1.

i) Design a competitive inhibitor of elastase, a serine protease (refer to the table on the right for useful information on specificity).

ii) Sketch the double reciprocal plot you would expect to obtain for steady-state data obtained in the absence and the presence of your inhibitor. Be sure to label your axis.

iii) How would you get $K_I$ from your plot?

A competitive inhibitor should be similar to the substrate, but cannot undergo a chemical reaction. We know that peptides with a leucine sidechain bind well to elastase (low $K_m$).

We cannot have a peptide bond after the leucine residue, because that would just be another substrate. Two modifications will prevent the enzyme from cleaving that bond (both of these are utilized in HIV protease inhibitors—see the question below).

a) Change the C=O to an alcohol, if the alcohol is attacked by the activated nucleophile (Ser or OH), it cannot easily go to the transition state because that would involve breaking the C-O bond.

b) Replacement of the N with C would reduce the partial positive charge on the carbon, making the carbon less likely to be a good target for a nucleophile.

d) Since this would be a competitive inhibitor, $V_{max}$ remains the same ($y$-intercept), only the slope will change.

ii) The ratio of the slopes gives $\alpha$, from which $K_I = [I]/(\alpha - 1)$

2. A patient who is infected with the HIV virus is no longer responsive to HIV protease inhibitors due to the generation of a mutant virus. You obtain the structure of the mutant HIV protease (you can view the mutant protease on Jmol).

Notes on the Jmol page:

- "Simple View"—will make it easy to identify the specificity pocket and the bound drug.
- "Unmodified" will load the wild-type enzyme, with the cyclohexane drug (drug shown on right).
- "Mut+cyclohexane" will load the mutant enzyme, with the cyclohexane drug.
- "Drug A" will load the mutant enzyme and drug A.
- "Drug B" will load the mutant enzyme and drug B.
- "Drug C" will load the mutant enzyme and drug C.
- Clicking on "#82" will highlight residue 82 in the enzyme.

i) What residue has been changed in the altered enzyme? In what way was it changed? Val82 to Asp

ii) You have the following three possible drugs that could be used to treat this patient. Use the drylab to find the $K_I$ for each of these drugs. Write you $K_I$ values in the table below. Collect data using substrate concentrations from 0 to 20 using an inhibitor concentration of 2 nM.

b) On the basis of the $K_I$, which drug would be a better inhibitor? Drug A, B or C?

c) View the Jmol page and explain how the interaction of the drug with the enzyme is related to the $K_I$ values. Look for favorable and unfavorable interactions. Briefly summarize your explanation in the table.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$k_{CAT}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-Ala</td>
<td>100 μM</td>
<td>$10^4$/sec</td>
</tr>
<tr>
<td>Leu-Ala</td>
<td>10 μM</td>
<td>$10^4$/sec</td>
</tr>
<tr>
<td>Phe-Ala</td>
<td>100 μM</td>
<td>$10^2$/sec</td>
</tr>
</tbody>
</table>

\[
y = 0.0151x + 0.005 \\
y = 0.007x + 0.005 \\
y = 0.005x + 0.005 \\
\]
The velocity data and double reciprocal plot, using [I]=2 nM:

<table>
<thead>
<tr>
<th>S</th>
<th>vi</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>66.7</td>
<td>28.4</td>
<td>52.3</td>
<td>58.4</td>
</tr>
<tr>
<td>1</td>
<td>99</td>
<td>50.4</td>
<td>84.1</td>
<td>91.7</td>
</tr>
<tr>
<td>2</td>
<td>134.3</td>
<td>79.4</td>
<td>116.8</td>
<td>124.1</td>
</tr>
<tr>
<td>10</td>
<td>180.2</td>
<td>152.5</td>
<td>173.9</td>
<td>177</td>
</tr>
<tr>
<td>20</td>
<td>190.4</td>
<td>174.3</td>
<td>187.4</td>
<td>189.1</td>
</tr>
</tbody>
</table>

The alpha values are obtained from the ratio of the slopes, from which $K_i = [I]/(\alpha-1)$.

