CARBONIC ANHYDRASE: KINETICS OF Zn(II) REMOVAL By 2,6-PYRIDINECARBOXYLATE

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Abstract

In the past decade, supervised activity recognition methods have been studied by many researchers, however these methods still face many challenges in real world settings. Supervised activity recognition methods assume that we are provided with labeled training examples from a set of predefined activities. Annotating and hand labeling data is a very time consuming and laborious task. Also, the assumption of consistent pre-defined activities might not hold in reality. More importantly, these algorithms do not take into account the streaming nature of data, or the possibility that the patterns might change over time. In this chapter, we will provide an overview of the state of the art *unsupervised* methods for activity recognition. In particular, we will describe a scalable activity discovery and recognition method for complex large real world datasets, based on sequential data mining and stream data mining methods.

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Introduction

Background

Carbonic anhydrase (CA) catalyzes the interconversion of carbon dioxide and carbonic acid/bicarbonate as follows:

$$CO_2 + H_2O \overset{CA}{\rightleftharpoons} H_2CO_3 \tag{1}$$

Lindskog and Coleman showed the catalytic activity of carbonic anhydrase to be most efficient at neutral pH.[1] In this experiment, neutral pH is maintained using phosphate buffer. As Figure 1 illustrates, the CA active site contains a Zn^{2+} cofactor (denoted as CA·Zn), upon which the enzyme relies for its catalytic activity. The zinc ion can be stripped from the enzyme using a Lewis base ligand, which donates electrons to the ion to form a covalent bond. The ligand being studied in this experiment is 2,6-pyridinecarboxylate, commonly called dipicolinate (or dipic). Figure 2 shows the structure of dipic. In this experiment, the rate of zinc removal by dipic will be measured.

Mechanism

When $[dipic] >> [CA]$, that is, when $\frac{[dipic]}{[CA]} \geq 25$, the removal of zinc is pseudo-first-order with respect to CA·Zn because the concentration of dipic, denoted as L, does not change appreciably. Thus the formation of the inactive enzyme, apoCA, can be modeled using the following rate equation:

$$\frac{d[\text{apoCA}]}{dt} = k_{obs}[\text{CA·Zn}] \tag{2}$$

The pseudo-first-order rate constant, k_{obs}, increases as [L] increases, but levels off at sufficiently high concentrations of L. Biochemists will recognize behavior similar to Michaelis-Menten enzyme kinetics in which the enzyme, CA·Zn, and the substrate, L, reversibly form a

