# Problem Set 3 – Practical Kinetics

BMB 178  Due 11/29/2017

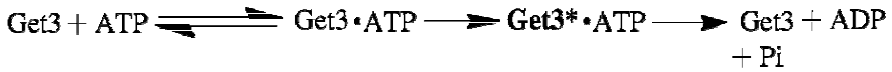
Please turn in the problem set by the end of the class. You can also email to hhsieh@caltech.edu to submit your work.

*There are four problems in this section. You may use any books, problem sets, Internet resources, or computer programs to solve these problems. If you get some ideas from a specific paper or website, please cite it as a reference.*

## Problem 1: Pre-steady state kinetics 1 (26 points)

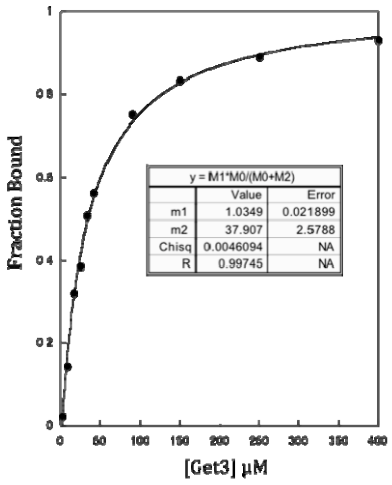
The cytosolic ATPase Get3 mediates targeting of tail-anchored (TA) membrane proteins to the endoplasmic reticulum (ER). Get3 functions as a molecular chaperone by binding to transmembrane domains of newly synthesized TA proteins and shuttling these proteins to a membrane docking complex (Get1/2) in the ER. It has been previously shown through structural studies that the nucleotide state of Get3 regulates its conformation and function. Upon binding ATP, Get3 undergoes a conformational change into a compact "closed" form (denoted as Get3*), which is thought to accommodate the substrate proteins and stimulate ATP hydrolysis. Although Get3 is a dimer, single-site binding and single turnover ATPase assays were done in order to determine the binding constants and nucleotide hydrolysis rates for individual Get3 monomers.

The proposed reaction mechanism for single-site ATP hydrolysis is as follows:




a. The fluorescence of mant-ATP changes upon binding to Get3. Using this assay, the following data was obtained to determine the equilibrium binding constant for the Get3-ATP complex. In this assay, mant-ATP is held constant at 0.4µM and Get3 is titrated. Plot the data below and use non-linear regression to determine the dissociation constant ($K_d$). (7 points)

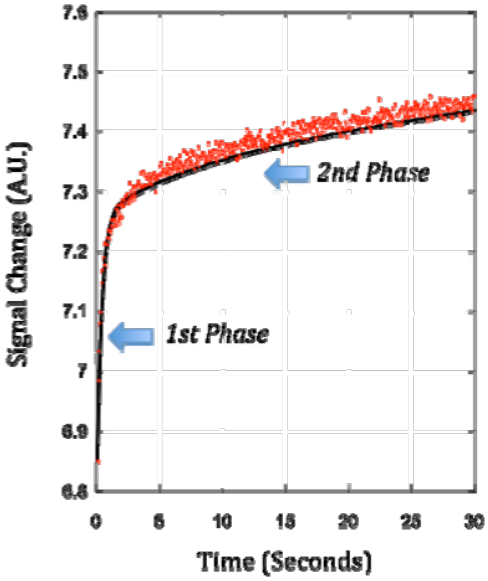
| [Get3] µM | Fraction Bound |
|---|---|
| 1.6715 | 0.021889 |
| 8.3572 | 0.14389 |
| 16.714 | 0.31894 |
| 25.0 | 0.38494 |
| 33.431 | 0.50622 |
| 41.786 | 0.55989 |
| 90.0 | 0.75 |
| 150.0 | 0.833 |
| 250.0 | 0.89 |
| 400.0 | 0.93 |




**The $K_d$ is ~ 38 µM.**

Due to the actual fitting code of different software, there might be some deviations. The exact number is not going to affect points you get as long as the fitting model is correct.

b. Using the same assay, the following data was obtained with a Stopped-Flow in order to determine the association and dissociation rate constants for nucleotide binding. ATP is held constant at 0.4µM and Get3 is titrated. Shown below is a plot from a typical association rate measurement, which displays two distinct phases.

