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Enzyme Kinetics

Abstract:

In component I we attempted to develop a novel model accounting for nonlinearity during initial rate measurements for urate oxidase. This nonlinearity is believed to partially arise from the quick accumulation of 5-hydroxyisourate. This directly interferes with the UV-vis measurements at this wavelength. Our model took into consideration a new simple kinetic mechanism for the reaction, specifically a rate constant for the degradation of 5-hydroxyisourate. We used this model to more accurately predict velocities of the urate oxidase enzyme. In another experiment we provided further evidence for a sequential kinetic mechanism of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by varying the concentration of a substrate, GAP, at set levels of another substrate, NAD^+. Lastly, we utilized ADP as a reversible inhibitor for GAPDH, in order to determine the inhibitor dissociation constant, the mechanism of inhibitions, and to further support the idea of a sequential kinetic mechanism.

Introduction:

During our investigation of enzyme kinetics we primarily focused on developing a novel mathematical model for correction of standard UV-vis absorbance changes in the urate oxidase reaction. We also attempted to confirm previously studied multi substrate binding patterns of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with respect to it's two substrates. In our final experiment we performed inhibition studies on the same GAPDH enzyme to elucidate the kinetic mechanism and to determine the dissociation constant of the inhibitor.

The urate oxidase enzyme converts urate, a waste product of purine degradation, into 5-hydroxyisourate. This 5-hydroxyisourate undergoes spontaneous hydrolysis to the next product allantoin. Urate oxidase is a well characterized enzyme, but no consensus on the mechanism has been established. Further relevant research on the enzyme is still of interest because of its proven successful treatment of both gout and Tumor Lysis Syndrome[1]. These treatments are typically expensive, challenging to produce, and

situational[1]. We have previously measured the rate of reaction of urate oxidase by measuring the disappearance of urate at 293 nm in a spectrophotometer. This is sufficient for qualitative activity assays, but shows significant nonlinearity when observed under initial conditions. These conditions are of particular interest when studying Michaelis-Menten kinetics due to the greatly simplified derivations of kinetic parameters. The reason for this nonlinearity is partially understood and is often attributed to the formation of 5-hydroxyisourate, which also shows some absorbance around 293 nm[2]. When we initially measured this reaction we maintained a pH near 9 in order to decrease the stability of 5-hydroxyisourate by supplementing base catalyzed hydrolysis. This partially improved linearity under initial measurement conditions; however, we decided to design a mathematical model to more effectively account for this phenomenon. In our model we input absorbance vs time data that is evaluated according to the following kinetic equation where P is 5-hydroxyisourate and Q is allantoin.

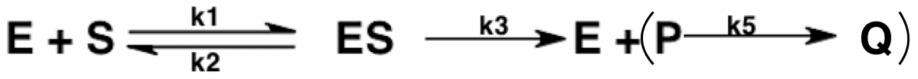


Figure 1:
The above graphic gives the equation we used for the mathematical model we designed. The E represents urate oxidase, the S represents urate, the P represents 5-hydroxyisourate, and the Q represents allantoin.

The k_5 of this model was determined experimentally from the change in absorbance of the reaction of urate oxidase at 316 nm whilst accounting for the absorbance of allantoin. Essentially, the k_5 rate constant allows for partial correction of the build up of 5-hydroxyisourate during initial velocity kinetic studies while measuring the disappearance of urate.

GAPDH is a tetramer of identical monomers and is directly implicated in glycolysis, transcription activation, and various other reactions.[3] That being said our interests focus on the primary reaction, in which GAPDH converts glyceraldehyde-3-phosphate into 1,3-biphosphoglycerate. This is a well characterized enzyme that will allow us to practice designing, performing, and analyzing enzymatic inhibition kinetics.

During our second experiment we varied the concentrations of one substrate, glyceraldehyde-3-phosphate (GAP), at three different concentrations of the second

substrate, nicotinamide adenine dinucleotide (NAD^+) for the enzyme GAPDH. This was done at 340 nm, the wavelength at which we can measure the formation of NADH, in a UV-vis spectrophotometer. The analysis of such data would lend itself to determining whether the enzyme follows a sequential or ping-pong based mechanism. According to preliminary research, it seems as though the consensus mechanism involves GAP binding to GAPDH after NAD^+ binds to the enzyme[4]. We also used this data to calculate macroscopic kinetic parameters V_{max} and K_m. Our third experiment also utilized GAPDH, constant [NAD^+], and varying [GAP] at three different concentration values of the inhibitor. After measuring the change in absorbance at 340 nm under the described parameters, we were able to determine a value of K_i, the dissociation constant of the inhibitor, and further support our discovery in the second experiment. This second experiment elucidated which bisubstrate mechanism the enzyme follows. We were also able to confirm the results from our references that GAPDH experiences mixed inhibition from ADP.