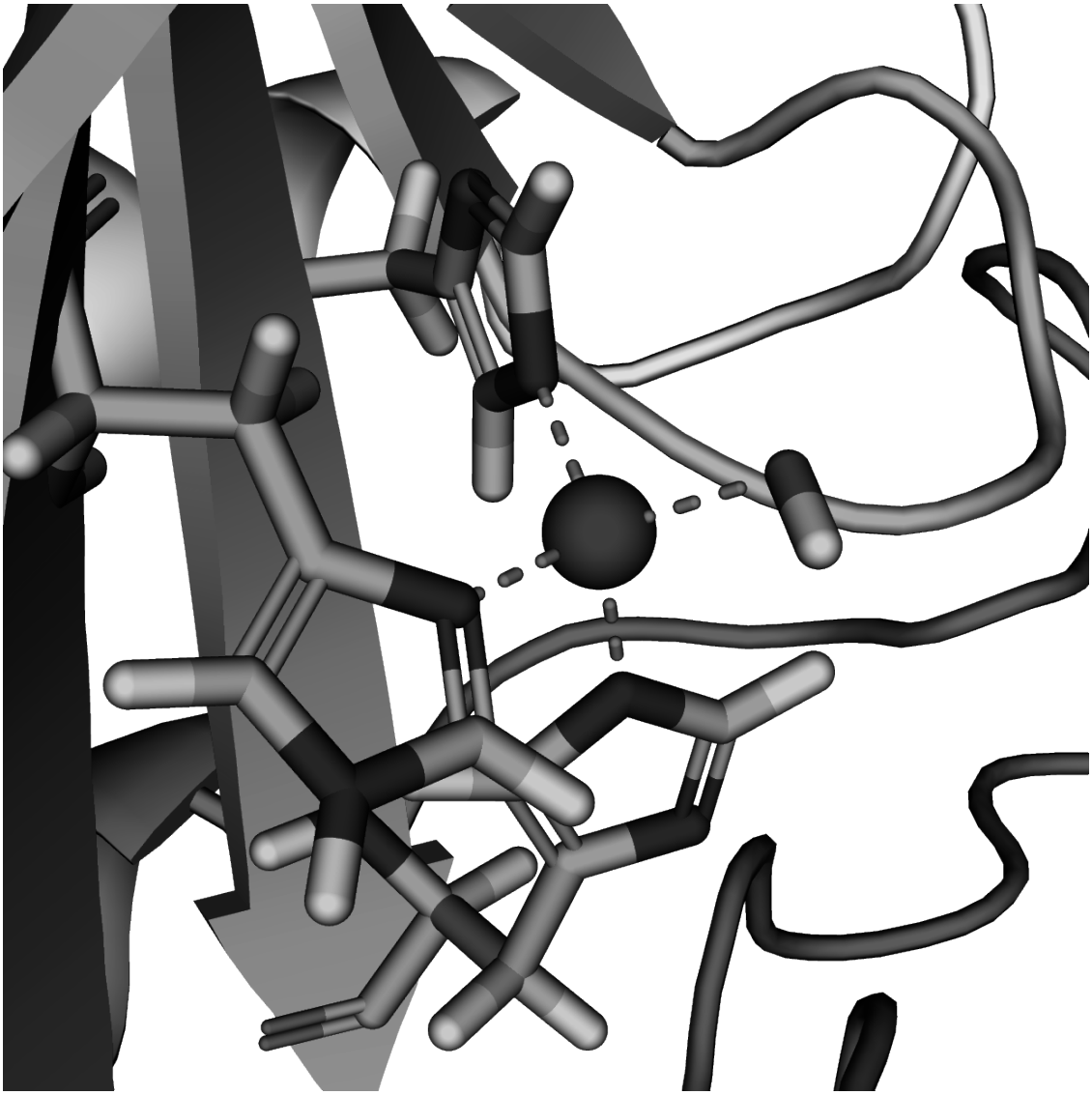


Figure 1: Zn^{2+} cofactor in the active site of human carbonic anhydrase II[2]

CA·Zn·L complex with association constant K_{EML} (EML stands for Enzyme-Metal-Ligand):

$$\mathrm{CA{\cdot}Zn} + \mathrm{L} \underset{}{\overset{\mathrm{K_{EML}}}{\rightleftharpoons}} \mathrm{CA{\cdot}Zn{\cdot}L} \tag{3}$$

This can be modeled as follows:

$$K_{EML} = \frac{[\mathrm{CA{\cdot}Zn{\cdot}L}]}{[\mathrm{CA{\cdot}Zn}][\mathrm{L}]} \tag{4}$$

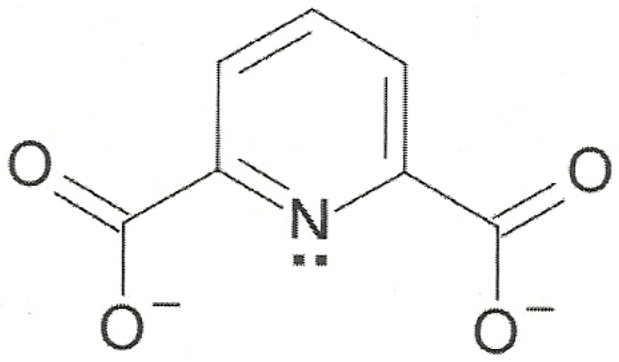


Figure 2: Structure of 2,6-pyridinecarboxylate (dipic)[3]

CA·Zn·L can either revert back to the original species or irreversibly convert to the inactive form of the enzyme, apoCA, and the covalently bound zinc-dipic molecule, ZnL:

$$\text{CA·Zn·L} \xrightarrow{\text{k}_\text{d}} \text{apoCA} + \text{ZnL} \tag{5}$$

This yields the following differential rate law:

$$\frac{d[\text{apoCA}]}{dt} = k_d[\text{CA·Zn·L}] \tag{6}$$

Note the difference in assumptions between this reaction and the Michaelis-Menten model; M-M kinetics assumes the enzyme is reformed and that only the substrate is modified, while Reaction (5) shows that both the substrate and the enzyme are permanently modified. Recall that [L] >> [CA·Zn], so [L] can be treated as a constant, $[\text{L}]_0$, which is substituted into a rearranged form of Equation (4),

$$[\text{CA·Zn·L}] = K_{EML}[\text{CA·Zn}][\text{L}]_0 \tag{7}$$

Carbonic anhydrase can exist in one of three forms: the metalloenzyme CA·Zn, the enzyme-metal-ligand complex CA·Zn·L, or the inactivated enzyme apoCA. Initially, all CA is tied up in the metalloenzyme, and none exists as CA·Zn·L or apoCA. As the activated

form of the enzyme gets bound to L and then inactivated,

$$[\text{CA}\cdot\text{Zn}] = [\text{CA}\cdot\text{Zn}]_0 - [\text{apoCA}] - \text{CA}\cdot\text{Zn}\cdot\text{L}, \tag{8}$$

which can be combined with Equation (7) to yield

$$[\text{CA}\cdot\text{Zn}] = [\text{CA}\cdot\text{Zn}]_0 - [\text{apoCA}] - K_{EML}[\text{CA}\cdot\text{Zn}][\text{L}]_0 \tag{9}$$

and rearranged as follows:

$$[\text{CA}\cdot\text{Zn}] = \frac{[\text{CA}\cdot\text{Zn}]_0 - [\text{apoCA}]}{1 + K_{EML}[\text{L}]_0} \tag{10}$$