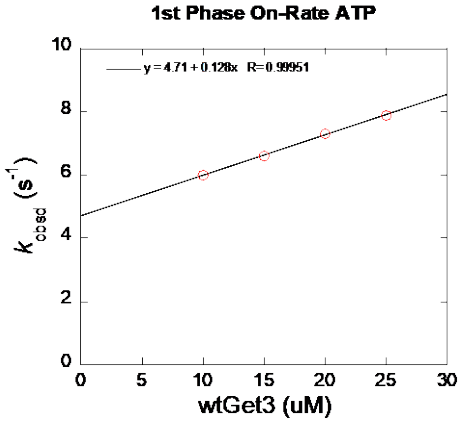



| [Get3] μM | $K_{obsd}(s^{-1})$ 1st Phase | $K_{obsd}(s^{-1})$ 2nd Phase |
|---|---|---|
| 10 | 6 | 0.0032 |
| 15 | 6.6 | 0.0031 |
| 20 | 7.3 | 0.0033 |
| 25 | 7.9 | 0.0032 |

(1) Propose an explanation for the molecular events that gives rise to both the 1st and 2nd phases. (7 points)

The first phase is dependent on Get3 concentration, suggesting a bi-molecular reaction (2 points). The second phase is independent of Get3 concentration, consistent with a unimolecular reaction (2 points). A working model consistent with the data is that the initial binding of Get3 to ATP gives the first phase, and a conformational change of Get3, the bound nucleotide, or both, could be responsible for the second phase. (3 points)

(2) Determine the $k_{on}$ and $k_{off}$ values for the Get3-nucleotide complex by plotting the observed rate constants from the first phase as a function of Get3 concentration. (6 points)




$K_{on} = 1.28 \times 10^5\ s^{-1}\ M^{-1}$

$K_{off} = 4.71\ s^{-1}$

$\frac{K_{off}}{K_{on}} = 36.7\ \mu M$

(3) How does this value compare with the dissociation constant from part 1? Does the result support the model you proposed in (1)?

(6 points)

. . The calculated $K_d$ is 36.7 µM, and this is close to the measured $K_d$ of 38.0 µM(3 points). This supports the model that the initial binding gives rise to the first phase (3 points).

## Problem 2: Pre-steady state kinetics 2 (28 points)

a. Assume you mixed 10 nM Enzyme (E) and 1µM substrate (S), and monitored the formation of product (P) as a function of time:

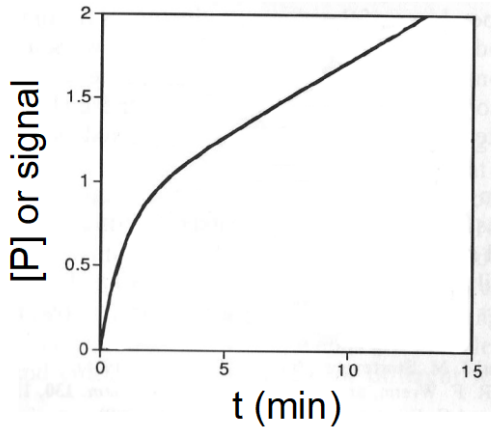



Figure 2A

(1) You can fit the data above to a bi-phasic exponential equation and extrapolate the $k_{obsd}$ for both phases. When you titrate the concentration of substrate and plot the $k_{obsd}$ against substrate concentration, you saw something like this:

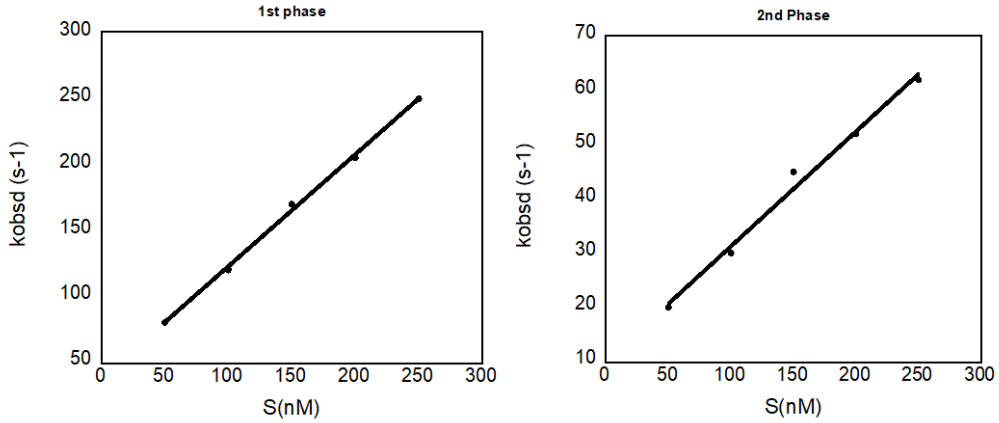
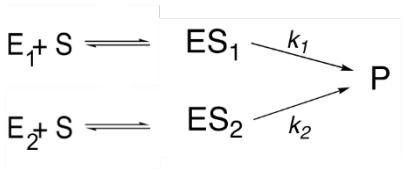



Figure 2B

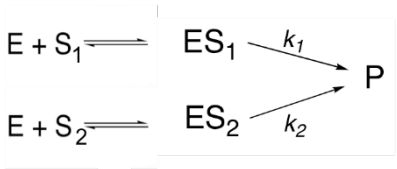
What could be the model(s) that account for the burst phase? Explain how you draw the conclusion. (6 points)

Answer key:

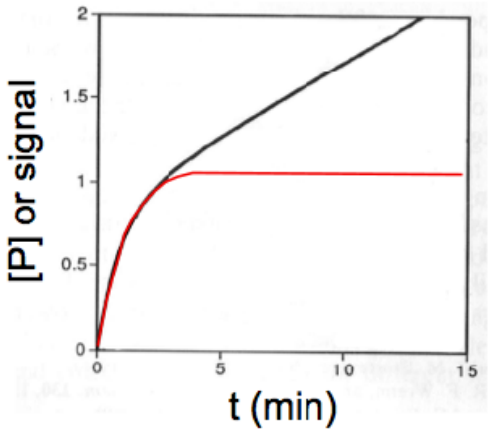
There could be heterogeneity in the sample, either in E or S. If there are two species of enzyme, or substrate, the two species form $ES_1$ and $ES_2$, which have different reaction rate to produce P. The burst phase comes from the more reactive $ES_1$. The reason is that both phases show a dependence on [S], indicating a bi-molecular reaction. The model could be:




or




b. Now you carried out the same experiment for a different reaction involving enzyme E, substrate S, and product P under both single turn-over and multiple turn-over conditions:




Black: multiple turnover
Red: single turnover

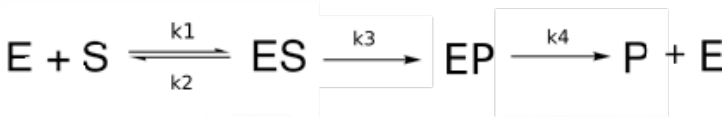
Figure 2C

(1) What could be the model that accounts for the burst phase in black line? Explain how you draw the conclusion. (6 points)

Answer key:

The slow phase is due to a rate-limiting step after chemistry. (3 points) The reason is that under multi-turnover condition ([E]<<[S]), multiple rounds of catalysis can happen. But in single-turnover condition ([E]>>[S]), all substrate is consumed in the first round of reaction. The rate constant in the burst phase (black curve) is similar to the rate constant in the red curve, while slower phase in the black phase is due to the steps after the first round of catalysis. (3 points)

(2) A chemical reaction involving E, S and P is described below. Several sets of reaction conditions and parameters are provided. Please choose two sets in which the kinetic parameters of the reaction could give rise to a burst phase during the time course. For these two sets, indicate which reaction condition gives a single turn-over measurement, and which one gives a multiple turn-over measurement. (4 points)




| set | $[E]_0$ (nM) | $[S]_0$ (nM) | $k_1$ ($nM^{-1}s^{-1}$) | $k_2$ ($s^{-1}$) | $k_3$ ($s^{-1}$) | $k_4$ ($s^{-1}$) |
|---|---|---|---|---|---|---|
| 1 | 10 | 100 | 0.1 | 1 | 1 | 0.1 |
| 2 | 10 | 100 | 0.1 | 1 | 0.1 | 1 |
| 3 | 100 | 10 | 0.1 | 1 | 0.1 | 1 |
| 4 | 100 | 10 | 0.1 | 1 | 1 | 0.1 |

Set 1 is the multiple turn-over while set 4 is the single turn-over condition. The other two are not rate-limited by steps after chemistry, and cannot produce the burst phase.

