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5.36 Biochemistry Laboratory
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SESSION 13 and SESSION 14 (lab open 1-5 pm each day)

Sign up for UV/Vis time with your TA. The class will be split into a 1-3 or a 3-5 pm slot.

During laboratory Sessions 13 and 14 you will

- Use a coupled phosphorylation assay to determine the specific activity of the **wild type Abl kinase domain 1)** in the absence of an inhibitor **2)** in the presence of the drug Gleevec (FDA approved in 2001) and **3)** in the presence of the drug Dasatinib (FDA approved in 2006)
- Use a coupled phosphorylation assay to determine the specific activity of the **H396P Abl kinase domain 1)** in the absence of an inhibitor **2)** in the presence of the drug Gleevec and **3)** in the presence of the drug Dasatinib

Researchers use a variety of strategies to monitor the activity of kinases. You will recall that kinases are enzymes that catalyze the phosphorylation of substrate peptides or proteins by transferring a phosphate group from a molecule of ATP to the substrate. Phosphorylation is spectroscopically silent, which means that we can monitor neither the formation of the phosphorylated product, nor the conversion of ATP to ADP directly using spectroscopy. This is because the products would appear identical to the starting materials due to identical absorption features and extinction coefficients. In order to monitor the activity of your wild type and mutant Abl kinase domains, you will therefore use a coupled assay, which “couples” the conversion of ADP to ATP with the conversion of NADH to NAD⁺, a process that can be detected by a decrease in absorbance at 340 nm. This coupling assay is outlined in **Fig.3**.

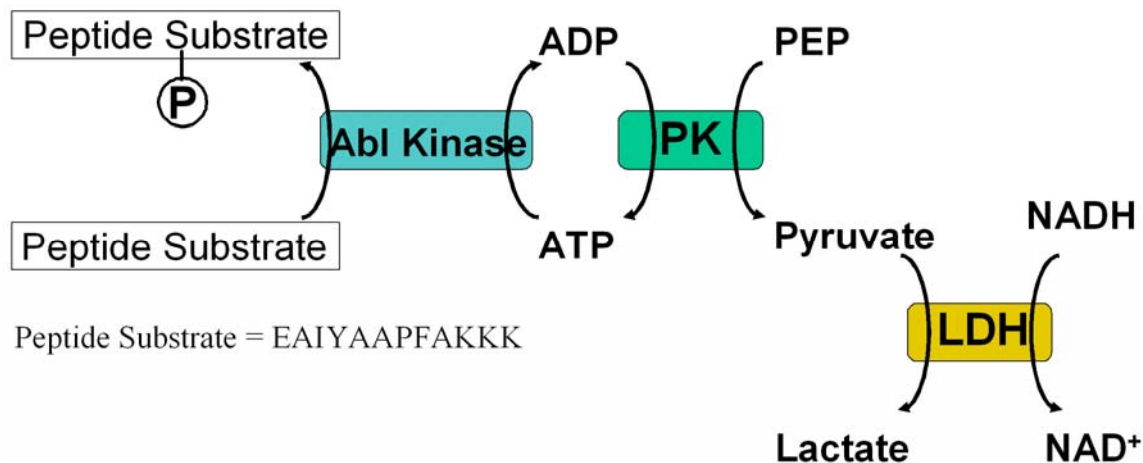


Figure 3. The Kinase Coupled Assay for Abl Activity

It is possible to use the kinase coupled assay to generate quantitative kinetic data on the kinase of interest. You will use this assay to determine the **specific activity** of the wt and mutant Abl kinase domains in the absence and presence of small molecule inhibitors, including Gleevec. Specific activity in biochemistry is defined as the amount of product formed by an enzyme in a given amount of time. Specific activity is often reported in

units (U) per mg of enzyme, where 1 unit is equal to 1 μmol of product formed per minute.

SESSION 13

1.) Determine the specific activity of the wt Abl kinase domain

To run the assay, you will set up a 120- μL reaction in a quartz cuvette and monitor the decrease in absorbance at 340 nm on the UV/Vis spectrometer. You will record the slope of the resulting line (decrease in absorbance over time) and calculate the specific activity of the kinase, as described below.

Combine the following in a 0.65 eppendorf tube:

- 86.4 μL of the thawed 1x assay buffer
- 9.6 μL of PK(928 U/mL)/LDH (1300 U/mL) for a final concentration of 74 U/mL // 104 U/mL.
- x μL of Abl kinase domain (typically 2-3 μL for wt Abl- check with your TA)
- 18-x μL of water

Gently mix the solution with tapping and with a pipette, and then transfer the mixed solution to a 100- μL capacity quartz cuvette. Record the initial slope for a background reading. *Note that for a proper reading the meniscus must not be visible in the cuvette window and the cuvette must be pushed down all the way in the holder.* Then add

- 6 μL of 20 mM peptide (for a final concentration of 1 mM) to start the reaction.

Let the reaction run for approximately 5 to 10 minutes. Record the slope and the R^2 value. Subtract your background slope from the slope after substrate addition to determine the slope (change in absorption per minute) from kinase activity.

Now you can calculate the specific activity of the Abl kinase using this slope determined in the coupled phosphorylation assay.