Methods:

Urate Oxidase:

Our urate oxidase absorbance vs time data was collected at 316 nm in a UV-vis spectrophotometer. Urate oxidase was isolated from *Bacillus subtilis* and was mutated at 253 from cysteine to threonine. The solutions contained 30 µL of urate oxidase solution and 770 µL of 80.9 µM urate solution in 50 mM CHES buffer. The data was analyzed by plotting it against our mathematical model in Mathematica. Our model and our data was then used to calculate the velocities of the reaction.

GAPDH:

Our GAPDH was purified from rabbit muscle tissue. The multi substrate data was collected in a UV-vis spectrophotometer at 340 nm. The solutions consisted of: 30 mM of Tris buffer, 10 mM of sodium arsenate, and three NAD^+ concentrations. These were 0.06 mM, 0.12 mM, and 0.24 mM. This data was then graphed using a Lineweaver-Burk plot and used to calculate V_{max} and V_{max}/K_m. The inhibition data was also collected at 340 nm. The inhibition solutions contained 50 mM Tris buffer, 10 mM of sodium arsenate, and

0.227 mM of NAD^+ at pH 8.5. The varying ADP concentrations were 0 mM, 10 mM, and 20 mM.

Results:

Urate Oxidase Model:

The following figures show how our urate oxidase model compares to the data we collected experimentally.

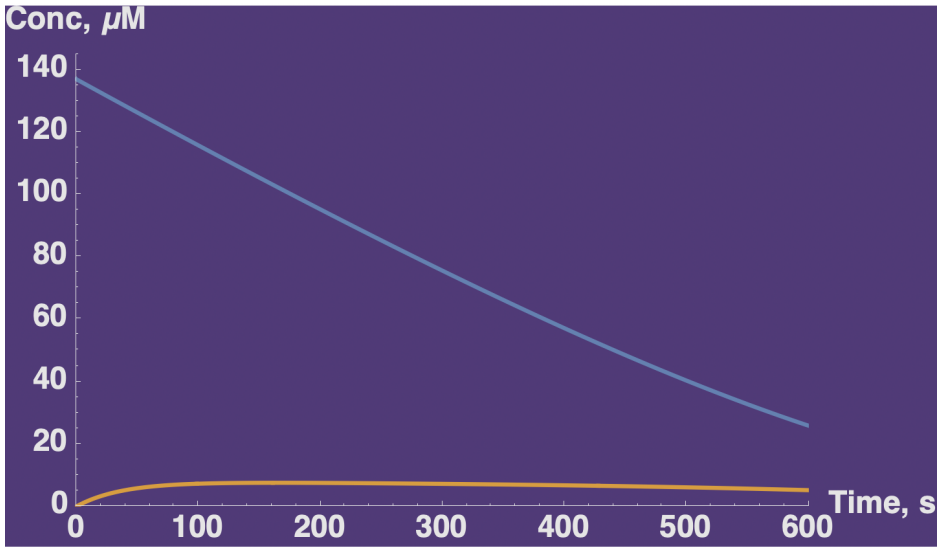


Figure 2: Model of [Substrate] vs Time
This figure shows the change in concentration over time of urate according to the mathematical urate oxidase model from our Mathematica code. The urate is shown in blue and the 5-hydroxyisourate is shown in orange.