Equations (6) and (7) can be combined to give

$$\frac{d[\text{apoCA}]}{dt} = k_d K_{EML}[\text{CA}\cdot\text{Zn}][\text{L}]_0 \tag{11}$$

Therefore, in a solution containing dipic in large excess, the rate of apoCA formation is first-order with respect to CA·Zn. Combining equations (11) and (10) yields

$$\frac{d[\text{apoCA}]}{dt} = k_d K_{EML}[\text{L}]_0 \frac{[\text{CA}\cdot\text{Zn}]_0 - [\text{apoCA}]}{1 + K_{EML}[\text{L}]_0} \tag{12}$$

Rearranging and integrating,

$$\begin{aligned} \int_{[apoCA]_0}^{[apoCA]_t} \frac{d[\text{apoCA}]}{[\text{CA}\cdot\text{Zn}]_0 - [\text{apoCA}]_t} &= \int_0^t \frac{k_d K_{EML}[\text{L}]_0}{1 + K_{EML}[\text{L}]_0} dt \\ &= \frac{k_d K_{EML}[\text{L}]_0}{1 + K_{EML}[\text{L}]_0} t \end{aligned} \tag{13}$$

The left side must be integrated using u-substitution:

$$u = [\text{CA}\cdot\text{Zn}]_0 - [\text{apoCA}]_t$$

$$du = -d[\text{apoCA}]$$

To change the integral boundaries,

$$u(t=0) = [\text{CA·Zn}]_0, \text{ since no inactivated enzyme has been formed}$$

$$u(t=t) = [\text{CA·Zn}]_0 - [\text{apoCA}]_t$$

Therefore,

$$\begin{aligned}\int_{[apoCA]_0}^{[apoCA]_t} \frac{d[\text{apoCA}]}{[\text{CA·Zn}]_0 - [\text{apoCA}]_t} &= -\int_{[\text{CA·Zn}]_0}^{[\text{CA·Zn}]_0 - [\text{apoCA}]_t} \frac{du}{u} \\ &= -ln(u)\Big|_{[\text{CA·Zn}]_0}^{[\text{CA·Zn}]_0 - [\text{apoCA}]_t} \\ &= -ln\left(\frac{[\text{CA·Zn}]_0 - [\text{apoCA}]}{[\text{CA·Zn}]_0}\right)\end{aligned} \tag{14}$$

Combining the evaluated integrals from equations (14) and (13) yields

$$ln\left(\frac{[\text{CA·Zn}]_0 - [\text{apoCA}]}{[\text{CA·Zn}]_0}\right) = -\frac{k_d K_{EML}[\text{L}]_0}{1 + K_{EML}[\text{L}]_0} t \tag{15}$$

Assuming [CA·Zn·L] is always negligible, $\frac{[\text{CA·Zn}]_0 - [\text{apoCA}]}{[\text{CA·Zn}]_0}$ is simply the fraction of CA·Zn remaining after reaction time t and can be referred to as $F_{\text{CA·Zn}}$:

$$ln\left(F_{\text{CA·Zn}}\right) = -\frac{k_d K_{EML}[\text{L}]_0}{1 + K_{EML}[\text{L}]_0} t \tag{16}$$

Since $-\frac{k_d K_{EML}[\text{L}]_0}{1+K_{EML}[\text{L}]_0}$ is a constant for a given concentration of dipic, Equation (16) exhibits linear behavior over time while the solution contains active enzyme. Therefore, a linear least squares regression procedure can be performed for measurements of $ln\left(F_{\text{CA·Zn}}\right)$ over time (until those measurements level off, which indicates that all the enzyme is used up), and the slope, denoted $-k_{obs}$, will be

$$-k_{obs} = -\frac{k_d K_{EML}[\text{L}]_0}{1 + K_{EML}[\text{L}]_0} \tag{17}$$

Taking the reciprocal of Equation (17) yields

$$\begin{aligned}\frac{1}{k_{obs}} &= \frac{1 + K_{EML}[\mathrm{L}]_0}{k_d K_{EML}[\mathrm{L}]_0} \\ &= \frac{1}{k_d K_{EML}[\mathrm{L}]_0} + \frac{K_{EML}[\mathrm{L}]_0}{k_d K_{EML}[\mathrm{L}]_0} \\ &= \frac{1}{k_d K_{EML}} \times \frac{1}{[\mathrm{L}]_0} + \frac{1}{k_d}\end{aligned} \tag{18}$$

