20.320 Exam 1
Thursday October 4th
9:35-10:55

Instructions:

0. Write your name of the front cover of the blue book.

1. Answer all questions in the blue books. This exam paper will not be graded.

2. All questions can be answered in at most a few sentences. We will deduct points for excessively long replies, even if they contain the right answer.

3. State all assumptions for each problem.

4. In order to pace yourself please note that the maximum possible score on this exam is 100 – note that there are 4 total questions.

Question 1. (25 points total)

Bromodomains are protein domains that bind to acetylated lysines (similar to SH2-phosphotyrosine interactions). Jay Bradner at Harvard has recently developed JQ-1, a cell-permeable small molecule (mass: 642 g/mol) that binds and inhibits a subset of bromodomains. In the lab, you would like to determine the affinity between JQ-1 and BrdX (mass: 24,354 g/mol), your bromodomain of choice.

A) (9 points) Design an SPR experiment to quantify the interaction affinity between JQ1 and BrdX, including proper controls and which molecule is immobilized. The details of exactly how SPR works are not critical to answer this question. Justify your immobilization selection by calculating the number of captured molecules needed to obtain a 10 RU signal change in both configurations. (Note: assume a 10 mm^2 chip surface area).

To maximize the signal obtained with each protein binding event, we want to immobilize the lighter protein (JQ-1) on the surface. During the SPR experiment, we then flow solution containing the larger protein over the plate and measure the RU vs. time profile.

One important control is to run solution containing the larger protein over a plate that doesn't have any immobilized protein. Changes in RU during this control may indicate potential non-specific binding.

Another important control is to run the experiment multiple times using different ligand concentrations. The results between these experiments should be consistent.

Immobilize BRDX: $10RU * \frac{1pg}{1\,RU} * 10mm^2 * \frac{10^{-12}g}{1\,pg} * \frac{1\,mol}{642g} * \frac{6.02*10^{23}\,molecules}{1\,mol} \quad 9.377 * 10^{13} molecules$

Immobilize $JQ-1$: $10RU * \frac{1pg}{1\,RU} * 10mm^2 * \frac{10^{-12}g}{1\,pg} * \frac{1\,mol}{24354g} * \frac{6.02*10^{23}\,molecules}{1\,mol} \quad 2.47 * 10^{9} molecules$

Clearly, by immobilizing JQ-1, we need far fewer binding events to obtain a 10RU shift and thus it is the logical choice.

B) (10 points) Given the following parameters:

$k_{on} = 3x10^{4}\ L\ mol^{-1}\ s^{-1}$

$k_{off} = 4x10^{-2}\ s^{-1}$

How long will it take to complete the experiment using a ligand concentration of 200 x 10^{-9}M, assuming the association reaches 90% of its equilibrium value before ligand stops and 90% of the associated ligand dissociates by the end of the experiment?

The time required is composed of the association time, τ_{assoc}, and the dissociation time, τ_{dissoc}.

For the association phase:

$$RU_{assoc} = RU_{eq}(1 - \exp(-k_{obs} \times t))$$

$$1 - \frac{RU_{assoc}}{RU_{eq}} = \exp(-k_{obs} * t)$$

$$k_{obs} = k_{on}L_0 + k_{off} = 3 * \frac{10^4 L}{mol * s} * 200 \times \frac{10^{-9} mol}{L} + 4 * 10^{-2} s^{-1} = 0.046 s^{-1}$$

$$\tau_{assoc} \quad \frac{\ln\left(1 \quad \frac{RU_{assoc}}{RU_{eq}}\right)}{k_{obs}} \quad \frac{\ln(1 \quad 0.9)}{0.046 s^{-1}} \quad 50.05619\ \text{sec}$$

For the dissociation phase:

$$RU_{dissoc} = RU_{eq}(\exp(-k_{off} * t))$$

$$\frac{RU_{dissoc}}{RU_{eq}} = \exp(-k_{off} * t)$$

$$t_{dissoc} \quad \frac{\ln\left(\frac{RU_{dissoc}}{RU_{eq}}\right)}{k_{off}} \quad \frac{\ln(0.1)}{4 * 10^{-2} s^{-1}} \quad 57.564627\ \text{sec}$$

Note that RU_{eq} corresponds to the value of RU after the association phase is terminated, not the actual equilibrium RU. Understanding the derivation for τ_{dissoc} was vital for understanding this point.