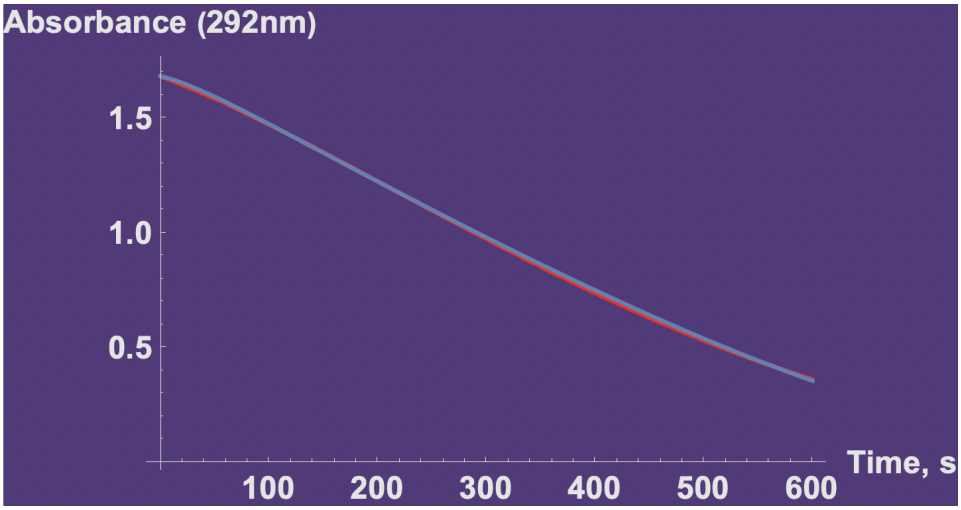


Figure 3: Model vs Data
This figure highlights the similarity in absorbance values between our mathematical urate oxidase model and the experimentally measured absorbances. The model is shown in blue and the measured values are shown in red.

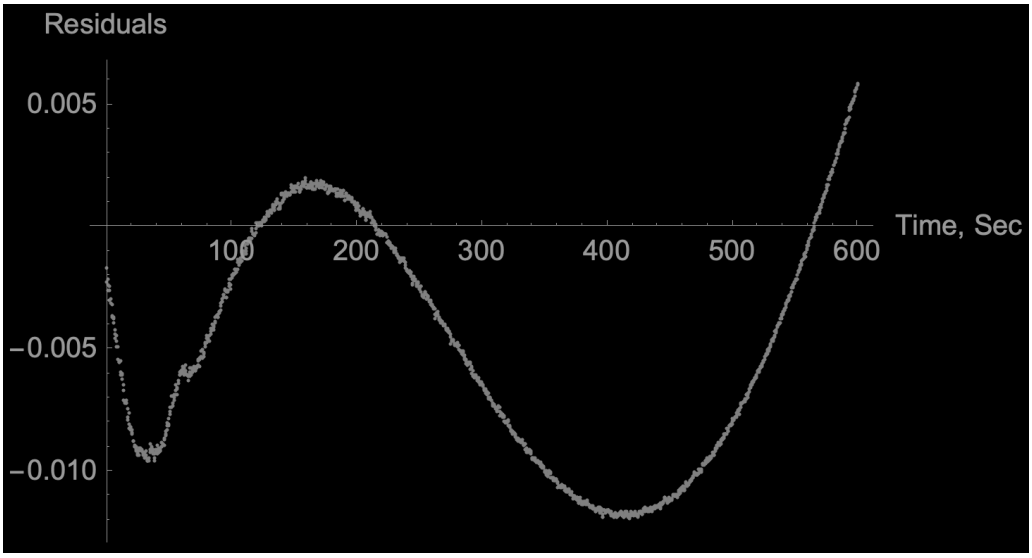


Figure 4: Model vs Data Fit Residuals
This figure gives the fit residuals of the absorbance data against the mathematical model.

GAPDH Multiple Substrate Kinetics:

The following figures show how the rate of reaction changed as a function of varying substrate concentration. Additionally, the kinetic parameters and their statistical error analysis are shown below. Lastly, the Lineweaver-Burk plot is shown for the three concentrations of NAD^+.

