Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2018

Lecture 22:

Questions from Quiz 2 and the Trypsin Digestion Experiment

29 March 2018

©David P. Goldenberg
University of Utah
goldenberg@biology.utah.edu

Quiz 2, Question 1

- For a newly discovered protease:

  $K_m = 13\,\mu M$

  $k_{cat} = 850\,min^{-1}$

  Molecular weight = 85,000 Da

- Part a: Calculate V_{max} when the total enzyme concentration is 1.5 μg/mL.

  $$V_{max} = [E]_T \times k_{cat}$$

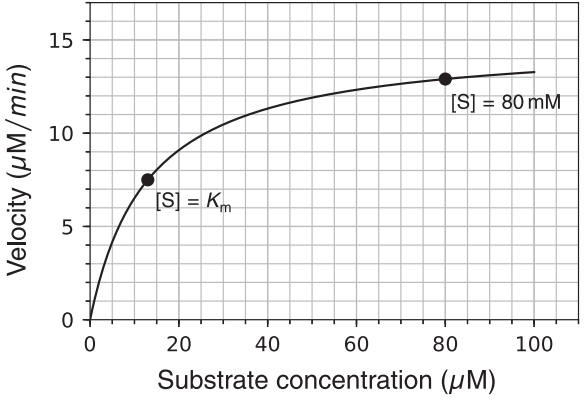
  $[E]_T$ must be expressed in M units!

- Part b: Calculate V when the total enzyme concentration is 1.5 μg/mL and the substrate concentration is 80 mM.

  $$V = \frac{V_{max}[S]}{[S] + K_m}$$

Quiz 2, Question 1

- Part c: Sketch the graph of V versus [S].



Quiz 2, Question 1

- Part d: Calculate the concentrations of the free enzyme (E) and the enzyme-substrate complex ($E \cdot S$) when the total enzyme concentration is $1.5\,\mu g/mL$ and the substrate concentration is $80\,mM$.
  - The ratio, V/V_{max} represents the fraction of enzyme with substrate bound.

$$\frac{V}{V_{max}} = \frac{[E \cdot S]}{[E] + [E \cdot S]} = \frac{[E \cdot S]}{[E]_T}$$

$$\frac{V}{V_{max}} = \frac{13\,\mu M/min}{15\,\mu M/min} = 0.87 = \frac{[E \cdot S]}{[E]_T}$$

$$[E \cdot S] = 0.87[E]_T = 0.87 \times 0.0176\,\mu M = 0.015\,\mu M$$

$$[E] = [E]_T - [E \cdot S] = 0.0176\,\mu M - 0.015\,\mu M = 0.0026\,\mu M$$

Quiz 2, Question 1

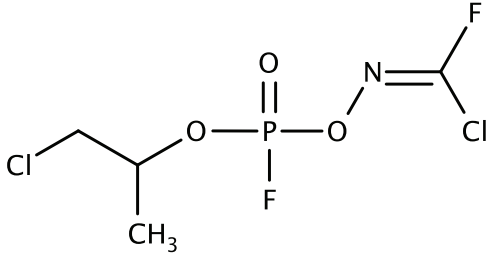
- Part d: Calculate the concentrations of the free enzyme (E) and the enzyme-substrate complex ($E \cdot S$) when when the total enzyme concentration is 1.5 μg/mL and the substrate concentration is 80 mM.
  - Another way: At any substrate concentration:

$$V = [E \cdot S] \times k_{cat}$$

$$[E \cdot S] = \frac{V}{k_{cat}} = \frac{13\,\mu M/min}{850\,/min} = 0.015\,\mu M$$

Quiz 2, Question 2

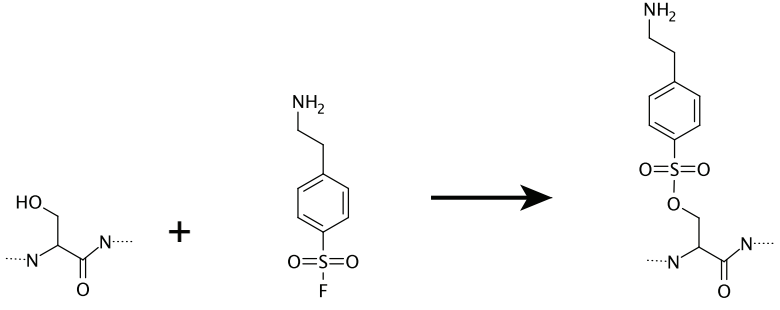
- A putative “Novichok” nerve agent:



  - Resembles diisopropyl fluorophosphate and reacts with acetylcholine esterase in the same way.
  - Draw the structure of the expected product of the reaction with acetylcholine esterase.

