Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2018

Lecture 12:

A Few Points on Quiz 1 and and More on Enzyme Kinetics

15 February 2018

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From Quiz 1, Problem 3

- Data from two spectrophotometers

	Spectrophotometer 1	Spectrophotometer 2
N	20	10
Average	0.523	0.565
SEM	0.0022	0.0031

- Part a: Calculate the sample standard deviation, s.
- The unstated question: What is the relationship between the standard error of the mean (SEM) and the sample standard deviation (s)?

Clicker Question #1

What does the standard error of the mean (SEM) represent?

1. The precision of the instrument used in the measurements.
2. The precision of the person making the measurements.
3. The overall precision of the measurements.
4. The precision of the mean derived from the measurements.

Clicker Question #2

What does the sample standard deviation (s) represent?

1. The precision of the instrument used in the measurements.
2. The precision of the person making the measurements.
3. The overall precision of the measurements.
4. The precision of the mean derived from the measurements.

Clicker Question #3

How are SEM and s related?

1. $\text{SEM} = s \cdot N$
2. $\text{SEM} = s/N$
3. $\text{SEM} = s\sqrt{N}$
4. $\text{SEM} = s/\sqrt{N}$

Calculating s from SEM

- $s = \text{SEM}\sqrt{N}$
- For spectrophotometer 1:

$$
\begin{aligned}
s &= \text{SEM}\sqrt{N} \\
&= 0.0022 \times \sqrt{20} \\
&= 0.0098
\end{aligned}
$$

- For spectrophotometer 2:

$$
\begin{aligned}
s &= \text{SEM}\sqrt{N} \\
&= 0.0031 \times \sqrt{10} \\
&= 0.0098
\end{aligned}
$$

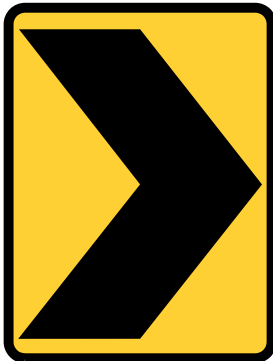
From Quiz 1, Problem 3

- Data from two spectrophotometers

	Spectrophotometer 1	Spectrophotometer 2
N	20	10
Average	0.523	0.565
SEM	0.0022	0.0031
s	0.0098	0.0098

- What can we say about the precision of the two instruments?
- What can we say about the accuracy of the two instruments?

Warning!



Direction Change

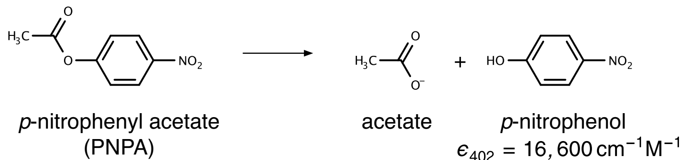
Back to enzyme kinetics.

Outline of Enzyme Kinetics Experiment

- This week:
  1. Measure velocity as a function of enzyme concentration.
  2. Determine enzyme concentration by titration with an inhibitor.
- Next week:
  1. Determine enzyme concentration by reaction with a “burst substrate”.
  2. Measure reaction velocity as a function of substrate concentration.
- Data analysis. Calculate:
  - K_{m}
  - V_{max}
  - k_{cat}

Experiment 3, Part C: Another Way to Measure the Trypsin Concentration

- Based on a historically important experiment:

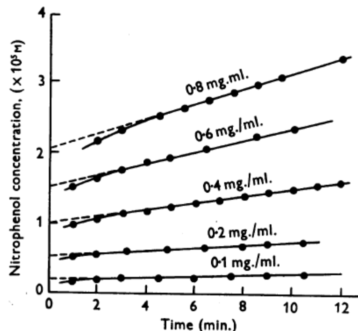


p-nitrophenyl acetate (PNPA) acetate *p*-nitrophenol

$\epsilon_{402} = 16,600\,\text{cm}^{-1}\text{M}^{-1}$

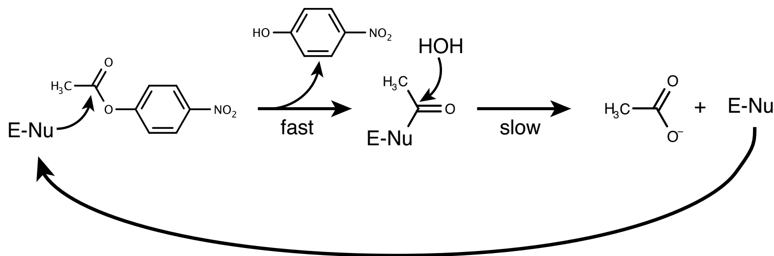
Reaction with chymotrypsin:

- PNPA isn't a very good substrate!
- Reactions are not linear with time.
- Initial displacement of curves increases with enzyme concentration.
- What is going on?



