Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2017

Lecture 12:

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

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From Quiz 1, Problem 1

- A buffer solution containing:
  0.2 moles HEPES (an acid, HA, $\mathrm{p}K_a = 7.5$)
  0.1 moles Na-HEPES (the conjugate base, A^-)
  $\approx 400\,\mathrm{mL}$ water
- What is the expected pH?

$$\mathrm{pH} - \mathrm{p}K_a = \log \frac{[A^-]}{[AH]} = \log \frac{\text{moles } A^-}{\text{moles AH}}$$

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{\text{moles } A^-}{\text{moles AH}}$$

$$= 7.5 + \log \frac{0.1 \text{ moles}}{0.2 \text{ moles}} = 7.5 + \log 0.5$$

$$= 7.2$$

From Quiz 1, Problem 1

- How much NaOH should we add to the buffer to increase the pH to 7.8?
- What happens when we add NaOH to the solution?
  - $NaOH \longrightarrow Na^+ + OH^-$
  - $OH^- + H^+ \longrightarrow H_2O \qquad [OH^-][H^+] = 10^{-14}\ M^2$
  - $AH \longrightarrow A^- + H^+$
- What is the $\frac{(\text{moles } A^-)}{(\text{moles } AH)}$ ratio at pH 7.8?

$$\text{pH} - \text{p}K_a = \log \frac{\text{moles } A^-}{\text{moles AH}}$$

$$\log \frac{\text{moles } A^-}{\text{moles AH}} = 7.8 - 7.5 = 0.3$$

$$\frac{\text{moles } A^-}{\text{moles AH}} = 10^{0.3} = 2.0$$

From Quiz 1, Problem 1

	Moles AH	Moles A^-
Initial	0.2	0.1
Change	$-x$	x
Equilibrium	$0.2 - x$	$0.1 + x$

- $x =$ moles NaOH added.
- At equilibrium:

$$\frac{\text{moles } A^-}{\text{moles AH}} = 2.0$$

$$\frac{0.1 + x}{0.2 - x} = 2.0$$

- Solve for x!

From Quiz 1, Problem 2

- Given:
  - A solution containing NAD^+ and NADH, with total concentration 0.4 mM.
  - For NADH, $\epsilon_{339} = 6,200\,\mathrm{cm}^{-1}\mathrm{M}^{-1}$
  - For NAD^+ $\epsilon_{339} = 0$
- Want to measure A_{339} to determine concentration of NADH.
- What other information do we need?
  - What is the first thing we do when measuring an absorbance? (after setting the wavelength)

From Quiz 1, Problem 2

- If $A_{339} = 0.89$, what fraction of light is absorbed?
- The definition of absorbance, A

$$A = \log \frac{I_0}{I}$$

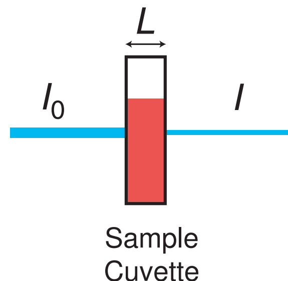
- Take logarithms and rearrange:

$$\frac{I}{I_0} = 10^{-A}$$

Is this the fraction absorbed?

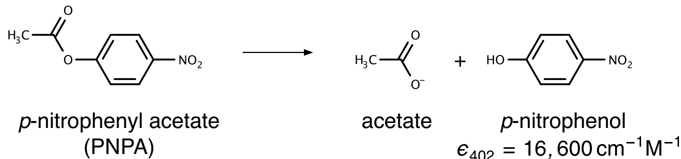
- The fraction absorbed:

$$f_{\text{abs}} = 1 - \frac{I}{I_0} = 1 - 10^{-A}$$



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

- Based on a historically important experiment:



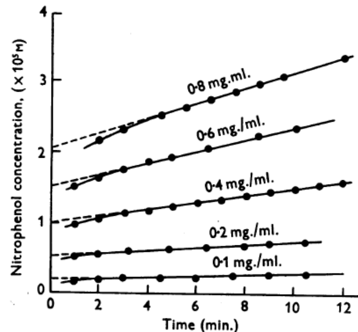
p-nitrophenyl acetate
(PNPA)

acetate

p-nitrophenol
$\epsilon_{402} = 16,600\,\mathrm{cm^{-1}M^{-1}}$

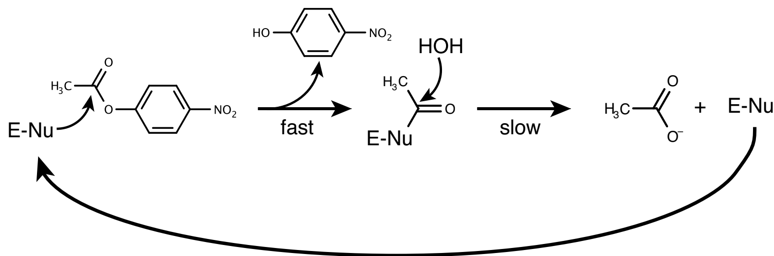
Reaction with chymotrypsin:

- 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

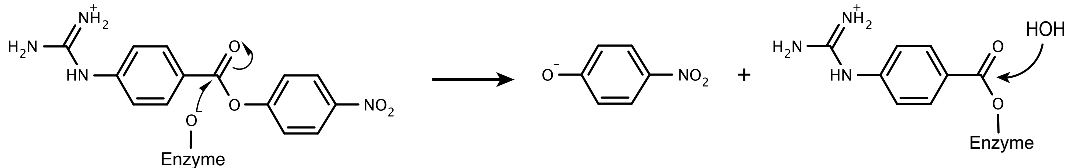
Proposed Mechanism to Explain "Burst Kinetics"



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

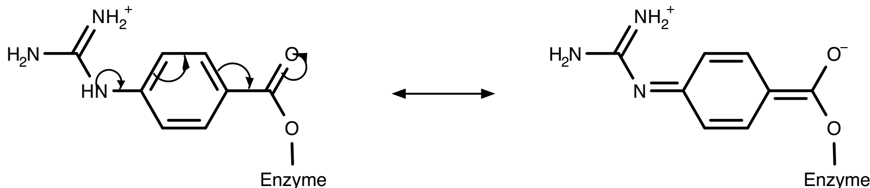
  Each molecule of enzyme produces one molecule of product in burst phase.
- Hydrolysis of covalent intermediate is much slower and is required to regenerate enzyme (turnover).
- Steady state rate of product formation is determined by the second step.
- Among the first evidence for a covalent intermediate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

A Designed Burst Substrate for Trypsin



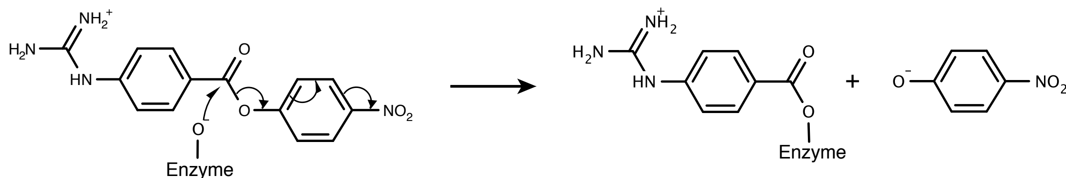
- Guanido group resembles arginine side chain.
- Hydrolysis of covalent intermediate is very slow. (half-time greater than ≈ 40 h)
- An (almost) irreversible inhibitor, or "suicide substrate".
- Why is hydrolysis so slow?

Resonance Stabilization of the Covalent Intermediate



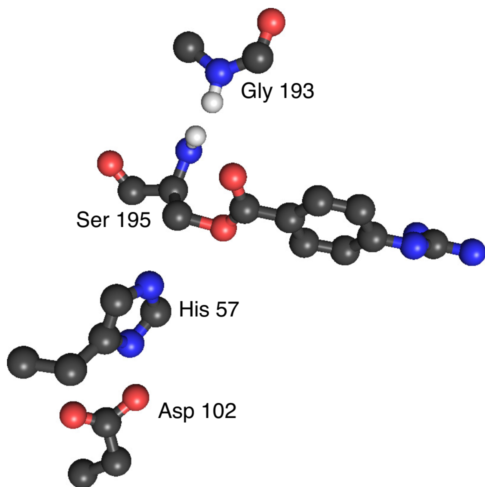
- Resonance structures shift electrons from phenyl ring to carbonyl carbon.
- Electron density on carbonyl carbon disfavors nucleophilic attack by water.
- Why doesn't the same effect prevent *formation* of the intermediate?