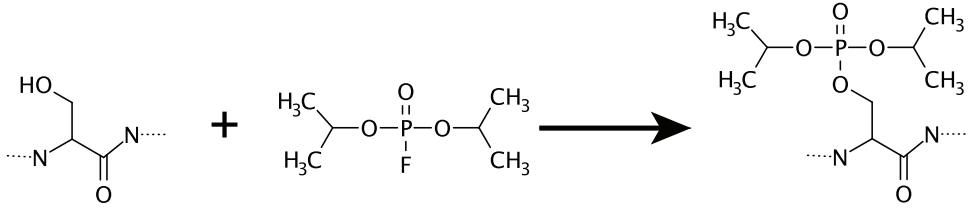
Irreversible Inhibition of Trypsin by AEBSF

4-(2-aminoethyl)-benzenesulfonyl fluoride

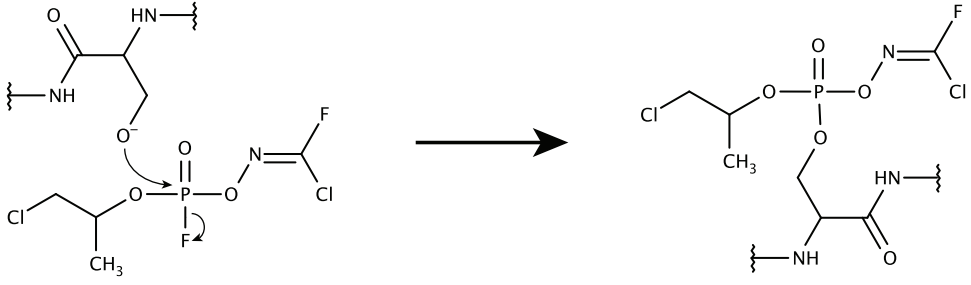


-Ser- AEBSF

Irreversible Inhibition of Serine Proteases by Diisopropyl Fluorophosphate



Expected Reaction of Novichok Agent With Acetylcholine Esterase



Warning!

Direction Change

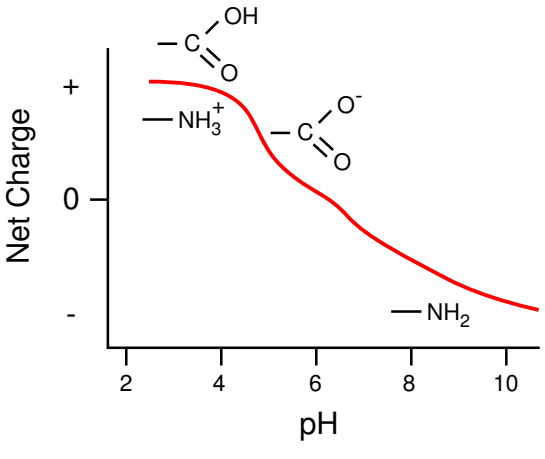
Back to Electrophoresis

Factors That Influence Mobilities in Non-Denaturing Gels

1. Net charge of protein
   - Amino acid sequence (relative number of acidic and basic residues)
   - Solution pH
   - Three dimensional structure (can influence pK_as)
2. Size and shape of protein
3. Concentration and degree of cross-linking in gel
   - Gel will generally reduce mobilities of proteins, relative to their free mobilities.
   - Larger molecules will be affected by the gel more than smaller ones.
   - Composition of the gel can be manipulated to fractionate molecules of different sizes.

- Conditions often have to be optimized for a particular protein.