(3) Use Berkeley Madonna to simulate the two reaction parameter sets you choose from (2). (8 points)

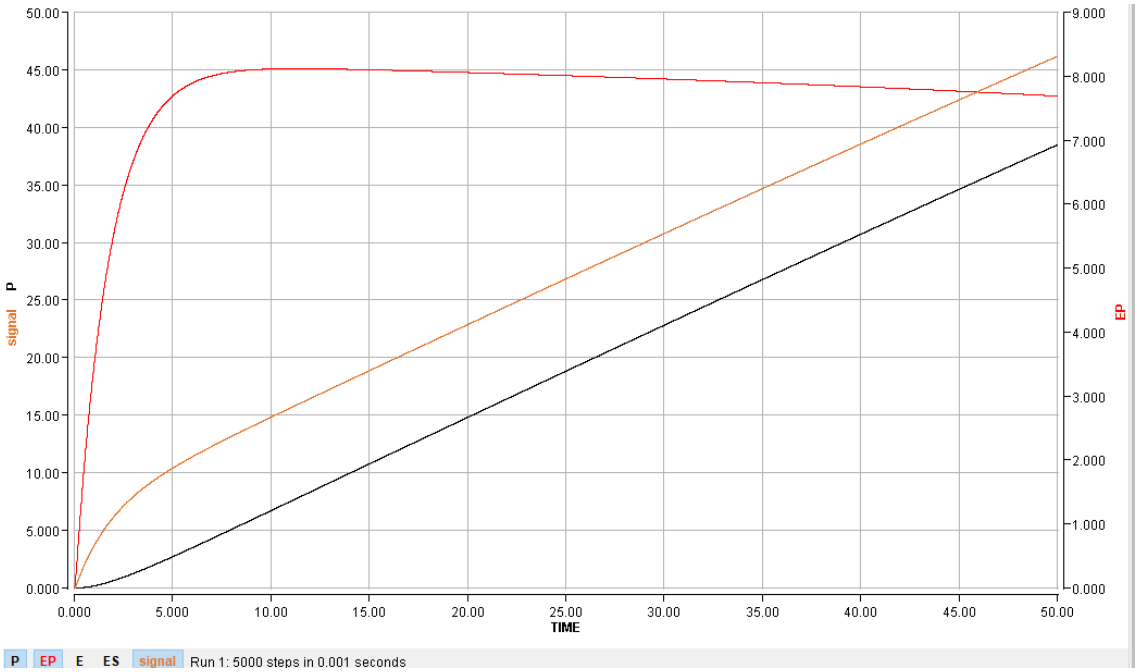
Make sure your units are consistent.

Use startime = 0; stoptime = 50s and t = 0.01s.

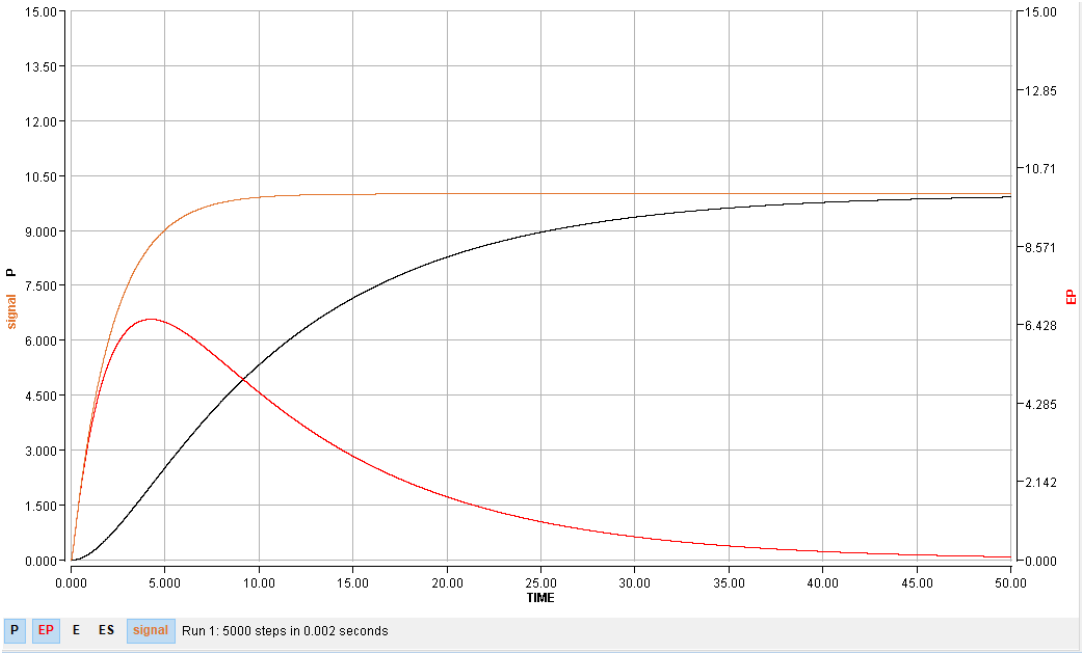
Add the script “signal=EP+P” in the equation window, and choose the variable “signal” under the graph option