Therefore, the total time is $\tau_{tot} \quad t_{assoc} \mid \tau_{dissoc} \quad 50.05619 \mid 57.564627 \quad 107.62\ \text{sec}$

C) (6 points) Although SPR is a very powerful approach, it does have some limitations. Provide two limitations and describe how you would know that they are affecting your measurements.

1. Must immobilize ligand on surface. Attachment can affect chemistry, such as blocking the binding site. You would know because you would not observe any binding even when you would expect to.
2. K_d must be between 10^{-4} and 10^{-12} M. You would know because below 10^{-12} you would see nearly instantaneous binding and no dissociation. Above 10^{-4} you would never get any binding.
3. Non-specific binding can lead to spurious results. You could see unreasonable values of K_d. You could run a control with the ligand solution before immobilizing the protein on the surface to see if any binding occurs. This binding would then indicate potentially spurious binding.

Question 2. (25 points total)

Overexpression or mutation of the Epidermal Growth Factor Receptor (EGFR) has been associated with a broad range of cancers. As discussed, there are many companies that have developed molecularly-targeted therapeutics, and several of these compounds are ATP analogs designed to inhibit EGFR. One of these compounds, Gefitinib, is currently approved for lung cancer patients with high EGFR expression or with selected activating mutations in EGFR. Km for the EGFR-ATP interaction is 50×10^{-6} M, kcat for EGFR-ATP is 10^6 sec-1, and K_i for the EGFR-Gefitinib interaction is 22×10^{-9}M. ATP concentration in the cell is 3×10^{-3}M.

a) (10 points) Due to dose-limiting toxicity, the intracellular concentration of Gefitinib is limited to 100×10^{-9}M. How effective is this dose at shutting down EGFR activity?

We can use the regular MM equation and the MM equation with a competitive inhibitor to determine the reduction in reaction rate as follows:

$$v_{without\ inhibitor} = \frac{v_{max} S_0}{K_m + S_0}$$

$$v_{with\ inhibitor} = \frac{v_{max} S_0}{K_m \left(1 - \frac{I}{K_i}\right) + S_0}$$

$$\frac{v_{with\ inhibitor}}{v_{without\ inhibitor}} = \frac{\frac{v_{max} S_0}{K_m \left(1 + \frac{I}{K_i}\right) + S_0}}{\frac{v_{max} S_0}{K_m + S_0}} = \frac{K_m + S_0}{K_m \left(1 - \frac{I}{K_i}\right) - S_0}$$

$$\frac{v_{with\ inhibitor}}{v_{without\ inhibitor}} = \frac{50 * 10^{-6} M + 3 * 10^{-3} M}{50 * 10^{-6} M \left(1 + \frac{100 * 10^{-9} M}{22 * 10^{-9} M}\right) + 3 * 10^{-3} M} = \frac{0.00305}{0.003277272} = 0.93065$$

This means that the rate of the reaction WITH the inhibitor is 93% that of the reaction WITHOUT the inhibitor. % Inhibition is given by 1-0.93. Therefore, it only reduces the rate by roughly 6.94%

b) (4 points) How much does EGFR inhibition change in cells expressing 10^6 copies of EGFR versus cells expressing 10^4 copies of EGFR?

The equation for v_i/v is not dependent on the enzyme concentration, so the percent inhibition is the same for both 10^4 and 10^6. However, the total number of inhibited receptors is different.

10^4: 700 receptors inhibited

10^6: 70,000 receptors inhibited (100 fold change)

c) (4 points) Why is a strong increase in expression of EGFR often associated with cancer, even in the absence of ligand stimulation?