Effects of pH on Protein Net Charge



- Curve represents a population average! (or a time average)
- Shape of curve will depend on amino acid sequence and structure of a particular protein.
- For each protein, there is a pH at which positive and negative charges are balanced and the molecules have an average net charge of 0. This is the isoelectric point, pI.

pK_a Values of Ionizable Groups in Proteins

Group	In peptides	Avg. in proteins	Low in proteins	High in proteins
Asp	3.9	3.5±1.2	0.5	9.2
Glu	4.3	4.2±0.9	2.1	8.8
His	6.5	6.6±1.0	2.4	9.2
Cys	8.6	6.8±2.7	2.5	11.1
Tyr	9.8	10.3±1.2	6.1	12.1
Lys	10.4	10.5±1.1	5.7	12.1
C-term	3.7	3.3±0.8	2.4	5.9
N-term	8.0	7.7±0.5	6.8	9.1

Grimsley, G. R., Scholtz, J. M. & Pace, C. N. (2008). A summary of the measured pK values of the ionizable groups in folded proteins. *Protein Sci.*, 18, 247–251. http://dx.doi.org/10.1002/pro.19

Clicker Question #1

For a protein containing:

- 3 Asp residues
- 6 Glu residues
- 4 His residues
- 6 Lys residues

At what pH will the mobility be greatest?

1. pH 2
2. pH 4
3. pH 6
4. pH 8
5. pH 10

All answers count for now.

Clicker Question #2

For a protein containing:

- 3 Asp residues
- 6 Glu residues
- 4 His residues
- 6 Lys residues

At what pH will the mobility be smallest?

1. pH 2
2. pH 4
3. pH 6
4. pH 8
5. pH 10

Potentially Charged Residues in RNAse A

- Potentially negatively charged:
  - Terminal carboxyl group: 1
  - Aspartic acid: 5
  - Glutamic acid: 5
- Potentially positively charged:
  - Terminal amino group: 1
  - Arginine: 4
  - Lysine: 10
  - Histidine: 4
- Only present in RCM form:
  - Carboxymethylated Cys: 8

- For native and RCAM forms:
  - Calculated isoelectric point: 8.6
  - Expected charge at pH 7: $+4$
- For RCM form:
  - Calculated isoelectric point: 5.6
  - Expected charge at pH 7: -4

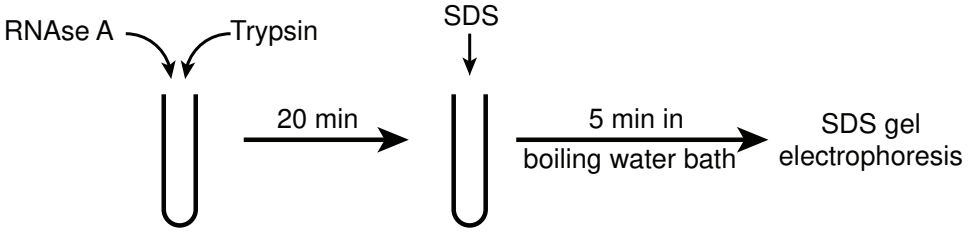
Conditions for Electrophoresis of Ribonuclease A

1. pH 4.4
   - All forms of RNAse A have a net positive charge.
   - Buffered with β-alanine and acetate.
2. All molecules migrate towards the negative electrode (cathode)
3. Gel is composed of 12% acrylamide, 0.032% bisacrylamide (cross-linker)
   (A relatively high concentration for a relatively small protein)

Trypsin Digestion Experiment

- The questions:
  - Are different forms of RNAse A substrates for trypsin?
  - Are some forms better substrates than others?
  - Can we compare RNAse A forms to chromogenic peptide as substrates for trypsin?
- The strategy:
  - SDS gel electrophoresis separates polypeptides on the basis of molecular weight. (number of amino acid residues)
  - Follow the disappearance of intact RNAse A to monitor hydrolysis.
- Possible experimental variables:
  - Time of reaction.
  - Enzyme concentration.
  - Substrate concentration.
  - Solution conditions: Temperature, pH, other solutes.
- We will only vary enzyme concentration.

Trypsin Digestion Experiment



- For each RNAse A form (N, RCM, RCAM):
  - Reaction with no trypsin.
  - Two reactions with different trypsin concentrations.
- Reactions stopped after 20 min by adding SDS and heating (95°C).
- RNAse A analyzed by SDS gel electrophoresis.
- Relative concentrations estimated by quantitative analysis of gel image.