Again, a linear relationship is observed; measuring k_{obs} at several different dipic concentrations enables one to perform a least squares regression procedure on $\frac{1}{k_{obs}}$ versus $\frac{1}{[\mathrm{L}]_0}$ to determine the slope, $m = \frac{1}{k_d K_{EML}}$, and intercept, $b = \frac{1}{k_d}$. k_d and K_{EML} are thus calculated as follows:

$$k_d = \frac{1}{b} \tag{19}$$

$$K_{EML} = \frac{b}{m} \tag{20}$$

Deriving k_d and K_{EML} using Equation (18) requires varying the dipic concentration while holding constant the concentration of carbonic anhydrase. As stated in the procedure, a certain volume, v_L, of dipic with a given concentration, $[\mathrm{L}]_{stock}$, is added to a solution of carbonic anhydrase and phosphate buffer in order to dilute the dipic to a desired concentration, $[\mathrm{L}]_0$. The final volume of solution is v_{soln}. In order calculate the needed volumes,

$$v_L = \frac{v_{soln} \times [\mathrm{L}]_0}{[\mathrm{L}]_{stock}} \tag{21}$$

The amount of phosphate buffer, v_{buff}, needed to bring the solution to v_{soln}, is simply

$$v_{buff} = v_{soln} - v_{CA} - v_L \tag{22}$$

where v_{CA} is the original carbonic anhydrase solution volume.

Rate Measurements

While Equation (17) seems to imply that the determination of k_{obs} requires knowing [CA·Zn] or [apoCA] at any point in time, in reality a plot of $f(t)$ vs t, where t refers to CA/dipic reaction time and $f(t)$ is proportional to $F_{\text{CA·Zn}}$, will still exhibit the same slope $-k_{obs}$. The Michaelis-Menten kinetics model states that, for sufficiently large substrate concentrations, the enzyme is fully saturated and the reaction velocity asymptotically approaches a constant rate,

$$V_{max} = k_{cat}[\text{CA·Zn}], \tag{23}$$

where k_{cat} (the "turnover number") is the amount of substrate that a single saturated enzyme molecule can convert to produce in a given unit of time.[4] Thus for sufficiently large concentrations of substrate, the enzyme catalytic activity fulfills the requirement of proportionality to $F_{\text{CA·Zn}}$. Ideally, the determination of the enzymatic activity from V_{max}, that is, taking an enzyme assay, can be done using a substrate which is cheap, readily available, and easily measurable. As Figure 3 shows, carbonic anhydrase happens to hydrolyze para-nitrophenyl acetate (pNPA) to form para-nitrophenol and acetic acid:

Figure 3: Carbonic anhydrase-catalyzed hydrolysis of para-nitrophenyl acetate (pNPA) to para-nitrophenol and acetic acid[3]

Para-nitrophenol absorbs strongly at 348nm. Recall that Beer's law states

$$A = \epsilon bc \tag{24}$$

where A is the measured absorbance at a particular wavelength, ϵ is the molar absorptivity, b is the measurement path length, and c is the concentration.[5] Since b and ϵ stay constant, A is dependent only on c. c varies with time, therefore the change in absorbance under enzyme-

catalyzed conditions, $\left(\frac{dA}{dt}\right)_{cat}$, is proportional to the para-nitrophenol formation velocity, which, as (23) demonstrates, is itself proportional to the amount of active carbonic anhydrase in solution. Because the reaction occurs to a nontrivial extent without enzymatic catalysis, a baseline correction is needed, $(\frac{dA}{dt})_{uncat}$, which can be obtained either from a solution containing only pNPA or a mixture of pNPA and apoCA (that is, an assay taken after sufficient time has passed to inactivate all extant carbonic anhydrase). In this experiment, $(\frac{dA}{dt})_{uncat}$ was determined from the solution of pNPA with no enzyme added. Thus, Equation (16) can be implemented using

$$ln\left(\frac{dA}{dt}\right)_{cat} = ln\left(\frac{\left(\frac{dA}{dt}\right)_t}{\left(\frac{dA}{dt}\right)_{uncat}}\right) \tag{25}$$

(note: this also fulfills the logarithm function's unitless requirement.)

Procedure

This experiment was performed using a Spectral Instruments model SI 440 spectrophotometer, which includes a CCD detector and fiber optic probe. The spectrophotometer was set to detect absorbance at wavelength 348 nm every five seconds over the course of two minutes. Device precison was set to "low," and a blank was obtained and locked. A sample of deionized water was measured to ensure absorbance stayed constant, preventing systematic errors arising from faulty instrumentation.

A 0.003 M aqueous pNPA solution was prepared by dissolving 25 mg solid pNPA in 1.5 mL acetone within a 250 mL Erlenmeyer flask, then slowly adding 50 mL deionized water while stirring vigorously to prevent precipitation. Assays were constructed by combining 1.7 mL deionized water, 0.3 mL HEPES buffer (0.25 M, pH 8.0), and 1.0 mL pNPA solution. To measure the uncatalyzed assay velocity $\left(\frac{dA}{dt}\right)_{uncat}$, 40 µL deionized water was added to an assay containing no carbonic anhydrase or dipic, and the absorbance was measured for two minutes.