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.

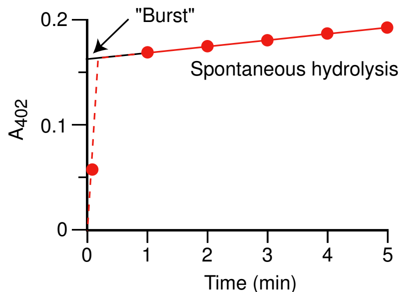
Three-dimensional Structure of *p*-guanido benzoate intermediate



- 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?

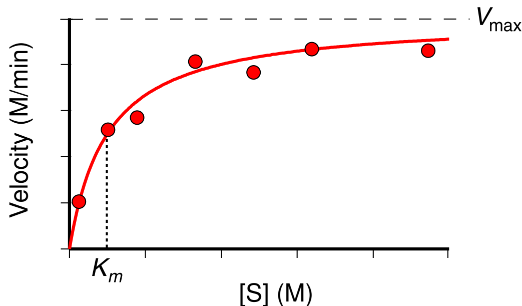
Mangel, W. F., Singer, P. T., Umland, T. C., Toledo, D. L., Stroud, R. M., Pflugrath, J. W. & Sweet, R. M. (1990). Structure of an acyl-enzyme intermediate during catalysis:(guanidobenzoyl)trypsin. *Biochemistry*, 29, 8351–8357. http://dx.doi.org/10.1021/bi00488a022

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



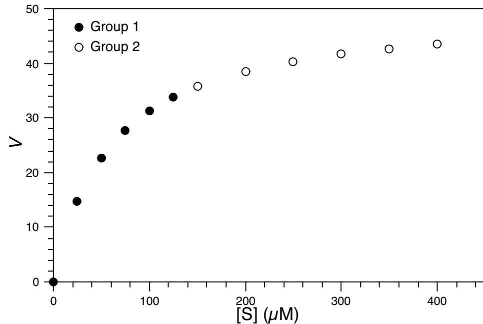
- Requires high enzyme concentration, since each enzyme molecule generates only one chromophore molecule.
- Tris buffer is bad for this experiment, because amines are nucleophilic and react with *p*-NPGB.
- Need to record both the absolute increase in absorbance and the rate of steady-state increase, 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.

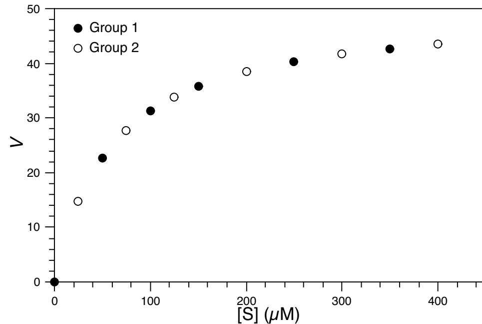
Experiment 3, Part C: Velocity as a Function of Substrate Concentration



- 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?

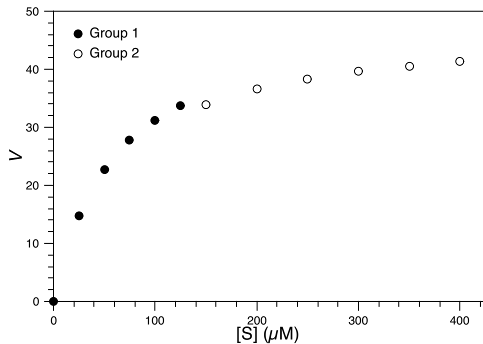
Two Ways to Group the Reactions

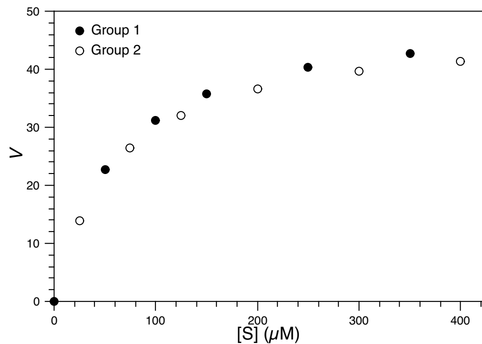




- Is one way better than the other?
- What if something changes between the reactions?

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.