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Transient State Kinetics Tutorial Using the Kinetics Simulation Program, KINSIM

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ABSTRACT This article provides an introduction to a computer tutorial on transient state kinetics. The tutorial uses our Macintosh version of the computer program, KINSIM, that calculates the time course of reactions. KINSIM is also available for other popular computers. This program allows even those investigators not mathematically inclined to evaluate the rate constants for the transitions between the intermediates in any reaction mechanism. These rate constants are one of the insights that are essential for understanding how biochemical processes work at the molecular level. The approach is applicable not only to enzyme reactions but also to any other type of process of interest to biophysicists, cell biologists, and molecular biologists in which concentrations change with time. In principle, the same methods could be used to characterize time-dependent, large-scale processes in ecology and evolution. Completion of the tutorial takes students 6–10 h. This investment is rewarded by a deep understanding of the principles of chemical kinetics and familiarity with the tools of kinetics simulation as an approach to solve everyday problems in the laboratory.

INTRODUCTION

Knowledge of kinetic constants frequently provides some of the most penetrating insights about most molecular mechanisms in biology and chemistry, but many molecular biologists are reluctant to take on the task of evaluating these constants (Maddox, 1993). Instead, most focus on the other information required to establish mechanisms: 1) a complete inventory of the molecular components of the system; 2) a list of the intermediates in the reactions of these components; and 3) the atomic structures of these components. A variety of biochemical, molecular biological, genetic, and biophysical methods are available to complete these tasks—but kinetic analysis is the only way to obtain the information about the rates of the transitions between the various possible intermediates. These rate constants are the key to understanding how things work, because a full set of rate constants allows one to decide which of the possible intermediates are used in a reaction mechanism, to appreciate the rates of the various steps, and to evaluate the free energy changes at each step. Johnson (1992) provides a particularly clear explanation of the strategy and methods used in transient kinetics analysis of enzyme mechanisms.

Given their fundamental importance, why do we generally know less about the kinetic constants than other features of our systems? Although everyone learns in biochemistry classes about the traditional tools used to evaluate the initial rates of enzyme reactions, this steady-state approach is often inadequate for detailed understanding of even simple enzyme mechanisms and is not applicable to a wide variety of processes in which enzyme reactions play little or no part. Examples of largely nonenzymatic processes include the assembly of cellular organelles, the cytoskeleton, and the extracellular matrix; the binding of cells to each other and the extracellular matrix; and the activities of most ion channels. Just like enzyme reactions, none of these processes will be understood until we work out their molecular mechanisms, including the evaluation of the rates of the transitions between the various intermediate states.

Transient kinetics

The goal of an analysis by transient kinetics is to understand the mechanism of a reaction. This approach can provide an inventory of the reactants, intermediates, and products along a reaction pathway together with the kinetic rate constants that determine the transitions between these chemical species. Both the identity of the intermediates and the values of the rate constants are important and interdependent in appreciating the mechanism. You can view the reactants, intermediates, and products as the roster of players in the game. The rate constants reveal not only the rate of the transitions between these intermediates, but also the pathway through the various possible intermediates between reactants and products. As a bonus, if one knows the forward and reverse rate constants for any step, their ratio gives the equilibrium constant for the reaction. This provides a powerful connection between kinetics and thermodynamics. From the equilibrium constant, one can calculate the free energy change, which provides valuable thermodynamic information about processes that may be inaccessible to evaluation by equilibrium methods.

The strategy is simple: just change the conditions of the system and watch the time course as it approaches a new equilibrium or steady state. This is called transient state or pre-steady-state kinetics, because one observes the transition from one equilibrium to another equilibrium or steady state,

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rather than the rate of a biochemical reaction running at a steady rate. Steady-state data are also useful, but transient data are frequently more informative. One frequently does steady-state experiments before transient experiments. First, the steady-state experiments generally require less material, particularly for an enzyme reaction. Second, the steady-state parameters will provide important clues required to design transient experiments.