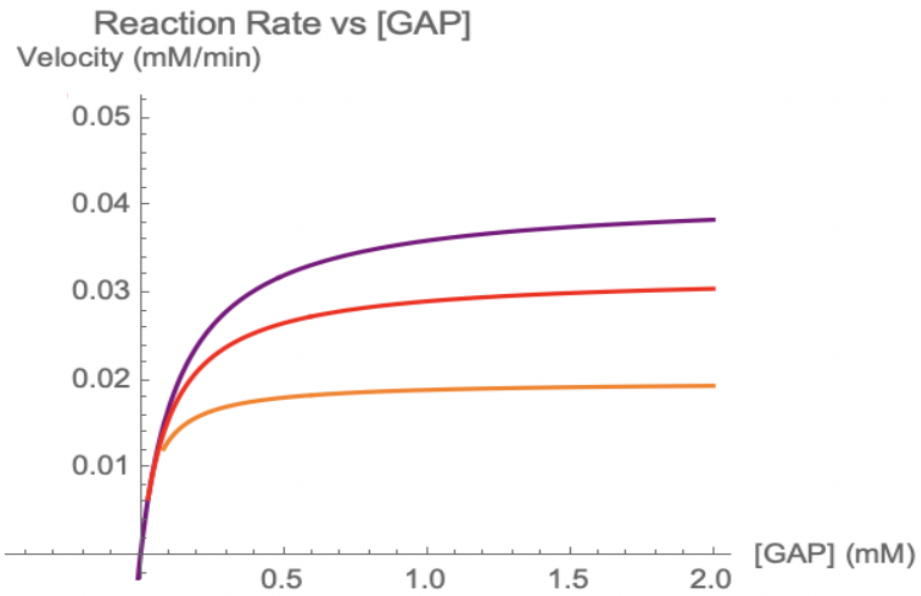


Figure 5: Multi Substrate Reaction Rates
This figure plots the best nonlinear fit for each three sets of data measured. 0.240 mM NAD^+ is shown in purple, 0.120 mM NAD^+ is shown in red, and 0.060 mM NAD^+ is shown in orange.

$$\frac{0.0250156\,x}{0.0179898 + 1.39054\,x},$$

	Estimate	Standard Error	t-Statistic	P-Value
Vmax6t2	0.0179898	0.00106379	16.9111	1.51602×10^{-7}
Km6t2	0.0129373	0.0110753	1.16812	0.276389

$$\frac{0.00998914\,x}{0.0321587 + 0.31062\,x},$$

	Estimate	Standard Error	t-Statistic	P-Value
Vmax12t2	0.0321587	0.000545502	58.9525	7.61385×10^{-12}
Km12t2	0.103531	0.00869928	11.9011	2.28344×10^{-6}

$$\frac{0.0120521\,x}{0.0410794 + 0.293384\,x},$$

	Estimate	Standard Error	t-Statistic	P-Value
Vmax24t2	0.0410794	0.00106911	38.424	2.31179×10^{-10}
Km24t2	0.140019	0.0161028	8.69535	0.000023853

Figure 6: Kinetic Parameters
This Figure highlights the estimated V_{max} and K_m values for each NAD^+ concentration. It also provides the standard errors of the fit, the t-Statistic value, and the P-Value for each parameter.