Hartley, B. S. & Kilby, B. A. (1954). The reaction of *p*-nitrophenyl esters with chymotrypsin and insulin. *Biochem. J.*, 56, 288–297. http://dx.doi.org/10.1042/bj0560288

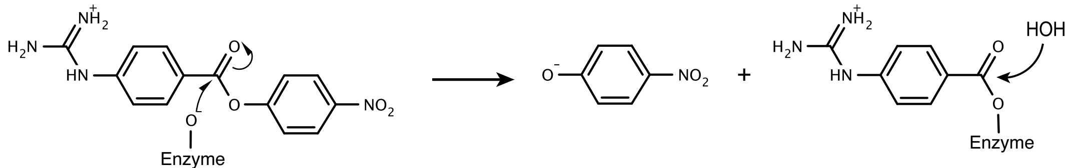
Proposed Mechanism to Explain “Burst Kinetics”



- Enzyme reacts rapidly with substrate to forms covalent intermediate and releases *p*-nitrophenol.

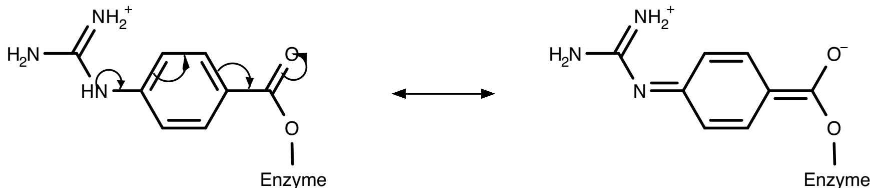
  Each molecule of enzyme produces one molecule of product in burst phase.
- Hydrolysis of covalent intermediate is much slower and is required to regenerate enzyme (turnover).
- Steady state rate of product formation is determined by the second step.
- Among the first evidence for a covalent intermediate in an enzyme-catalyzed reaction.

A Designed Burst Substrate for Trypsin



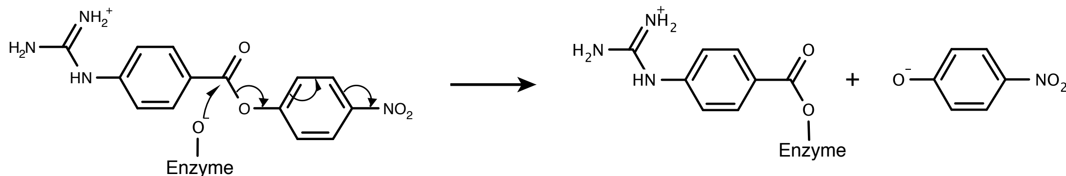
- Guanido group resembles arginine side chain.
- Hydrolysis of covalent intermediate is very slow. (half-time greater than $\approx$ 40 h)
- An (almost) irreversible inhibitor, or "suicide substrate".
- Why is hydrolysis so slow?

Resonance Stabilization of the Covalent Intermediate



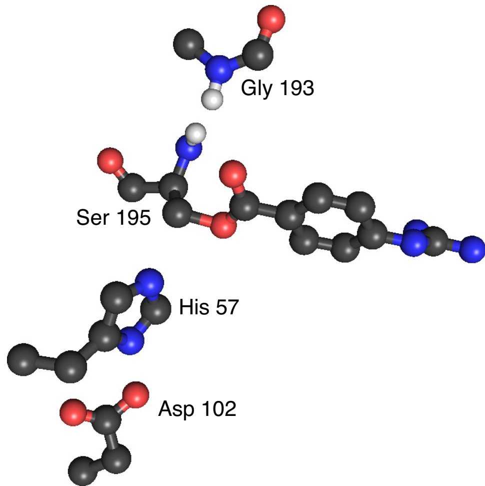
- Resonance structures shift electrons from phenyl ring to carbonyl carbon.
- Electron density on carbonyl carbon disfavors nucleophilic attack by water.
- Why doesn't the same effect prevent *formation* of the intermediate?

Resonance Also Favors Formation of the Covalent Intermediate



- Resonance shifts electrons from carbonyl carbon to nitrophenyl ring.
- Makes the nitrophenyl group a better leaving group.
- *Favors* nucleophilic attack in the first step of the reaction.