Five dipic concentrations were tested: 0.20 M, 0.10 M, 0.05 M, 0.032 M, and 0.016 M. For each [dipic], a 500 µL solution was prepared consisting of 250 µL aqueous bovine carbonic anhydrase (10^{-4}M in 0.125 M phosphate buffer, pH 7.5) and the required volumes of phosphate buffer (0.125 M, pH 7.4) and dipic (0.4 M in 0.125 M phosphate buffer, pH 7.4) needed to dilute to the specified [dipic] (see Equations (21) and (22)). In each run, the carbonic anhydrase and buffer solutions were mixed and placed in a 25°C water bath to equilibrate for several minutes. Dipic was added just prior to starting measurements. After one minute, and then again at regular intervals over the course of one hour (or until $\left(\frac{dA}{dt}\right)_t$ measurements stopped decreasing, signifying full holoenzyme depletion), a 40 µL aliquot of CA/dipic solution was transferred to an assay cuvette, and a two-minute $\left(\frac{dA}{dt}\right)_t$ measurement was recorded.

Sample Calculations

In each experiment, a 500 µL solution ($v_{soln} = 500$ µL) was prepared consisting of 250 µL of carbonic anhydrase solution, a certain volume (v_{buff}) of phosphate buffer, and, just before measurements were started, a certain volume (v_L) of 0.4 M dipic ($[\mathrm{L}]_{stock} = 0.4$ M) needed to dilute the dipic to a specific concentration ($[\mathrm{L}]_0$). To achieve 0.10 M dipic, as per Equation (21),

$$\begin{aligned} v_L &= \frac{v_{soln} \times [\mathrm{L}]_0}{[\mathrm{L}]_{stock}} \\ &= \frac{500\ \mu\mathrm{L} \times 0.10\ \mathrm{M}}{0.4\ \mathrm{M}} \\ &= 125\ \mu\mathrm{L} \end{aligned}$$

Equation (22) gives the amount of phosphate buffer, v_{buff}, added to the carbonic anhydrase solution (volume v_{CA}) before placing in the water bath to equilibrate:

$$\begin{aligned} v_{buff} &= v_{soln} - v_{CA} - v_L \\ &= 500\ \mu\mathrm{L} - 250\ \mu\mathrm{L} - 125\ \mu\mathrm{L} \\ &= 125\ \mu\mathrm{L} \end{aligned}$$

As Equation (25) demonstrates, a baseline correction is needed to convert the raw absorbance velocity, $\left(\frac{dA}{dt}\right)_t$, to a proportion which can take the place of $F_{\mathrm{CA{\cdot}Zn}}$ in Equation (16). $\left(\frac{dA}{dt}\right)_{uncat}$ was found to be $3.18 \times 10^{-5}\ sec^{-1}$. One data point that was measured for the 0.10 M dipic concentration experiment was $\left(\frac{dA}{dt}\right)_{t=1.08\ min} = 0.00295\ sec^{-1}$. Therefore,

$$\begin{aligned} ln\left(\frac{dA}{dt}\right)_{cat} &= ln\left(\frac{\left(\frac{dA}{dt}\right)_t}{\left(\frac{dA}{dt}\right)_{uncat}}\right) \\ &= ln\left(\frac{0.00295\ sec^{-1}}{3.18 \times 10^{-5}\ sec^{-1}}\right) \\ &= 4.530 \end{aligned}$$

Recall from Equation (17) that $-k_{obs}$ is the slope obtained from a least-squares regression of multiple assay $ln\left(\frac{dA}{dt}\right)_{cat}$ readings versus CA/dipic reaction time. For 0.10 M dipic, this was found to be $-k_{obs} = -0.090 min^{-1}$ (so $k_{obs} = 0.090 min^{-1}$).

Equation (18) implies that a least-squares regression of $\frac{1}{k_{obs}}$ versus $\frac{1}{[\mathrm{L}]_0}$ gives a slope, $m = \frac{1}{k_d K_{EML}}$, and intercept, $b = \frac{1}{k_d}$, which can be used to calculate k_d and K_{EML} for the dipic/CA reaction. Empirically, m was found to have a value of 0.5372 $M \cdot mins$ (0.50 $M \cdot mins$ rounded to accepted precision from error analysis), and b was found to be 8.513 $mins$ (9.0 $mins$ rounded to accepted precision from error analysis). Thus,

$$\begin{aligned} K_{EML} &= \left(\frac{b}{m}\right)\ M^{-1} \\ &= \frac{(9.0\ mins)}{(0.5\ M \cdot mins)} \\ &= 20\ M^{-1} \end{aligned} \tag{26}$$

$$\begin{aligned} k_d &= \left(\frac{1}{b}\right)\ mins^{-1} \\ &= \frac{1}{(9.0\ mins)} \\ &= 0.1\ mins^{-1} \end{aligned} \tag{27}$$