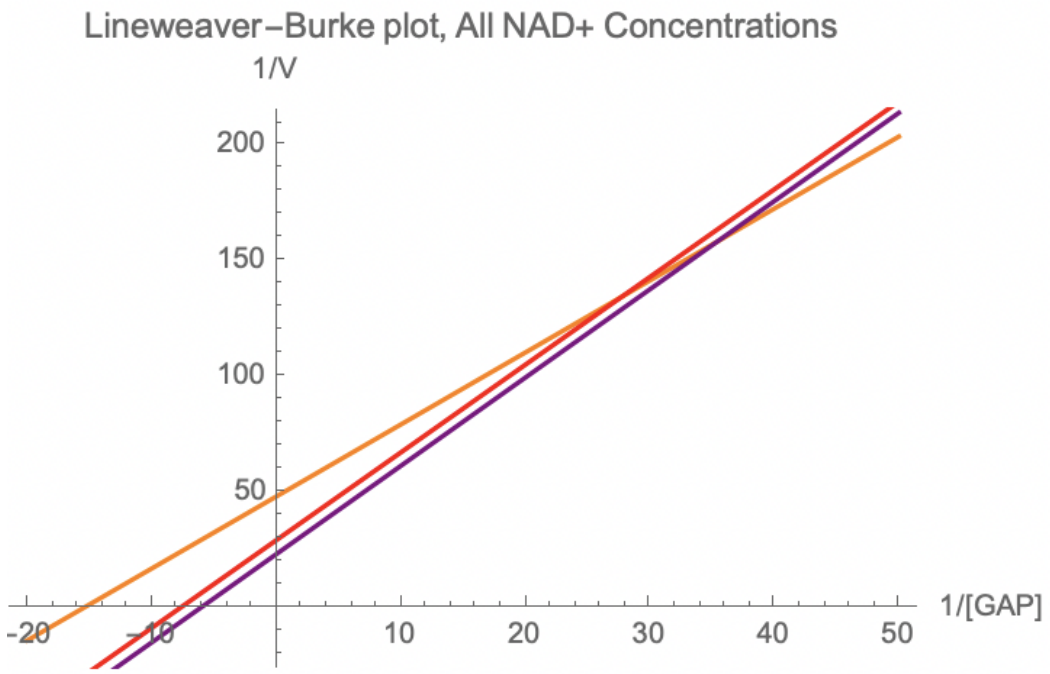
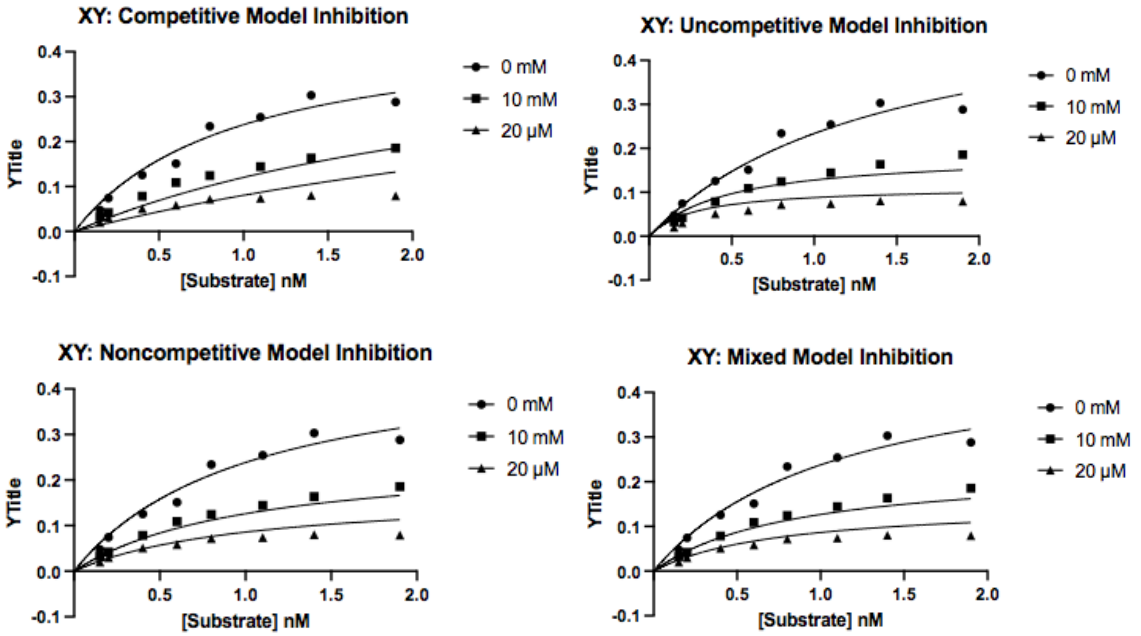


Figure 7: Lineweaver-Burk Plot
This figure shows the Lineweaver-Burk plot (Double-Reciprocal plot) of the three data sets. 0.240 mM NAD^+ is shown in purple, 0.120 mM NAD^+ is shown in red, and 0.060 mM NAD^+ is shown in orange.

GAPDH Inhibition Studies:

The inhibition data was plotted using 4 different inhibition models from Graphpad Prism 9 (Figure 8). The mixed model of inhibition had the highest regression coefficients and thus represented the chosen model for further analysis. Figure 9 shows the equations used in the mixed inhibition model.