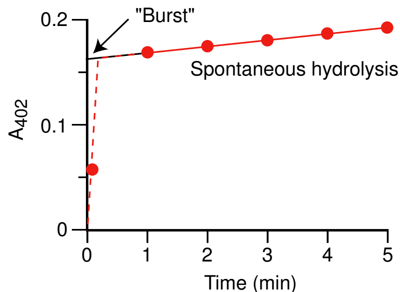
Three-dimensional Structure of *p*-guanido benzoate intermediate



- As predicted, Ser 195 O_γ forms ester with *p*-guanido benzoate.
- Amide groups of Ser 195 and Gly 193 form "oxyanion hole" that stabilizes negative charge on carbonyl oxygen in transition state.
- Where would the water molecule be for hydrolysis of the intermediate?

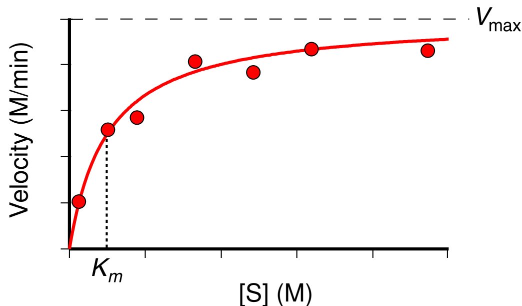
Mangel, W. F., Singer, P. T., Umland, T. C., Toledo, D. L., Stroud, R. M., Pflugrath, J. W. & Sweet, R. M. (1990). Structure of an acyl-enzyme intermediate during catalysis:(guanidobenzoyl)trypsin. *Biochemistry*, 29, 8351–8357. `http://dx.doi.org/10.1021/bi00488a022`

Experiment 3, Part C:
Measurement of Trypsin Concentration with Burst Substrate



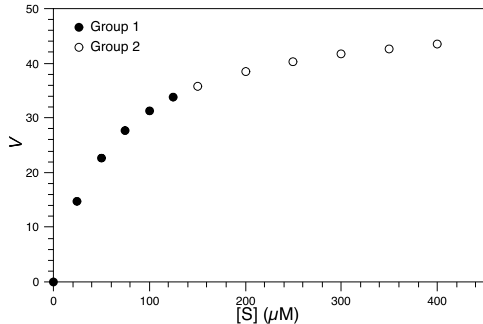
- Requires high enzyme concentration, since each enzyme molecule generates only one chromophore molecule.
- Tris buffer is bad for this experiment, because amines are nucleophilic and react with *p*-NPGB.
- Need to record both the absolute increase in absorbance and the rate of steady-state increase, in order to extrapolate initial burst phase.
- Ignore first data point, since it is recorded slightly after reaction starts, but probably before the burst is complete.

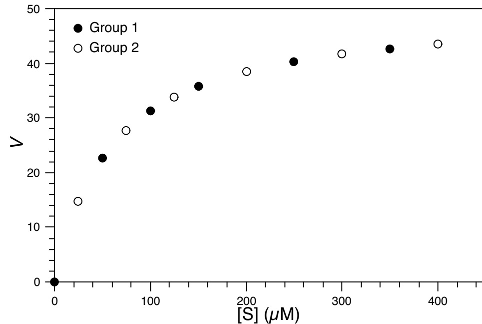
Experiment 3, Part C: Velocity as a Function of Substrate Concentration



- To reliably estimate both K_m and V_{max}, substrate concentrations must cover range both below and above K_m.
- We will use eleven substrate concentrations, plus a control without substrate.
- Two groups of six reactions.
- How should the reactions be grouped?

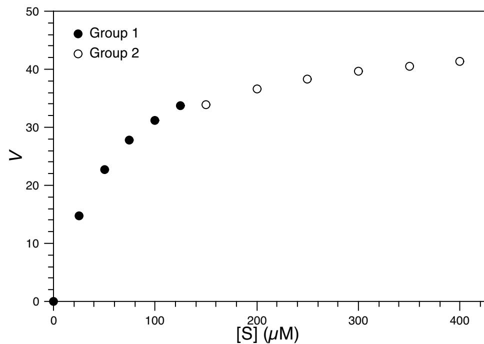
Two Ways to Group the Reactions

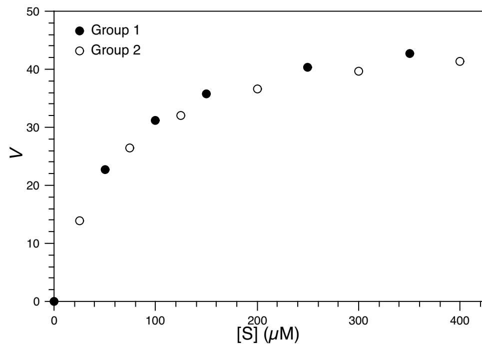




- Is one way better than the other?
- What if something changes between the reactions?

Two Ways to Group the Reactions





- 1–6 grouping: changes between reaction groups may be hard to detect.
- Odd–even grouping: changes between reaction groups are easier to detect. Fitting data together averages effects more evenly.