Error Analysis

To determine k_{obs}, readings of $ln\left(\frac{dA}{dt}\right)_{cat}$ were plotted against CA/dipic reaction time, and a least squares regression was performed. From the analysis of 0.10 M dipic, $m = -0.0936\ mins^{-1}$ and $s_m = 0.0207\ mins^{-1}$. There were 5 data points taken while active CA enzyme activity was detected, so 3 degrees of freedom were used for the 95% confidence t-factor, $t_{95} = 3.182$. From this, a 95% confidence interval was calculated:

$$\begin{aligned} -k_{obs} &= \left(-0.0936 \pm \frac{0.0207 \times 3.182}{\sqrt{5}}\right)\ mins^{-1} \\ &= (-0.090 \pm 0.029)\ mins^{-1} \end{aligned}$$

To calculate k_d and K_{EML}, values of $\frac{1}{k_{obs}}$ retrieved from all five experiments were plotted against $\frac{1}{[\mathrm{L}]_0}$. A least squares regression determined $m = 0.5372\ mins \cdot M$, $s_m = 0.1675\ mins \cdot M$, $b = 8.513\ mins$, and $s_b = 5.507\ mins$. Since 5 runs were performed, there were 3 degrees of freedom. The 95% confidence t-factor was $t_{95} = 3.182$. Therefore,

$$\begin{aligned} m_{95} &= \left(0.5372 \pm \frac{0.1675 \times 3.182}{\sqrt{5}}\right)\ mins \cdot M \\ &= (0.50 \pm 0.24)\ mins \cdot M \end{aligned} \tag{28}$$

$$\begin{aligned} b_{95} &= \left(8.513 \pm \frac{5.507 \times 3.182}{\sqrt{5}}\right)\ mins \\ &= (9 \pm 8)\ mins \end{aligned} \tag{29}$$

Equation (20) shows how to calculate K_{EML} from the slope and intercept determined by Equations (28) and (29):

$$K_{EML} = \left(\frac{b}{m} \pm \lambda_{K_{EML}}\right)\ M^{-1}$$

Therefore, a 95% confidence interval can be established as follows:

$$\begin{aligned}
\lambda_{K_{EML}} &= \sqrt{\left(\frac{\partial\ K_{EML}}{\partial\ m}\right)^2 \lambda_m^2 + \left(\frac{\partial\ K_{EML}}{\partial\ b}\right)^2 \lambda_b^2} \\
&= \sqrt{\left(-\ \frac{b}{m^2}\right)^2 \lambda_m^2 + \left(\frac{1}{m}\right)^2 \lambda_b^2} \\
&= \sqrt{\left(-\ \frac{(9.0\ mins)}{(0.50\ M \cdot mins)^2}\right)^2 \times (0.24\ M \cdot mins)^2 + \left(\frac{1}{(0.50\ M \cdot mins)}\right)^2 \times (8.0\ mins)^2} \\
&= 18\ M^{-1}
\end{aligned} \tag{30}$$

To calculate k_d, use the y-intercept of the line passing through a plot of $\frac{1}{k_{obs}}$ versus $\frac{1}{[\mathrm{L}]_0}$ as determined by Equation (29). From Equation (19),