	Mixed Inhibition	Competitive Inhibition	Uncompetitive Inhibition	Noncompetitive Inhibition
Inhibition Constant, Ki (mM)	16.070	5.009	4.965	11.230
Regression Coefficient for 0 mM ADP	0.959	0.957	0.954	0.958
Regression Coefficient for 10 mM ADP	0.939	0.890	0.889	0.944
Regression Coefficient for 20 mM ADP	0.561	0.128	0.427	0.492
α	0.536	N/A	N/A	N/A

Figure 8: Inhibition Models

This figure highlights the four inhibition models in Graphpad Prism 9. The four graphs visualize the fit, while the table below gives regression coefficients for each data set relative to the model.

$$V_{maxApp} = \frac{V_{max}}{\frac{(1+I)}{\alpha \times K_i}} \qquad K_{mApp} = K_m \times \frac{\frac{(1+I)}{K_i}}{\frac{(1+I)}{\alpha \times K_i}}$$

Figure 9: Built In Equations

This figure gives the equations used in Graphpad Prism 9's mixed inhibition model. Where **α** is a mechanism parameter. According to the model **α** close to but greater than 0 indicate uncompetitive inhibition while **α** close to 1 indicate noncompetitive inhibition.

Discussion:

Urate Oxidase Model:

Our model provides an alternative to simply measuring the conversion from urate to 5-hydroxyisourate at a high pH. That is, it intuitively corrects for the formation of 5-hydroxyisourate, the presumed source of nonlinearity. Since we are measuring the rate of disappearance of urate in this experiment, we can effectively use the k_5 rate parameter in order to remove the 5-hydroxyisourate that appears at the same wavelength as urate. Figure 2 simply shows that the model is logical, that is the 5-hydroxyisourate quickly reaches equilibrium and the concentration of urate continuously decreases as product forms. Figure 3 provides little quantitative information, but does show that the correction occurs initially and that it should not be a huge correction because [S] >> [E] and thus [S] >> [ES]. Furthermore, figure 4 gives quantitative values to show how the model differs at given times. The fit residuals are logical and have very small magnitudes. Despite this, there is a fairly significant discrepancy at around 400 seconds. This discrepancy is likely caused by measurement errors, but in the case that it is not it could be caused by our model not accounting for an additional rate constant. For example, at this time the concentration of 5-hydroxyisourate is approximately 10% of that of urate. This may cause the value of a hypothetical k_4, that converts 5-hydroxyisourate back to the enzyme substrate complex, to become analytically relevant. So, a potential k_4 would increase the accuracy of our model, specifically in that time range.

Overall, our model is underdeveloped. This is likely due to our lack of understanding of the mathematical principles behind complex enzymatic kinetics, our limited experience using Mathematica to develop said principles, and our poorly planned initial attempts where we made the model more complicated than it needed to be. This was our third attempt at a model for this task. The first was much too complicated to start with. Our code was illegible and we did not understand the implications of our proposed mechanism. Our second was slightly too complicated still, but pointed us in the right direction. In the future, I would suggest that we start by analyzing the simplest model of our mechanism possible. Slowly and incrementally, I would suggest we add more rate constants as is practical.

GAPDH Multi Substrate Kinetics:

Figure 5 provides a valuable insight into the design of our experiment. It shows how decreasing the fixed concentration of one substrate does not affect the $V_{initial}$, but does affect the V_{max} of the reaction. For understanding of the mechanism see figures 6 and 7. These provide some quantitative data to suggest both an ordered sequential and a ping-pong mechanism. The double reciprocal plot appears to show two parallel lines and another line that intersects both of the others. The table on the other hand shows that an increase in V_{max} does not necessarily correspond with a change in K_m. For this reason, I suggest three things be considered. First, that the double reciprocal plot is fairly zoomed out and gives a less accurate representation of the data then the table does. Secondly, similar experiments in literature suggest that the reaction follows an ordered sequential mechanism.[4] Thirdly, our experiment lacked sufficient data, while the literature experiments did not. If we performed the same analysis at 3 more concentrations of NAD^+ we would be able to determine outliers. In our case any one of the NAD^+ concentration values could be disproportionately hurting the precision of our data. That is, we would be able to eliminate sets of NAD^+ concentration that vary significantly because we could identify them as outliers. Our concentrations of NAD^+ are also on the lower side. The other data set was not analyzed in this document for time's sake, but was analyzed on the side and showed significantly better correlation to an ordered sequential mechanism. That is changes in slope and not changes in intercept . For this reason, the literature data, and the favorability of the table to the Lineweaver-Burk plot, we can fairly comfortably state that GAPDH follows an ordered sequential mechanism.

