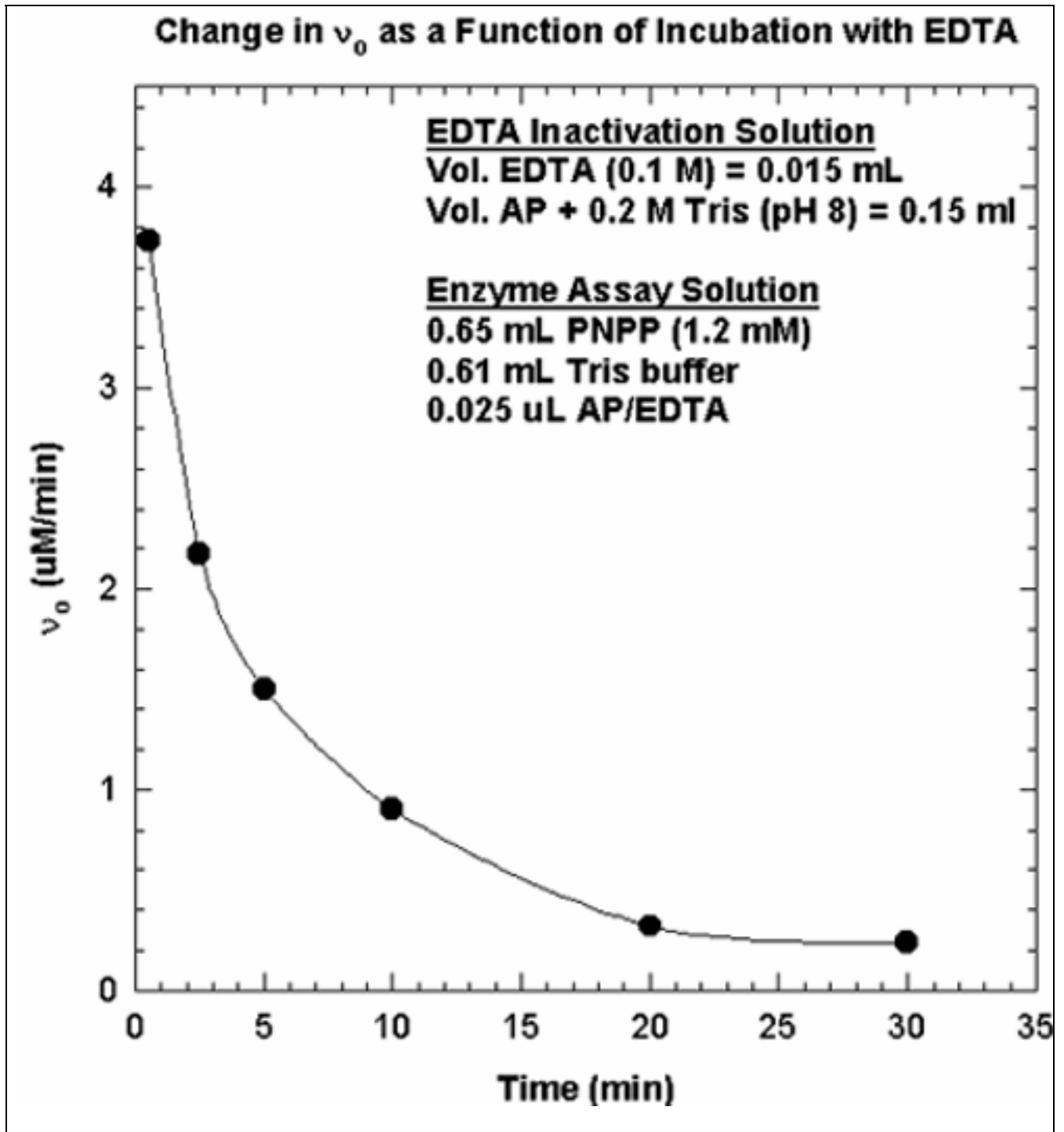
Thus, the general method is to incubate AP with excess EDTA (pseudo first-order conditions) and use an aliquot of this reaction mixture for the determination of catalytic activity in a PNPP assay. As EDTA inactivates the enzyme, we should observe a decrease in the activity of the enzyme, measured by a decrease in v_o (= V_{\max}), as a function of incubation time. Figure 1 is a representative kinetic trace of the change in v_o when AP is incubated with EDTA under the conditions given in the figure legend. The value for $t_{1/2}$ can be derived directly from the kinetic trace, which then enables the calculation of k_{obs} at that concentration of EDTA

Assay Procedure

Each reaction was initiated by addition of a specific volume of EDTA (0.1M) to 0.15 mL of AP (0.2 μM). The time of addition is considered to be $t = 0$ min. 25 μL aliquots of this reaction solution were withdrawn and used to determine the initial velocity (v_o in μM product/min) for the catalytic hydrolysis of PNPP in an assay solution containing 0.65 mL of 1.2 mM PNPP and 0.61 mL Tris buffer (pH 8). Initial velocity measurements were based upon the change in absorbance at 410 nm over a time interval ≤ 20 seconds and using an extinction coefficient of $0.018 \text{ } \mu\text{M}^{-1} \text{ cm}^{-1}$ for para-nitrophenolate, the product of the catalytic reaction.