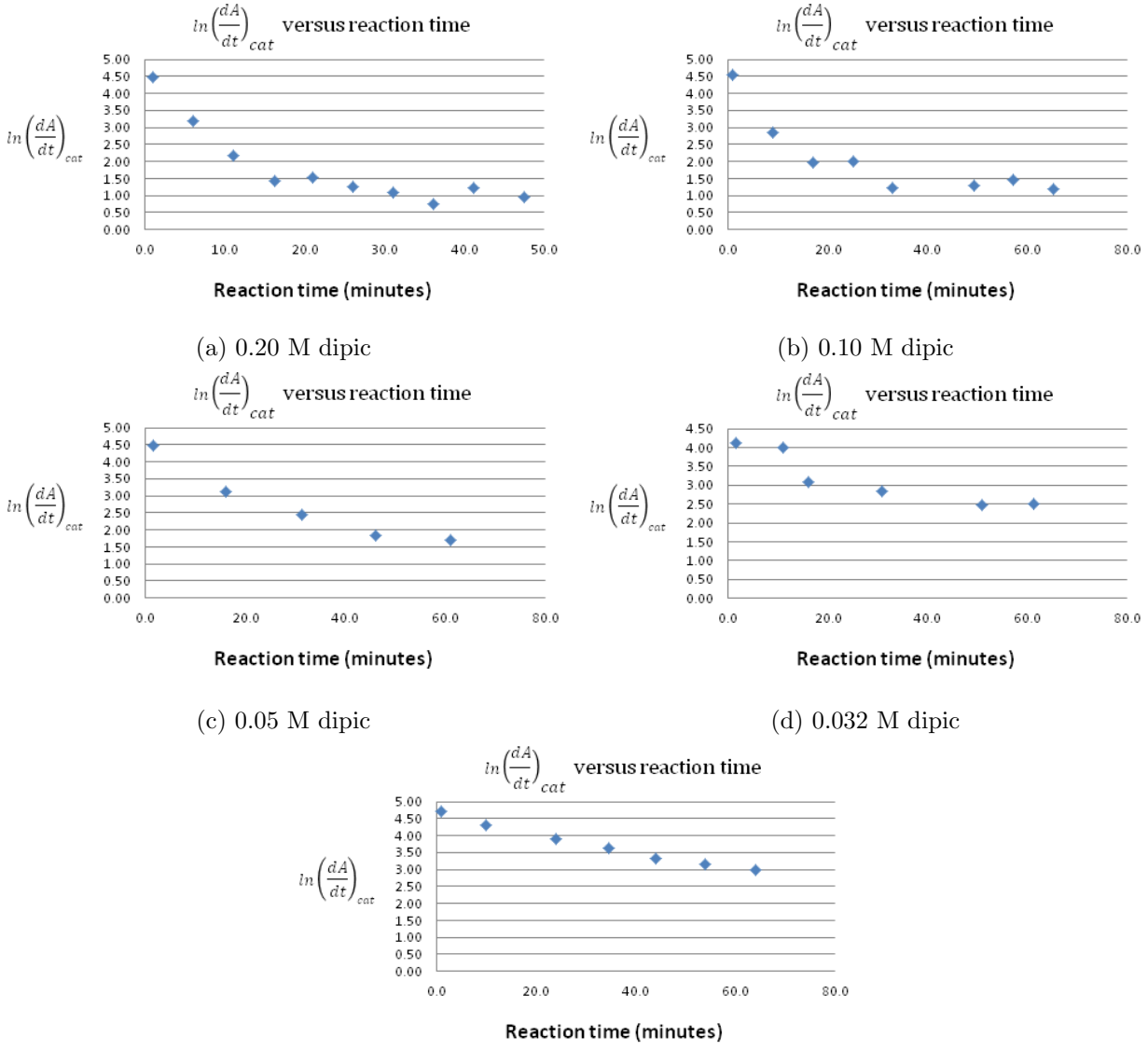
$$k_d = \left(\frac{1}{b} \pm \lambda_{k_d}\right)\ mins^{-1}$$

These can be combined to give a 95% confidence interval:

$$\begin{aligned}
\lambda_{k_d} &= \sqrt{\left(\frac{\partial\ k_d}{\partial\ b}\right)^2 \lambda_b^2} \\
&= \sqrt{\left(-\ \frac{1}{b^2}\right)^2 \lambda_b^2} \\
&= \sqrt{\left(-\ \frac{1}{(9.0\ )^2}\right)^2 \times (8.0\ )^2} \\
&= 0.10\ mins^{-1}
\end{aligned} \tag{31}$$

Data and Results

Five experiments were performed using a constant amount of carbonic anhydrase and varying concentrations of dipic. For each experiment, several measurements of CA-catalyzed pNPA hydrolysis velocities, $ln\left(\frac{dA}{dt}\right)_{cat}$, were taken using spectrophotometry and plotted against the CA/dipic reaction time. The results of these experiments are shown in Figure 4.



(a) 0.20 M dipic

(b) 0.10 M dipic

(c) 0.05 M dipic

(d) 0.032 M dipic

(e) 0.016 M dipic

Figure 4: $ln\left(\frac{dA}{dt}\right)_{cat}$ measurements versus CA/dipic reaction time for various concentrations of dipic. Slopes of the descending portions of these plots were used to assign values for k_{obs}.

From the descending portions of the plots shown in Figure 4, five values of k_{obs} were calculated using the slopes determined by least squares regression. The reciprocals of k_{obs} and $[\mathrm{L}]_0$ (dipic concentration) were plotted against each other, as shown in Figure 5.

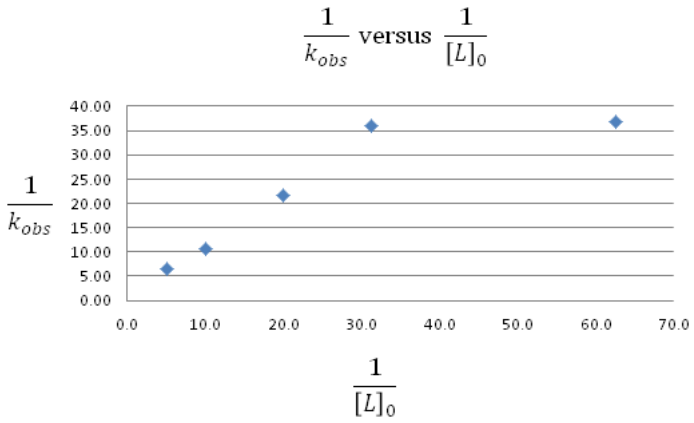


Figure 5: Plot of $\frac{1}{k_{obs}}$ versus $\frac{1}{[\mathrm{L}]_0}$. The slope and intercept of the line passing through these points were determined by least squares regression and used to calculate K_{EML} and k_d, demonstrated by Equations (26) and (27) respectively.