The increase in the number of proteins allows the proteins in the cell to sample more conformations. Thus, more EGFR receptors spontaneously open their active site and allow ATP to enter even in the absence of the activating ligand. As a result, these cells are more prone to dimerizing and activating the EGFR pathway and its associated anti-apoptotic and pro-survival prognosis.

Other answers accepted included references to EGFR activating mutations, altered role of feedback in cancer such that more and more receptor is generated, etc. Stating that EGFR drives proliferation which runs rampant in cancer IS NOT enough here.

d) (3 points) Describe how the mechanism of action of a Type II inhibitor differs from that of an ATP analogue.

An ATP analogue binds competitively to the ATP binding pocket, blocking activation of EGFR by preventing interaction with its necessary co-factor, namely ATP. A Type II Inhibitor binds the DFG out motif when the enzyme is in the inactive state and blocks the opening of the activation loop, which is necessary for the enzyme to bind ATP and substrate. With respect to ATP, Type II inhibitors can be considered a non-competitive inhibitor although it is more complicated then this in reality.

e) (4 points) Given a cancer cell line with an activating EGFR mutation, would you use a Type II inhibitor or an ATP analogue inhibitor and why?

Due to the mutation, the kinase will always be active. As a result, a type II inhibitor will be completely ineffective as its function is to prevent the kinase from transitioning into the active conformation. As a result, the ATP analogue would be the correct treatment option

Question 3. (35 points total)

As discussed in lecture, the mitogen-activated protein kinase (MAPK) cascade is ultrasensitive, demonstrating very strong response to low-level input. Many other signaling systems also feature ultrasensitivity. One of these is the CDK1 (kinase) - CDC25 (phosphatase) interaction, which regulates the cell cycle. In a recent manuscript (PNAS, 2012), Lu et al. developed a computational model describing multi-site phosphorylation of CDC25 by CDK1, where the multiply-phosphorylated CDC25 phosphatase is fully active. For simplicity in this model, assume 2 CDK1-dependent phosphorylation sites on CDC25. In the parts that follow, please designate CDK1 as A and CDC25 as B and use the nomenclature from class to describe complexes and phosphorylated species (BP is B that's been phosphorylated once, BPP is double-phosphorylated). Remember to state your assumptions!

A. (6 points) Write out the chemical reactions for the above system. You can start with phosphorylated, activated CDK1.

$$A + B \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} A*B \xrightarrow{k_{cat1}} A + B - P$$

$$A + B - P \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} A*B - P \xrightarrow{k_{cat2}} A + B - PP$$

What we looked for:

- Michaelis menten kinetics, with both a reversible binding step and an irreversible catalysis step for each overall protein-substrate interaction.
- Active CDK1 (A, A*, or AP; nomenclature could go many ways) acting as a kinase, putting phosphate groups on B one at a time. These phosphates came from environmental ATP.
- That CDK1 acted as an enzyme, not a reagent. This means that it IS NOT CHANGED BY THE REACTIONS IT CATALYZES. That is, it does not transfer its own phosphates to B, ever. Kinases don't do that.

B. (12 points) Write out ODEs for each species.

We graded ODEs based on whether they correctly reflected the changes in whichever model you wrote in part A, even if the model was incorrect. We searched for proper mass-action kinetics.

$$\frac{d[A]}{dt} = -k_1[A][B] - k_2[A][B-P] + (k_{-1} + k_{cat1})[A*B] + (k_{-2} + k_{cat2})[A*B-P]$$

$$\frac{d[B]}{dt} = -k_1[A][B] + k_{-1}[A*B]$$

$$\frac{d[B-P]}{dt} = k_{cat1}[A*B] - k_2[A][B-P] + k_{-2}[A*B-P]$$

$$\frac{d[B-PP]}{dt} = k_{cat2}[A*B-P]$$

$$\frac{d[A*B]}{dt} = k_1[A][B] - (k_{-1} + k_{cat1})[A*B]$$

$$\frac{d[A*B-P]}{dt} = k_2[A][B-P] - (k_{-2} + k_{cat2})[A*B-P]$$

C. (4 points) What are the key points in this system that lead to ultrasensitivity?

The 2-step distributive phosphorylation of CDC25: When CDK1 performs the first reaction, it is increasing (rather than decreasing) the concentration of its potential substrates and the overall reaction rate goes up. Separating out the process into consecutive steps like this gives it ultrasensitivity.