Save an image of your simulation plot of signal, [EP], and [P] versus time and attach it to your problem set. Make sure to label the graph and select thicker points for easier viewing. (6 points)

Set1: (3 points)




Set4: (3 points)



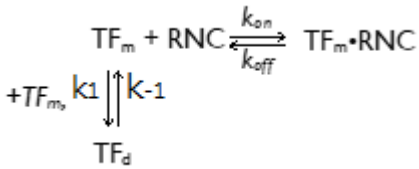

(3) Suppose you are an experimentalist who obtained these data, but do not know about the rate constants of the underlying microscopic reaction steps. Describe how you would extract the values of $k_3$ and $k_4$ from these data (there are multiple ways to do this, and any solution that works reasonably is fine). (6 points)

The signal in burst phase is mainly contributed by building up of EP and would reflect $k_3$. On the other hand, the linear phase, which is in steady state, is due to EP dissociating into E and P. Therefore, one can extract $k_4$ from the linear phase.

One can fit the signal in multiple turnover to $\text{signal}(t) = [E]_0(k_4 t + (1 - e^{-k_3 t}))$. Alternatively, one can fit the steady-state phase in multiple turnover to $[E]_0 k_4 t$ and fit the trace from single turnover to $[E]_0(1 - e^{-k_3 t})$.

## Problem 3: Kinetic Simulation (23 points)

TF is a co-translational chaperone. TF's affinity for translating ribosomes was shown to depend on the length and hydrophobicity of the nascent polypeptide chain on the ribosome. Moreover, TF monomers (TFm) can also dimerize to form homodimers (denoted as TFd). The rate constants during TF dimerization are $k_1$ = 0.015 $nM^{-1}$ $s^{-1}$ and $k_{-1}$ = 0.015 $s^{-1}$. A FRET assay has been developed to monitor the interaction of TF with ribosome nascent chain complexes (RNCs). The on and off rates of TF for a specific RNC was determined as shown below:



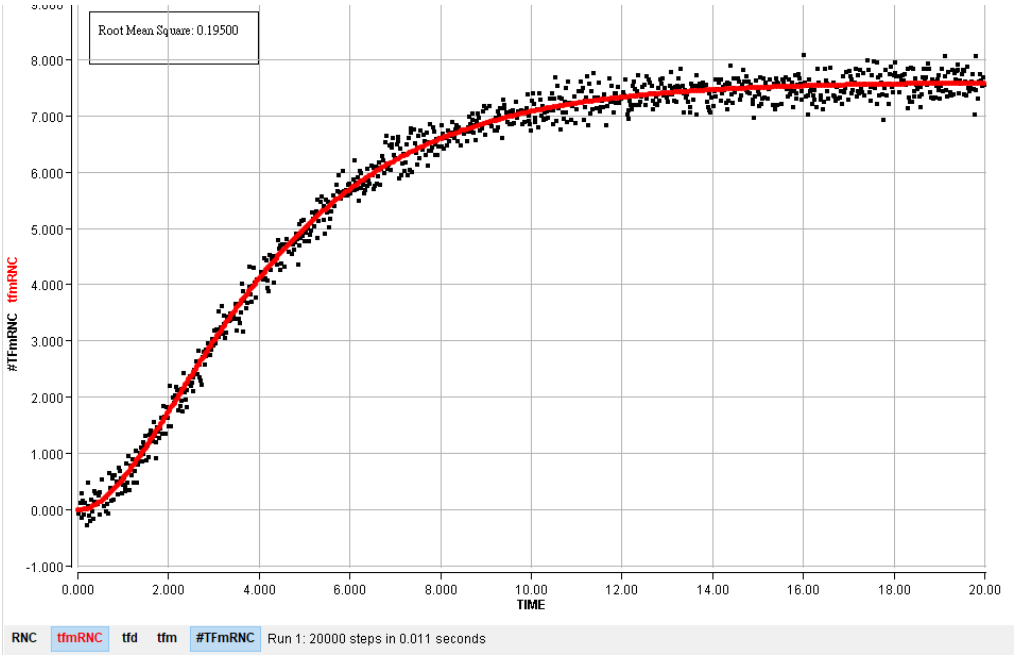

a. Using a stopped-flow device, the formation kinetics of [TFm-RNC] is monitored. RNC and concentrated TF are quickly mixed and the FRET change of the donor on RNC is recorded. The concentration of TF is high and mixing is rapid, such that TF is

>99% in the dimer form immediately after mixing. Thus, the initial conditions of the reaction are: $[RNC]_0$= 20 nM and $[TFd]_0$ = 160 nM. The initial concentrations of other species can be assumed to be 0. The duration of experiment is 20s. The data set is given in the .csv file attached. Use the curve fitting function in Berkeley Madonna to derive $k_{on}$, $k_{off}$. (7 points)

Set $k_{on}$ and $k_{off}$ as free variables. If the result does not make sense, you need to play with guess#1 and guess#2 to try different initial conditions. Do not set guess#1 and guess#2 as the same since the software will hit a bug. A good range to play with is 0.001 to 1. Set the tolerance to <0.0001 for accurate result.

In addition to $k_{on}$ and $k_{off}$, be sure to attach the figure of fitting result to your problem set answer.

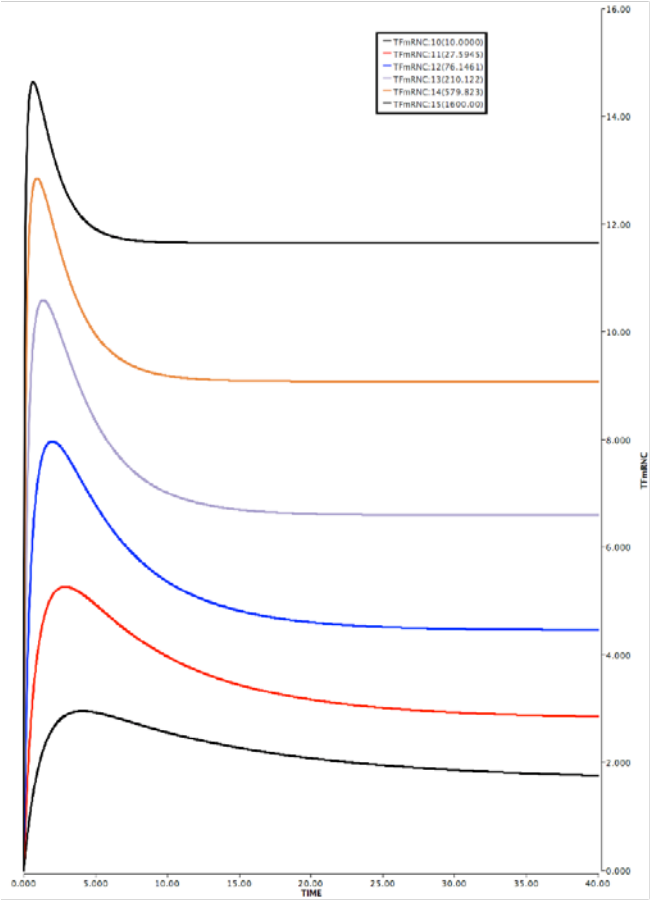
$k_{on}$ and $k_{off}$ are roughly 0.015 ($nM^{-1}$ $s^{-1}$) and 0.3 ($s^{-1}$), respectively.




b. Using the "batch run" function and the $k_{on}$ and $k_{off}$ you just derived, simulate with a different set of initial conditions: $[RNC]_0$= 20nM, varying $[TFm]_0$ from 10 to 1600 nM. Set the initial concentration of all other species to 0. Monitor the change in [TFmRNC].