One has many options when changing conditions to initiate a transient. Changing the concentration is a method for systems with two reactants or products, since mass action will drive it toward a new equilibrium. For example, one can simply dilute such an equilibrium system and watch what happens. Or one can mix two reactants and watch for the disappearance of reactants or the formation of products. Another strategy is to change the environment of the system, for example, by changing the solution conditions (pH, ionic strength, etc.), the temperature, or the pressure. Slow reactions taking seconds can be initiated by hand. Fast reactions on a millisecond time scale require rapid mixing equipment to change the conditions. "Stopped-flow" devices mix two solutions and inject them into a spectroscopic cuvette in ~2 ms for measurement of the time course of changes in absorption, light scattering, or fluorescence.

In each case, you learn about the system from watching it approach a new equilibrium or steady state, so you need some way to observe what is happening. Fortunately, one can usually find a good assay for the concentrations of reactants, intermediates, and/or products—the more assays the better.

Since many reactions are fast, spectroscopic assays are particularly useful. For example, one can follow absorbance, light scattering, fluorescence, fluorescence polarization, and (recently) even circular dichroism, electron spin resonance, or x-ray diffraction. If the system does not have an intrinsic optical signal, you can add one. For example, fluorescent probes can be covalently attached to the reactants or products to monitor their behavior, or an indicator dye can be added to the solution to measure pH or ion concentrations. With optical probes, one can usually follow concentration changes continuously during the reaction.

If optical signals are not available, virtually any chemical assay will work, but one is forced to stop a series of identical reactions at various time points to measure the change in concentrations. Reactions can be stopped chemically with acid or a denaturant or physically by freezing. Slow reactions can be stopped by hand, but fast reactions require rapid mixing and quenching that can be achieved mechanically in a "quenched-flow" device. Even complicated chemical assays like electron microscopy and gel electrophoresis are useful, providing that the reaction can be stopped and the various chemical species are stable over the time course of the assay. For example, the products of a single step in DNA synthesis have been followed on a millisecond time scale by gel electrophoresis (Johnson, 1992).

Having set up an assay system and watched what happens when the system approaches a new equilibrium, one needs tools to extract the kinetic constants from the observed time courses. The strategy at this point depends on the complexity of the mechanism. If things are really simple, one can get the answer with a pencil and paper. If the mechanism is complicated with one or more intermediates between reactants and products, more robust methods are required to calculate the rate constants. The traditional approach seeks an analytical solution, an equation with all of the rate constants. Formulation of these equations requires a good working knowledge of calculus and algebra. This approach is elegant but limited, because simplifying assumptions are usually required to complete the analysis. The Michaelis-Menten analysis of enzyme mechanisms is an example of an analytical solution using simplifying assumptions (rapid equilibrium binding of substrate to enzyme, no enzyme product complex, and no back reactions). The required simplifying assumptions are rarely all valid. Furthermore, the mathematical requirements of the analytical approach have limited the number of biologists capable of using transient kinetics to analyze the mechanisms of their favorite processes.

An alternative approach is to use numerical integration by computer to simulate the kinetic data and calculate the rate constants. In principle, numerical integration can solve any mechanism without simplifying assumptions, being limited only by computer speed. Fortunately, powerful computer programs can now run on readily available computers to take almost all of the work out of the analysis. One can now decipher mechanisms on a lap top computer, a feat which a decade ago was not possible. The purpose of this tutorial is to show you how to do it without knowledge of calculus.

Before working on the methods of analyzing kinetic processes, we need a word about the data required to analyze a mechanism. If your reaction is simple, perhaps only one step, you will be able to solve the puzzle with a minimum of measurements. For example, knowledge of the equilibrium constants and a single transient experiment showing the time course of the concentration change of either the reactant or product as the system approaches a new equilibrium may be enough. On the other hand, if the mechanism includes two or more intermediates or competing side reactions, you will need to measure the time course of the concentration change of several species (as many as possible). Even complicated mechanisms can be solved with incomplete knowledge of the internal steps, providing the full time course of the reaction for those accessible species can be measured over a range of initial concentrations. This is possible because you can use a computer to find a unique set of rate constants that simulate these kinetic curves.