- Drug A: $\alpha=0.0151/.005=3.0$, $K_i = 2/(3-1) = 1$ nM
- Drug B: $\alpha=0.0070/.005=1.4$, $K_i = 2/(1.4-1) = 5$ nM
- Drug C: $\alpha=0.0060/.005=1.2$, $K_i = 2/(1.2-1) = 10$ nM

<table>
<thead>
<tr>
<th>Drug A</th>
<th>Drug B</th>
<th>Drug C</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Drug A" /></td>
<td><img src="image" alt="Drug B" /></td>
<td><img src="image" alt="Drug C" /></td>
</tr>
<tr>
<td>$K_i$</td>
<td>1 nM</td>
<td>5 nM</td>
</tr>
<tr>
<td>Mol. Explan.</td>
<td>The positive charged amine group on the drug interacts favorably with the negative charge on Asp82.</td>
<td>Although the NH group on the indole (tryptophan) can form a favorable hydrogen bond with Asp82, it is too bulky and will give unfavorable VDW.</td>
</tr>
</tbody>
</table>
Biochemistry I  Tutorial 7 – Part B - Answers  March 27, 2017

1. Use the online software (Practice version) to purify protein B from a mixture of the following four proteins. The total amount of each protein is 100 units. Sketch out your purification scheme using a flow chart.

<table>
<thead>
<tr>
<th>Protein</th>
<th>[Ammonium Sulfate] that precipitates 50% of protein*</th>
<th># Residues (Mol Wt)</th>
<th>#Asp (pK_a=4.0)</th>
<th>#His (pK_a=6.0)</th>
<th>#Lys (pK_a=9.0)</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.0 M</td>
<td>120 (13,200 Da)</td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>76 u/mg</td>
</tr>
<tr>
<td>B</td>
<td>1.5 M</td>
<td>120 (13,200 Da)</td>
<td>4</td>
<td>4</td>
<td>12</td>
<td>76 u/mg</td>
</tr>
<tr>
<td>C</td>
<td>4.0 M</td>
<td>120 (13,200 Da)</td>
<td>4</td>
<td>3</td>
<td>12</td>
<td>76 u/mg</td>
</tr>
<tr>
<td>D</td>
<td>6.0 M</td>
<td>240 (26,400 Da)</td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>38 u/mg</td>
</tr>
</tbody>
</table>

*Concentrations 1 M below will leave all of the protein in solution. Concentrations 1M above will ppte all the protein.

**General process for each purification step:**

a) Select and apply scheme. Possible purification methods are:

- **Precipitation by ammonium sulfate.** Concentrations of ammonium sulfate 1 M below the solubility point will leave all of the protein in solution. Concentrations 1M above will ppte all the protein.

- **Gel Filtration Chromatography:** separate by molecular weight, collect sample in tubes (fractions), larger proteins are found in earlier tubes.

- **Cation chromatography** – separates by positive charge, proteins with larger positive charge are found in later tubes.

- **Anion chromatography** – separates by negative charge, proteins with larger negative charge are found in later tubes.

b) Assay fractions to determine location of target protein by highlighting tube and clicking on “Do Enzyme Assay”

c) Pool fractions with target enzyme by highlighting tubes and clicking on “Collect Samples”

d) Compare specific activity to the specific activity of the pure protein (right column of table) to determine if additional step(s) are required.

Before developing a scheme, you need to look at the known properties to see if there are differences in size, charge, or solubility in ammonium sulfate that could be used to separate the proteins.

**Step 1: Solubility in Ammonium Sulfate:** C and D have different solubility in ammonium sulfate than A and B. If an ammonium sulfate concentration of 2.5M was used, all of A and B would precipitate while none of C or D would. The precipitate would then be dissolved before the next step.

After this step the specific activity is 38 units, therefore the protein is not pure and a second step is required.

**Step 2: Ion exchange.** Since size exclusion cannot be used (A and B are the same size) we have to resort to ion exchange. At pH=6 (the pKa of His, making the calculation easy) the net charge on A is +8 and B is +10. This is sufficient to allow separation. Since the charge on the protein is +, we need to do a cation exchange column. The elution profile is shown below, and the second peak was the target protein, as expected since its charge is higher.
Tubes 42-48 were collected, giving 99 units total activity (yield 99%) and a specific activity of 76, a pure protein.

2. You have isolated an inhibitor of HIV integrase from algae and you want to determine whether it is a competitive or a mixed type inhibitor.
   a) Use the dry lab to collect initial velocity data, recommended inhibitor concentration is 2 nM.
   b) Plot a velocity plot (v versus [S]) and a double reciprocal plot (1/v versus 1/[S]). Based on these plots, what type of inhibitor is this, competitive or mixed type? What are the $K_I$ and $K'_I$ values?
   c) What is the $K_D$ for binding of the inhibitor to the free enzyme.
   d) Assuming that the inhibitor concentration was 5 nM in the patient, what fraction of the enzyme would have inhibitor bound (e.g. calculate fractional saturation).
   e) HIV patients are already treated with a drug that is a competitive inhibitor of integrase, would this new drug be a useful addition to the arsenal of HIV anti-viral drugs, or not? Why?