A least squares regression procedure was performed on the data shown in Figure 5 to retrieve values for the slope and intercept of the line passing through those points. Equations (26) and (27) show how these were used to calculate K_{EML} and k_d. Table 1 lists these values and their 95% confidence intervals, as well as accepted values reported in literature.

Table 1: Values of K_{EML} and k_d, as determined in this experiment as well as accepted values from literature

Constant	Empirical Value	Accepted Value
K_{EML}	$(20 \pm 18)\ M^{-1}$	$(7.7 \pm 1.5)\ M^{-1}$ [6]
k_d	$(0.10 \pm 0.10)\ mins^{-1}$	$(0.43 \pm 0.08)\ mins^{-1}$ [6]

Conclusion

In this experiment, kinetics were investigated for the removal of Zn^{2+} from the active site of carbonic anhydrase by 2,6-pyridinecarboxylate (dipic). Concentrations of dipic were varied for constant concentrations of carbonic anhydrase, and the resulting catalytic activities were measured periodically over the course of holoenzyme depletion using spectrophotometry.

Dipic forms an enzyme-metal-ligand complex at the carbonic anhydrase active site, and then either reverses back to the holoenzyme and dipic or proceeds to covalently bond with zinc, stripping it from the active site and converting the enzyme to the inactive form, apoCA:

$$\mathrm{CA{\cdot}Zn + L} \overset{\mathrm{K_{EML}}}{\rightleftharpoons} \mathrm{CA{\cdot}Zn{\cdot}L}$$

$$\mathrm{CA{\cdot}Zn{\cdot}L} \xrightarrow{\mathrm{k_d}} \mathrm{apoCA + ZnL}$$

K_{EML} was determined to be $(20 \pm 18)\ M^{-1}$, a relatively wide error range which includes the literature value, $(7.7 \pm 1.5)\ M^{-1}$.[6] The enzyme-metal-ligand complex is therefore thermodynamically favorable over the separated enzyme and the dipic ion by approximately a factor of ten. k_d was measured at $(0.10 \pm 0.10)\ mins^{-1}$, while the accepted value from literature, $(0.43 \pm 0.08)\ mins^{-1}$, is slightly outside of that range. What this means is that of the enzyme-metal-ligand complex extant at any point, approximately $25 - 40\%$ is converted into apoCA and ZnL within a minute. A consequence of these data is that the assumption that $[\mathrm{CA{\cdot}Zn{\cdot}L}] = 0$ needed to convert Equation (32) into (16) may not necessarily be accurate. Instead, Equation (32) would translate into

$$ln\left(\frac{[\mathrm{CA{\cdot}Zn}]_0 - [\mathrm{apoCA}]}{[\mathrm{CA{\cdot}Zn}]_0}\right) = ln\left(\frac{[\mathrm{CA{\cdot}Zn}]_t + [\mathrm{CA{\cdot}Zn{\cdot}L}]}{[\mathrm{CA{\cdot}Zn}]_0}\right) \tag{32}$$

where $[\mathrm{CA{\cdot}Zn{\cdot}L}]$ is a constant due to steady state approximation. The final effect on the accuracy of K_{EML} and k_d is difficult to determine and should be investigated further. It may

turn out to be negligible.

Besides the source of uncertainty just discussed, an obvious source of uncertainty is the subjective selection of usable data required. In order to determine values for k_{obs}, assay measurements were selected or discarded visually based on their conforming to a descending straight line, and due to the data set's relative sparsity it was difficult to determine the exact range of enzyme activity. One way to correct for this uncertainty is to simply collect more assay measurements. Rather than having five or six data points within the window of holoenzyme activity, using ten or twenty would increase the precision of the least squares regression. Instead of scrambling to quickly perform assays, the temperature may be lowered so that the reactions occur slower.

One more possible source of error was the way in which the assay absorbance velocities were determined. $\frac{dA}{dt}$ was calculated by subtracting the difference in absorbance at the start and end of the two minute assay and didn't account for the uncertainty involved in individual absorbance readings. This has more to do with the limitations of the spectrophotometer software. To correct for this in the future, all absorbance readings from the two minute assay could be exported to a data file and loaded into a spreadsheet application, which can be used to establish the slope through least squares regression. The calculated error from this linear regression could then be further propagated throughout the calculations.

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