A final point deals with the range of processes accessible to analysis by transient kinetics. Most of our examples involve relatively rapid molecular interactions, processes taking place on a millisecond to second time scale. On the other hand, exactly the same strategy can be used to study processes on much slower time scales. One of our examples is from clinical medicine: a childhood cancer which develops over a period of months.

KINETICS TUTORIAL

You will learn how to use transient kinetics by doing some problems. You will use a kinetics simulation program originally written by Barshop et al. (1983) for use on a VAX computer. Their KINSIM program has been ported to desk top computers. The PC version was written by G. Hua and B. V. Plapp of the University of Iowa. D. Wachsstock wrote the Macintosh version called HopKINSIM. Carl Frieden maintains a public domain archive of the latest versions of the KINSIM family of programs, including an automatic fitting program called FITSIM, at Washington University in St. Louis. FITSIM is not yet available for Macintosh. See Frieden (1993, 1994) for directions on how to log on to WUARCHIVE by anonymous FTP. This tutorial is available from his resource. This tutorial, HopKINSIM, and the HopKINSIM manual are also available on diskette from T. D. Pollard at The Johns Hopkins University School of Medicine, Baltimore, MD (please include $5 for production and handling) and on the BJ Internet server at the University of Minnesota. See directions for access by anonymous FTP or Gopher in the BJ.

To use the Macintosh version of KINSIM, you will need a Macintosh computer with a math coprocessor (IICi, Powerbook 170, SE30, or more powerful recent models) and a text editor (Apple's TeachText, or you can use any word processor as long as you remember to save your file as a text file). To do the problems you will need either a graphing program like Cricket Graph or graph paper and pencil. To get printouts, you can either open the output files in your graphing program and print them there, or get a screen dump of the HopKINSIM screen by setting the screen up the way you like it and pressing ⟨command⟩⟨shift⟩⟨3⟩ and then printing the file named Picture 1 with the Finder. To get quick estimates of half-times, a ruler to place on the screen is helpful.

To do the following problems you must know the definitions of rate constants and the relation of rate constants to equilibrium constants. Appendix 1 will provide you with these relationships as well as the physical basis for rate constants. You will also need to know how to run the HopKINSIM program. You will acquire most of these skills during the Tutorial. For details, consult the Manual provided with each version of the program.

The problems start easy and become more difficult. If you are not challenged by the first problems, skip ahead to the later problems. Open up the KINSIM Tutorial on the disk. You can either follow the tutorial on the screen or print out the tutorial, leaving the screen free for displaying kinetic data. All of the answers are given in Appendix 2.

Problem 1: KINSIM practice and properties of first-order reactions

In this problem we will examine an artificial situation to get a feel for both KINSIM and rate constants. The reaction is a simple first-order change in a protein molecule, A, to a conformation with a higher intrinsic fluorescence, A′.

Reaction 1. A ---> A′

In real life an equilibrium would exist between states A and A′, but for our introduction we will pretend that the transition from A to A′ is irreversible. This is a first-order reaction, because there is only one reactant. In a first-order transition like this, the rate of change of the concentration of reactants and products at any point in time is simply the product of the first-order rate constant (k) and the concentration.

$$\text{Rate} = k_{+}(\text{A})$$

Tasks

1.1. Open up your text editor and write out the mechanism of Reaction 1. Your mechanism should look like this:

A==A′ (return)
*Output (return)
A (return)
A′ (return)

Note: In KINSIM a double equal (==) represents a reversible kinetic reaction. A single equal (=) is a rapid equilibrium, used where the rate constants are not known. Generally KINSIM runs faster with a reversible kinetic reaction. *Output signals that the concentrations of the following species will be graphed vs. time. We have decided to view the concentrations of both A and A′.