Results and Data Analysis

Figure 1 shows the change in v_0 vs. time of incubation of EDTA with alkaline phosphatase when 15 μL of 0.1 M EDTA is added to 0.15 mL of 0.2 μM of AP.



First, extrapolate the curve drawn through the data points in Figure 1, to $t = 0$ min, and $t = 8$ and calculate the difference in v_0 , $\Delta v_0 = v_0(t = 0) - v_0(t = 8)$. The first half-life (time), $t_{1/2}$, is the time required to reach $\Delta v_0/2$.

Table 1 lists the volumes of EDTA added and the first half-life ($t_{1/2}$) for the decay of v_0 at each [EDTA]. Complete the columns in Table 1, calculating [EDTA] for each reaction, and the value of k_{obs} at each [EDTA], using the $t_{1/2}$ values given in the table and the value you determined from Figure 1.

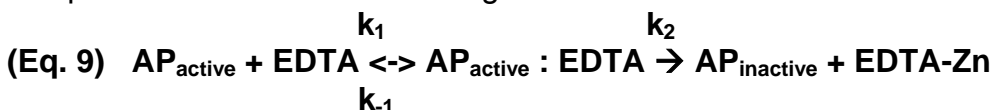
Table 1.

Vol. EDTA (uL)	[EDTA]	$t_{1/2}$ (min.)	k_{obs} (min ⁻¹)
2.3	_____	10.66	_____
3.8	_____	7.45	_____
7.5	_____	4.5	_____
10	_____	3.8	_____
15 (see Fig.1)	_____	_____	_____
17.5	_____	2.71	_____
24.5	_____	2.43	_____

Once you have completed Table 1, plot k_{obs} vs. [EDTA] as Figure 2.

Discussion

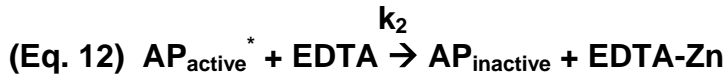
If the plot of k_{obs} vs. [EDTA] is not a simple linear plot, you must consider a more complicated mechanism than that given by Eq. 1. In fact, based on the data plotted in Figure 2, it is apparent that k_{obs} becomes independent of EDTA at high concentrations and reaches a limiting value, k_{limit} . This suggests that there exists an **[EDTA]-independent first-order process that becomes rate limiting**. There are two possible mechanisms that can explain these observations. The first mechanism is similar to that observed for enzyme substrate reactions, in which a complex is formed between AP_{active} and EDTA in a rapid equilibrium step, followed by a first order inactivation reaction within the complex that becomes rate limiting:



where k_1 is the second order rate constant for complex formation, k_{-1} is the first-order rate constant for complex dissociation, and k_2 , represents the rate constant for the rate limiting first-order reaction, which is [EDTA] independent, by which inactive AP is produced:

$$\text{(Eq. 10) } k_{limit} = k_2$$

The second mechanism is based upon a pre-equilibrium reaction involving the enzyme alone, which exists as either a closed, AP_{active} , or an open, AP_{active}^* , conformation. EDTA can only react with the open conformation of AP (Eq. 12) by which it gains access to the active site metals, resulting in a second-order inactivation reaction:



where k_1 and k_{-1} are the first-order rate constants for the interconversion of the two forms of AP and k_2 is the second-order rate constant for the bimolecular inactivation of AP^* by EDTA. Thus, in this mechanism, the EDTA concentration independent first-order reaction is determined by the conformational equilibrium involving AP alone. Assuming that k_1 is not excessively greater than k_{-1} , then the limiting value for k_{obs} is:

$$\text{(Eq. 13) } k_{\text{limit}} = k_1 + k_{-1}$$

The conformational equilibrium controlling this second mechanism is often referred to as a “breathing” process.

Homework Assignment

Referring to your plot of k_{obs} vs. [EDTA] suggest a way in which you could distinguish between the two mechanisms given above. Hint: consider EDTA as a member of the family of chemical agents called metal chelators. How might the limiting value for k_{obs} be affected by changing the chemical properties of a variety of metal chelators?