<table>
<thead>
<tr>
<th>[S]</th>
<th>v</th>
<th>v (+ inh)</th>
<th>1/[S]</th>
<th>1/v</th>
<th>1/v (Inh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>66.4</td>
<td>26.9</td>
<td>2</td>
<td>0.01506</td>
<td>0.037175</td>
</tr>
<tr>
<td>1</td>
<td>100.6</td>
<td>45.7</td>
<td>1</td>
<td>0.00994</td>
<td>0.021882</td>
</tr>
<tr>
<td>5</td>
<td>165.3</td>
<td>99.1</td>
<td>0.2</td>
<td>0.00605</td>
<td>0.010091</td>
</tr>
<tr>
<td>10</td>
<td>181</td>
<td>117.1</td>
<td>0.1</td>
<td>0.005525</td>
<td>0.00854</td>
</tr>
<tr>
<td>20</td>
<td>189.1</td>
<td>128.1</td>
<td>0.05</td>
<td>0.005288</td>
<td>0.007806</td>
</tr>
</tbody>
</table>

b) This is a mixed type inhibitor because $V_{max}$ is reduced, this can be seen in the double reciprocal plot (Y-intercept is higher) AND the velocity curve – the velocity is reduced at high substrate.

$K_I = [I]/(\alpha'-1)$; $\alpha'$ = ratio of slopes = 0.015/0.005 = 3; $K_I = 2 \text{nm}/(3-1) = 1 \text{nm}$

$K'_I = [I]/(\alpha'-1)$; $\alpha'$ = ratio of y-int = 0.007/0.005 = 1.4; $K'_I = 2 \text{nm}/(1.4-1) = 5 \text{nm}$

c) $K_D = K_I = 1 \text{nM}$

d) $Y = [L]/(K_D + [L]) = 5/(1+5) = 5/6 = 0.83$

e) Yes, because it is a mixed type inhibitor it wouldn’t bind in the active site; mutations in the integrase that affected binding of the existing competitive inhibitor would not affect binding of the new inhibitor.
Competitive versus Mixed Type Inhibition – Which is better?

Download the spreadsheet PredictV. This will allow you to obtain velocity data for integrase in the absence of an inhibitor and in the presence of the competitive and mixed-type inhibitor (from problem 2). The spreadsheet will automatically calculate and plot the points for you.

a) Obtain the velocity versus substrate concentration data for 10 substrate concentrations (fill in the yellow highlighted boxes). Your selection of [S] values should allow you to determine \( V_{\text{MAX}} \) and \( K_M \) – i.e. you want values at high [S] and values of [S] that give you \( v \approx V_{\text{MAX}}/2 \) (you won’t actually obtain these values, these instructions were given so that you will generate a complete velocity curve. Sketch the curves in the plot on the right. See plot on right –>

b) Which inhibitor is best at low [S] ([S]<20), competitive or mixed type? Why?

The inhibitor with the lowest velocity is the better inhibitor, for [S]<20, it is the competitive inhibitor. This is because the low concentration of substrate allows the inhibitor to bind in the active site.

c) Which inhibitor is best at high [S] ([S]>50), competitive or mixed type? Why?

Again, the inhibitor with the lowest velocity is the better one - in this case it is the mixed type inhibitor. The substrate concentration is now high enough that it is becoming more difficult for the competitive inhibitor to bind, thus it is less effective than the mixed type.

4. A protein contains 2 small subunits (20 kDa), 1 large subunit (60 kDa), with both small subunits connected to the large subunit by a disulfide bond. (heterotrimer, \( \alpha_2\beta \)).

Sketch the SDS-PAGE gel without (left gel) and with (right gel) BME. The molecular weight standards are 10 and 100 kDa.

BME (\( \beta \)-mercaptoethanol) breaks disulfide bonds.

The SDS-PAGE gel without BME would leave the disulfide bonds intact, giving an overall molecular weight of \(~100\) kDa.

If BME is present, the disulfides are broken and there would be one chain with a MW of 60 kDa and another with 20kDa. The 20kDa band would be twice as thick, because the ratio of the \( \alpha \)-chains to the \( \beta \)-chain is 2:1. You can determine the exact migration distances using a calibration curve using the standards. A calibration curve that was obtained with the two standards (two additional points are also plotted).

\[
\log (20,000) = 4.31, \text{ this corresponds to } \sim 5.7 \text{ cm migration.}
\]

\[
\log (60,000) = 4.78, \text{ this corresponds to } \sim 2.3 \text{ cm migration.}
\]