1.2. Name and save the mechanism as a text file and then close the word processor.

1.3. Next, open up the HopKINSIM program. Inside HopKINSIM, open up your reaction mechanism. The computer will quickly compile the mechanism into the mathematical equations required to calculate the time course of the reaction.

1.4. Under RUN, select CHANGE followed by the various parameters that you need to adjust. Start with RATES. For k_{+1} (the forward rate constant) select 10. The units for a first-order rate constant are s^{-1}. Leave k_{-1} (the reverse rate constant) at 0, the default value. Under CONCENTRATIONS set A to 1 (the units are μM) and leave A′ at 0. Under TIME CONSTANTS select 1 (second) for the total time and 0.01 (second) for the interval between time points (called Δ time). Optional: Under TIME CONSTANTS you can also modify the size of the time steps and other parameters that affect the rate of the simulation. For purposes of this tutorial, use the default parameters. If you need to speed up a simulation, read the section on TIME CONSTANTS in the manual. Optional: Under AXES, set the y axis (concentration) to 1 μM and leave the x axis (time) at 1 s. The default settings will automatically adjust the axes for each simulation if you do not set them ahead of time.

1.5. Under PREFERENCES under the FILE menu set the Response time to 1000 ms. This will speed up the

calculations at the cost of having to wait 1 s to interrupt the program while running.

1.6. Also under PREFERENCES, uncheck the Save Output Data check box. If this is checked, the output from each simulation will be saved as a tab-delimited text file that your graphing program can read, and you will be asked to name the file each time. For the problems here, that may not be necessary, but you can recheck it at any time. If your graphing program is Cricket Graph 1.3, check that box in PREFERENCES. If your graphing program is something else, use your text editor to remove the first line of the sample data files (it contains an asterisk that Cricket Graph 1.3 requires). Eventually you will want read the entire Manual to learn the details of how HopKINSIM works, but the brief instructions in this tutorial should be sufficient for now.

1.7. Under RUN select GO to start the reaction. In a few seconds, the computer will display the time course of the reaction. (*Note*: The slope of the curve is the rate of the reaction. The slope is maximal early in the reaction when the concentration of A is highest and then declines steadily as A is depleted in favor of A′. As explained in Appendix 1, the curve is an exponential function.)

1.8. What is half-time for the reaction? It is the time when half of A is converted to A′. It may be helpful to change the axes (under the CHANGE menu in the RUN menu) to see the time precisely. Divide 0.693 by this half-time to estimate the observed rate constant (k_{obs}).

1.9. What is the value of k_{obs}? It should be the same as the k_{+1} that you entered, 10 s^{-1}.

1.10. Note the half-time for the reaction starting from the first half-time, the time for the reaction to proceed from 0.5 to 0.75 μMA′. What do you find? What is the half-time for the reaction from 0.75 μM to 0.875 μM? Why do you get the same answer for every part of the reaction?

1.11. Return to the CHANGE dialogue box and vary the value of k_{+1}. Change it to values between 5 and 25 s^{-1}. For each value selected, estimate the half-time and calculate the k_{obs} to reinforce the important relationship between the half-time and the rate constant.

1.12. Try a simulation with a lower concentration of A, like 0.2 μM. What happens to the half-time for the reaction? Why? (*Note:* If the screen become cluttered, you can delete or hide any of the data with the EDIT CURVES command under the RUN menu. Holding down the option key lets you delete all the data curves at once. If you need to take a break from KINSIM, select QUIT to exit HopKINSIM.)

Problem 2: Rate constants for a reversible first-order reaction

Now that you are familiar with the properties of a first-order reaction, we will try a simple reversible reaction. We will start with a simple imaginary reaction: a reversible conformational change. Protein A exists in two states, A and A′, with an equilibrium between these two species

Reaction 2: A == A′.