D. (3 points) From a design perspective, how could you increase the responsiveness of the system?

You could add more levels to the cascade (e.g., add another phosphorylation-activated protein to the chain). You could add a positive feedback loop. You could also add cooperativity, giving CDK1 a much higher affinity for the phosphorylated version of CDC25.

E. (10 points) One of the substrates of CDC25 is the CDK1 kinase. Add this reaction into your system and add in the additional ODE(s) reflecting this

reaction. What biological principle does this introduce into the system and why is it useful.

$$A + B - PP \underset{k_{-3}}{\overset{k_3}{\leftrightarrow}} A * B - PP \xrightarrow{k_{cat3}} A^{inactive} + B - PP$$

We'd mentioned that A was in an active, phosphorylated state. If B, a phosphatase, has A as a substrate, then B deactivates A by removing the phosphate. We looked for models that reflected B deactivating A, with Michaelis-Menten kinetics. Again, B is an enzyme in this case. It is not changed by the reaction it catalyzes. A, however, is now a substrate and *is* changed.

We need to alter the ODEs for A and B-PP and add additional ODEs for the new complex and the inactive form of CDC25:

$$\frac{d[A]}{dt} = -k_1[A][B] - k_2[A][B-P] + (k_{-1} + k_{cat1})[A*B] - (k_{-2} + k_{cat2})[A*B-P] - k_3[A][B-PP] + k_{-3}[A*B-PP]$$

$$\frac{d[B-PP]}{dt} = k_{cat2}[A*B-P] - k_3[A][B-PP] + (k_{-3} + k_{cat3})[A*B-PP]$$

$$\frac{d[A*B-PP]}{dt} = k_3[A][B-PP] - (k_{-3} + k_{cat3})[A*B-PP]$$

$$\frac{d[A^{inactive}]}{dt} = k_{cat3}[A*B-PP]$$

The addition of this reaction results in the introduction of a negative feedback loop into the system because B-PP deactivates A. Negative feedback loops attenuate responses and, most importantly, turn them into a short-lived pulse rather than a continued activation. They can be used to create transient activation waves.

Question 4. (15 points total)

Short answers – one to three sentences.

a) (3 points) Describe what information you can obtain from ITC and not from SPR and vice versa.

From ITC, one can obtain ΔH and ΔS which are not attainable from SPR. Note, however, that ΔG can be obtained from both types of experiments.

From SPR, one can obtain the kinetic rate constants k_{on} and k_{off}, which one cannot calculate from an ITC experiment.

Both can determine K_D.

b) (3 points) How does strong positive feedback affect the duration and amplitude of the kinase signaling cascade?

Strong positive feedback increases both the amplitude and duration of the kinase signaling cascade.

c) (3 points) Name one reason why the quantitative interaction affinities and kinetics generated by surface plasmon resonance (SPR) may not be applicable to modeling reactions in cells.

Immobilizing a protein could change its conformation, potentially leading to an incorrect K_D value if the binding site is blocked/altered. Also since the protein is immobilized, we are not able to account for any multimerization, cooperativity, or competition with other binders that may arise normally in the cells.

d) (3 points) Describe the pseudo first order approximation (PFOA). Is this applicable in ITC and SPR? Why or why not.

The PFOA assumes that the bulk concentration of substrate does not change and therefore remains at its initial value S_0. In order for this assumption to be valid, the amount of substrate in the bulk to begin with must greatly exceed that which will participate in binding or reaction events. It is not applicable in ITC because very small volumes of ligand are injected. In contrast, it is valid in SPR because an excess of ligand-containing solution flows over the surface.

e) (3 points) Describe one technique that can be used to determine if two proteins interact in vitro.

We were looking for a valid description of techniques such as co-IP, FRET and PLA. See class notes on details of these techniques.

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