GAPDH Inhibition Studies:

Figure 8 provides all the data from our measurements, along with the justification for our inhibition model, and our calculated results for the inhibition dissociation constant. None of the four described nonlinear fits correlated well with the 20 mM NAD^+, as seen from the regression coefficients. Despite this, there were no significant outliers in this dataset. This implies that the data was collected poorly. We found that the mixed inhibition model had the highest regression coefficients across the board, while the competitive inhibition model showed the least correlation. This fits well with the explanation of the **α** term.

Essentially, this term is a coefficient that determines the most similar inhibition model based on its value. When **α** >> 1 then competitive inhibition is dominant[5]. This is the opposite of our results where **α** is nearly exactly between 0 and 1. At 1 noncompetitive inhibition is dominant and at 0 uncompetitive inhibition is dominant[5]. The data we collected suggests that GAPDH exhibits mixed inhibition with respect to ADP. The mathematics were not carried out by hand, but instead we used Graphpad Prism 9's models. They are accurate and easy to use. Using these models saved a significant amount of time and yielded relatively favorable results relative to literature values[6]. Of course our results could be improved in several ways. First and foremost, the quantity of data collected needs to be increased in order to propose any results with great confidence. We need more data at each inhibition concentration and more inhibition concentration data sets. Also, from what I have seen our concentrations of sodium arsenate seem high. In nearly all other cases I have observed concentrations less than 5 mM. This may not be significant with regards to our results, but certainly is a noticeable difference in experimental design.

Conclusion:

We successfully modeled urate oxidase to attempt to account for initial nonlinearity in Michaelis-Menten analysis, but did not have any way to confirm its validity except through our own experimental rates from a mutant variant. We also accounted for the effects of varying concentrations of substrate against fixed concentrations of another substrate in order to identify the mechanism of substrate addition and product release in GAPDH. We compared this result to literature values and discussed improvements in experimental design and data collection. We also performed inhibition studies on GAPDH using ADP. ADP showed mixed inhibition in our results and was overall our most conclusive experiment in this lab component.

Additional Question:[7,8]

Enzyme kinetics and inhibition question:

Luciferases are oxidative enzymes that are responsible for some bioluminescence. They behave similarly to most enzymes in that they react with a substrate, luciferin, to produce a product, light, at a given rate. Green fluorescent protein (GFP) similarly produces light, but requires no molecular substrate. One proposed mechanism for GFP suggests that incoming visible light (~390 nm) causes a proton transfer event that eventually leads to release of visible light (~510 nm). Select all of the following that are true?

a. Rate data for GFP cannot be modelled by a Michaelis-Menten curve because GFP has no molecular substrate.
b. A strong and dark dye is an inhibitor for GFP because it decreases the rate of light reacting with the enzyme.
c. UV-vis spectrophotometry is capable of directly measuring the rate of reaction of both enzymes.
d. GFP's kinetic mechanism cannot be elucidated by inhibition studies because it has no molecular substrate.

Answer Strategy:

In general for this question I would read the questions carefully and consider the definitions of the main concepts very carefully. Take answer choice B for example, an inhibitor is often defined as covalently reacting with an enzyme. The approach is similar for A. For C one must consider what conditions are required to use UV-vis spectrophotometry quantitatively, and for D one must consider how an inhibitor may bind to a protein and how the change that incurs can be measured and help to explain the mechanism.

Educational Value:

I think this question is relatively straightforward for answers A, B, and C. They require an understanding of what we are studying with some additional critical thinking. I think answer D requires the most understanding and has a few additional tricks to divert the focus from the main question. I intentionally designed parts of this question to be on the easier side because I find the questions in this class to be challenging. In general, I like

multipart questions that have some easier and some harder components. It helps to separate students who do not have sufficient knowledge, from those with some knowledge and from those who went above and beyond in their studies. I also know that this class is full of very smart students who will most likely come up with many difficult questions.

Answer Key:

I don't know if you want the answer key, but I will say them here anyways:

A True - Michaelis-Menten analysis is heavily focused on substrate saturation, which is not a function of a consistent independent variable in this case.

B False - Dye would inhibit the function of the enzyme by decreasing the frequency of interaction of the enzyme and the light, but the dye does not interact with the enzyme chemically

C True - Both proteins emit visible light. This light can be measured by a spectrophotometer in order to determine the rate of the reaction

D False - While GFP's kinetic mechanism may not be determinable by inhibition studies, it is not because GFP has no molecular substrate. For example, it could have an allosteric inhibitor

References:

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