The reaction is characterized by two first-order rate constants k_{+1} for the forward reaction and k_{-1} for the reverse reaction. The equilibrium constant is K_{eq}.

$$K_{eq} = k_{+1}/k_{-1}. = (A'_{eq})/(A_{eq}),$$

where A'_{eq} and A_{eq} are the concentrations at equilibrium.

If these relationships are not clear to you, consult Appendix 1.

Tasks

2.1. Write out and compile this reaction in KINSIM.

2.2. Observe the time course of the reaction using the following parameters: A = 1 μM; $k_+ = 10\ s^{-1}$; $k_- = 5\ s^{-1}$. At equilibrium, what are the concentrations of A and A′? Calculate the equilibrium constant from these concentrations and confirm that it matches the ratio of the rate constants.

2.3. By checking the successive half-times, confirm that the time course of this reaction follows a single exponential, even though there are two reactions (forward and reverse).

2.4. Estimate the value of the apparent first-order rate constant (k_{obs}) from the half-time of the reaction and confirm its value by running a simulation with a first-order reaction and single rate constant ($k_+ = k_{obs}$ and $k_- = 0$). From the value of k_{obs}, can you guess how k_{obs} is related to the two rate constants for this reversible reaction?

2.5. To test your idea, run several more simulations of the reversible reaction, each time varying k_{-1} by a factor of 2. Each time observe the shape of the curve and estimate the values of k_{obs} from the half-times. What is the relationship of k_{obs} to the rate constants? As a hint, when k_{-1} is reduced to 2.5 s^{-1}, what happens to k_{obs}?

2.6. Note the effect of changing k_- on the equilibrium concentrations of A and A′. Verify that all the equilibrium concentrations of A and A′ agree with the equation given above.

2.7. Also vary the value of k_+. What are the consequences? By now you should be confident about how k_{obs} is related to the two rate constants. It is the sum of the two rate constants, as expected from the analysis in Appendix 1.

Problem 3: Calculation of the rate and equilibrium constants for a conformational change

We have a protein that can exist in two conformations. A′ has a higher fluorescence than A, so that we can measure its concentration spectroscopically. Our first experiment is to measure the equilibrium constant by measuring the concentrations of A and A′ in samples containing a total of 100 μM A + A′. We collect the following data:

Conditions	(A)	(A′)
pH 7	10 μM	90 μM
pH 8	99 μM	1.0 μM
pH 9	99.9 μM	0.1 μM

Tasks

3.1. From the data in the table calculate the equilibrium constants at the three pH values.

3.2. At pH 7, what happens to the concentrations of A and A′ when the sample is diluted 100-fold, giving a total of the these two species of 1 μM? What has dilution done to the ratio of the two species in equilibrium?

Our experiment will be to mix an equilibrium mixture of A and A′ at pH 9 with some acid to lower the pH to a physiological value of 7. The equilibrium shifts in the direction of A′, so that A′ is produced at the expense of A. The total concentration of A + A′ is 100 μM.

3.3. Write out and compile the reaction described in the experiment in KINSIM.

3.4. Display in KINSIM the data for this experiment filed on this disk under "Datafile3." Do this by selecting DATA in the OPEN submenu in the FILE menu.

3.5. Use the half-time to estimate k_{obs} and confirm the value with KINSIM.

3.6. Select values for the rate constants k_{+1} and k_{-1} that fit the equilibrium and kinetic data. Remember that the ratio of the rate constants must be equal to the equilibrium constant. The challenge is to select the two rate constants consistent with the equilibrium constant that best fit the data. Your estimates should be very close to the actual values if you understand the principle. To be sure, confirm the values by running a simulation.

Problem 4: A second-order reaction with an excess of one reactant—a pseudo-first-order reaction

We will now consider a bimolecular reaction where two molecules bind together to form a complex. We will use the binding of molecule A to molecule B as our example. We will use an excess of one of the reactants, B. This simplifies the analysis, because the concentration of B remains more or less constant, so that only the concentration of A changes during the reaction. This will make a second-order reaction look like a first-order reaction.