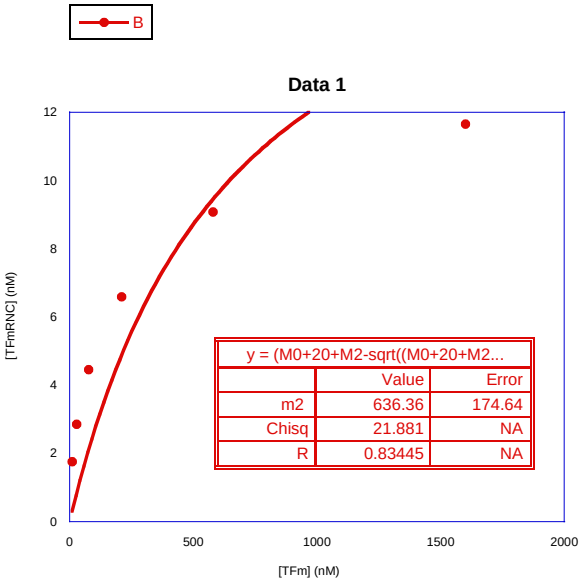
(1) Use the geometric series mode and simulate six runs. Keep the runs separate. Increase the stoptime to make sure that you have reached the end point in your simulated reactions. Make sure to attach your graph to your problem set. (5 points)

Increase run time to 50s, by which the reaction reaches equilibrium. We can get the concentration of TFmRNC for each [TFm] used.



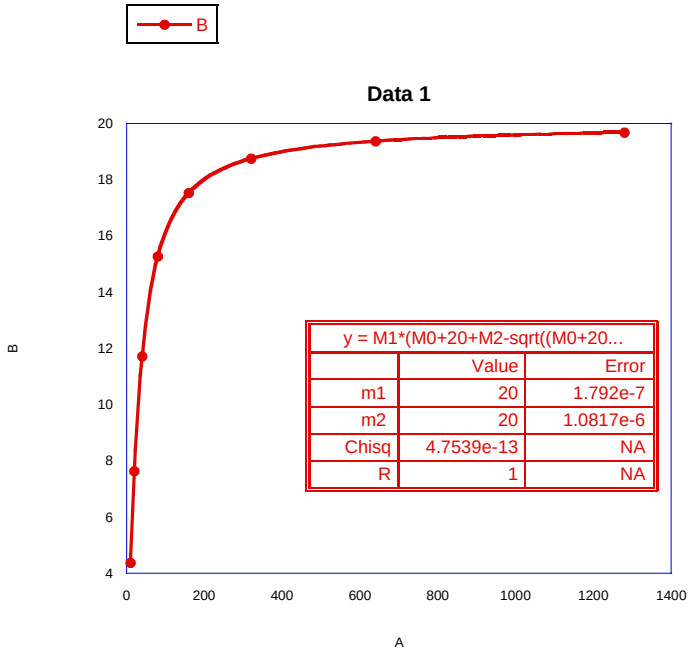

The equilibrium concentration of [TFmRNC] is 1.76, 2.86, 4.46, 6.59, 9.08, 11.66 nM when [TFm] is 10, 27.6, 76.2, 210, 580, 1600 nM, respectively.

(2) Plot steady state [TFmRNC] as a function of $[TFm]_0$. Use quadratic equation to determine the apparent $K_d$. Does this agree with the $K_d$ of TFm for this particular RNC expected from the rate constants? (4 Points)



The actual $K_d$ of TFm to RNC is 20nM <<apparent $K_d$=626nM. This does not agree with each other because of the dimerization of TFm to TFd.