Calculating the Specific Activity (this can be calculated in lab or at home)

$$\text{Specific Activity} = \text{U/mg} = \frac{\text{\u00b5mol product formed}}{\text{min}} \div \text{total mg of enzyme added}$$

To find specific activity, you need to determine two values: **A)** the amount of product (phosphorylated peptide) formed per minute (in $\mu\text{mol}/\text{min}$) by the Abl kinase and **B)** the amount of Abl enzyme (in mg) that was used in the assay.

A) Calculate the amount of product (phosphorylated peptide) formed per minute.

Using Beer's Law ($\text{Abs} = \epsilon cl$) and the slope determined in the assay, calculate the concentration of product formed per minute:

Beer's Law can be written: $\Delta A/\text{min} = (\Delta c/\text{min})(l \epsilon)$

where, $\Delta A/\text{min} = \text{change in absorption per minute} = |\text{slope}|$

$l = \text{the path length of the cuvette} = 1 \text{ cm}$

$\epsilon = \text{the extinction coefficient of your chromophore (here NADH)}$
 $\epsilon \text{ of NADH at } 340 \text{ nm} = 6,220 \text{ cm}^{-1}\text{M}^{-1}$

$\Delta c/\text{min} = \text{change in concentration per minute. Solve for this value.}$

Solving for $\Delta c/\text{min}$ gives the concentration of product formed in units of M/min. Multiply this value by 10^6 to get an answer in units of $\mu\text{M}/\text{min}$.

Remember that M = molarity (mol/L). Since the answer should be in terms of $\mu\text{mol}/\text{min}$, NOT $\mu\text{M}/\text{min}$, we need to convert μM to μmol . To do this, multiply your answer by the total volume of the reaction.

Now you have an answer in terms of $\mu\text{M}/\text{min}$, or units (U).

B) Calculate the amount of enzyme used in the assay.

The Calbiochem wild-type Abl has a concentration of 0.01 mg/mL. If you used 3 μL of this kinase stock, the total amount of enzyme in the reaction would be 0.00003 mg. If you used a different stock of enzyme, use the concentration and the volume added to determine the amount of enzyme (in mg).

Now you can divide your $\mu\text{M}/\text{min}$ of product formed by the total mg of kinase to determine your specific activity.

2.) Determine the specific activity of the wt Abl kinase domain in the presence of 1 μM Gleevec.

Follow the procedure described in part 1, but include Gleevec in the reaction mixture:

Combine the following in a 0.65 eppendorf tube:

- 86.4 μL of the thawed 1x assay buffer
- 9.6 μL of PK(928 U/mL)/LDH (1300 U/mL) for a final concentration of 74 U/mL // 104 U/mL.
- x μL of Abl kinase domain (typically 2-3 μL for wt Abl- check with your TA)
- 1.2 μL of a 100 μM solution of Gleevec dissolved in DMSO
- 16.8-x μL of water

Gently mix the solution with tapping and with a pipette, and then transfer the mixed solution to a 100- μ L capacity quartz cuvette. Record the initial slope for a background reading. Then add

- 6 μ L of 20 mM peptide to start the reaction.

3.) Determine the specific activity of the wt Abl kinase domain in the presence of 1 μ M of another kinase inhibitor, Dasatinib.

Follow the procedure described in part 1, but include Dasatinib in the reaction mixture:

Combine the following in a 0.65 eppendorf tube:

- 86.4 μ L of the thawed 1x assay buffer
- 9.6 μ L of PK(928 U/mL)/LDH (1300 U/mL) for a final concentration of 74 U/mL // 104 U/mL.
- x μ L of Abl kinase domain (typically 2-3 μ L for wt Abl- check with your TA)
- 1.2 μ L of a 100 μ M solution of Dasatinib dissolved in DMSO
- 16.8-x μ L of water

Gently mix the solution with tapping and with a pipette, and then transfer the mixed solution to a 100- μ L capacity quartz cuvette. Record the initial slope for a background reading. Then add

- 6 μ L of 20 mM peptide to start the reaction.

SESSION 14

Repeat steps **1.)** through **3.)** from Session 13 with your **H396P Abl(229-511)**. For the x μ L of Abl kinase solution, use x = 10 μ L for H396P Abl (double check this value with your TA).

In your laboratory report, use the specific activity data to address the following questions:

Is Gleevec an effective inhibitor of (wild type) Abl kinase activity in vitro? Is Gleevec an effective inhibitor of mutant H396P Abl kinase activity in vitro? Is Dasatinib an effective inhibitor of (wild type) Abl kinase activity in vitro? Is Dasatinib an effective inhibitor mutant H396P Abl kinase activity in vitro?

Note on data analysis: In the laboratory report you will compare the specific activity in the absence and presence of inhibitors for both the wt and the H396P mutant Abl kinase domains. You will NOT compare absolute values of specific activities between the wt and mutant. A comparison of wt versus mutant absolute specific activities is not valid with this experimental design because of differences in purity and storage of the (commercially available) wt and the (bacterially expressed and singly purified) mutant domains. In addition, measurements for the wt and mutant domains are taken on different days and possibly on different instruments. A 2006 study demonstrated that the H396P mutant has decreased in vitro kinase activity compared to the wild type (Griswold et al, 2006).