(3) TFx is a mutation that blocks the dimerization of TF. Perform the same analysis as in (2) and vary $[TFx]_0$ from 10 to 1600 nM and determine the $K_d$ for TF binding to RNC by fitting to quadratic equation. Does this value agree with the $K_d$ of TFm for this particular RNC? (4 Points)




The apparent $K_d$ is 20nM which agrees with actual $K_d$=20nM,

(4) Why are the $K_d$ values obtained in (2) and (3) different? (3 Points).

The dimerization of TFm has been blocked. So all input TF is in monomeric form that can bind RNC. But WT TFm, which can forms dimer, the actual TFm concentration is lower than input, so it need more input to saturate the binding of RNC. This results in higher Km.

**Problem 4 Single molecule kinetics (23 points)**

a. φ29 is a model system for investigating viral packaging. This motor packages a 19.3-kilobase pair genome into a capsid that is 40 nm in diameter and 50 nm in height, and can exert forces beyond 60 pN. The motor complex has an ATPase domain that generates the driving force for genome packaging. Each packaging cycle is composed of a dwell phase and a burst phase that results in the translocation of 10

bp of DNA in four 2.5 bp steps. More recently, it was shown that during burst phase, the φ29 motor rotates dsDNA during translocation. Read the paper from Bustamante lab (doi:10.1016/j.cell.2014.02.034) and answer the questions:
(1) What are the evidences to show that the motor rotates dsDNA during packaging? (5 points)

The movement of the attached third rotor bead was tracked as the motor packages DNA (2 points). The rotor bead rotated around the DNA-helical axis in a left-handed direction, or as a negative angle. The translocation pause is concomitant with the rotation pause (3 points).

(2) Under low capsid filling (<50%) conditions, explain the biological significance of dsDNA rotation with respect to the motor. (5 points)

Under low capsid filling condition, the burst size of motor is 10bp for each translocation cycle, while B-DNA has a helical pitch of 10.4bp (3 points). This means that DNA has to rotate with respect to the motor in order to sustain the interaction. The 0.4bp difference corresponds to 14 degree, which is compensated by the rotation of ~1.5degree/bp(2 points). This makes sure that every bp can always perfectly contact motor subunit.

b. During translation, the ribosome progressively charges aminoacylated tRNA onto mRNA molecule and synthesizes protein. The ribosome contains three tRNA-binding sites corresponding to three adjacent codons. Incoming aminoacylated tRNA is positioned at A site, which is then oriented to react with peptidyl-tRNA in the P site. Deacylated tRNA is released from E site. The movement of ribosome on mRNA is catalyzed by the GTPase EF-G. Read the paper from Puglisi lab (doi:10.1038/nature08925 ) and answer the following questions:

(1) How is fMet-(Cy3)tRNA bound ribosome immobilized in ZMW? (2 points)

The mRNA is biotinyated, which can bind streptavidin-coated PEG slides.

(2) What would you expect to see if the release of deacylated tRNA from the E site is tightly coupled to the binding of A-site tRNA for M(FK)6? (3 points)

Tight coupling means that the exit of E-site tRNA occurs at the same time as the binding of the A site tRNA. So we would not be able to resolve the arrival and departure of individual tRNAs (3 points). Instead of seeing bursts of red and blue in the non-coupling mode, we would see that the time traces for the red and blue tRNAs overlay on one another.

(3) Based on the real-time translation of M(FK)6, what seems to be the slowest step during elongation? What could be the reason for that? (4 points)

The slowest step is the arrival of the first Lys-tRNA (2 points). The model said that the E-tRNA has to be dissociated before the binding of the A-tRNA. One possible explanation is that the first translocation is very slow, or the exit of the first fMet is very slow, so it inhibits the arrival of the first Lys. The departure of the fMet might not be accurately reflected in the experiment due to the bleaching of Cy3.  Other

reasonable explanations are also welcome (2 points).

(4) The rate of bacterial translation is 10-20 amino acids per second in vivo. What is the rate of translation measured in 500nM EF-G in this paper? What could contribute to the discrepancy of translation rates measured in this paper from that in vivo? (4 points)

The rate is about 0.4 amino acids per second, 50 fold slower than that in vivo (2 points). Possible reasons could be: the effective concentration of the proteins, mRNA, tRNA or other reagents are much higher in vivo; the reaction temperature for e.coli in vivo is usually 37℃; this mRNA might has a intrinsically low translation speed. Other reasonable explanations are also welcome (2 points).