Reaction 4: A + B == AB

Tasks

4.1. Write out and compile the mechanism in KINSIM. Output only the concentration of AB.

4.2. Set the parameters as follows: A = 1 μM, B = 10 μM, $k_+ = 1\ \mu M^{-1}s^{-1}$, $k_- = 0$.

4.3. Run the reaction.

4.4. What is the shape of the curve? Set the y axis to 1 to see the curve well. Is it a single exponential? Verify this in the usual way by checking the half-times along the curve and by simulating the curve with a first-order reaction.

4.5. What is k_{obs}? How is it related to the rate constants that you used for the simulation?

4.6. What is k_{obs} when $k_+ = 0.5$ or 0.75 or 2 or 4 or 5 $\mu M^{-1}s^{-1}$? How does k_{obs} depend on k_+?

4.7. Now set k_+ to 0.5 $\mu M^{-1}s^{-1}$ and vary B in the range of 10–40 μM and observe what happens to k_{obs}. Confirm that $k_{obs} = k_+B$.

4.8. Now vary k_- in the range of 1 to 10 s^{-1} with k_+ set at 1 $\mu M^{-1}s^{-1}$ and B at 10 μM. What happens to the equilibrium concentration of AB and to k_{obs}? How does k_{obs} depend on k_-?

4.9. What is the general expression for k_{obs} as a function of k_+ and k_-?

4.10. Vary B = 10, 20, 30, 40 μM with $k_+ = 1\ \mu M^{-1}s^{-1}$ and $k_- = 10\ s^{-1}$. Make a plot of k_{obs} vs. B. What is the equation of the line? Confirm that the slope is k_+ and the y intercept is k_-.

4.11. *Unknown:* Now load Datafile4. In this experiment A binds to B to form complex AB. A = 0.1 μM and B = 2, 4, 6, 8, and 10 μM. What are k_+ and k_-?

Problem 5: A second-order reaction with limiting concentrations of both reactants

Again we will examine a bimolecular association reaction of two molecules A and B. The mechanism is the same as Problem 4, but this time we will use similar concentrations of the reactants.

Tasks

5.1. Load and run the reaction with the following parameters: A = 1 μM, B = 1 μM, $k_+ = 4\ \mu M^{-1}s^{-1}$, $k_- = 0.2\ s^{-1}$. Under TIME CONSTANTS, set the total time to 5 s and the Δ time to 0.05 s.

5.2. What is the shape of the curve? Is it a single exponential? Can one speak of k_{obs}?

5.3. How can you analyze the reaction and obtain the rate constants under these circumstances?

5.4. *Unknown:* Now load Datafile5. In this experiment A binds to B to form complex AB. A = 1 μM and B = 0.5, 1, and 2 μM. What are k_+ and k_-?

Problem 6: A two-step reaction mechanism—binding followed by a conformational change

This is an example of a very common reaction in biology. Two molecules bind together and then undergo a conformational change. This is simply a second-order bimolecular reaction (like Problems 4 and 5) followed by a first-order reaction (like Problems 1–3). Few new concepts are needed to understand the mechanism, but some interesting and important new properties emerge from coupling the reactions. One is that the two reactions can be separated by choosing appropriate conditions. Another is how the overall equilibrium depends on the properties of the two reactions.

Reaction: A + B == AB == AB′

Note that we now must deal with four rate constants: k_{+1}, a second-order rate constant with units of $M^{-1}s^{-1}$, and three first-order rate constants k_{-1}, k_{+2}, and k_{-2} with units of s^{-1}.

Tasks

6.1. Load and run the reaction with the following parameters: A = 1 μM, B = 10 μM, k_{+1} = 2 $\mu M^{-1}s^{-1}$, k_{-1} = 5 s^{-1}, k_{+2} = 100 s^{-1}, k_{-2} = 50 s^{-1}. Total time should be 0.25 s; Δ time should be 0.001 s.

6.2. Examine the shape of the AB′ curve. Is it a first-order reaction? Why is there a lag at the outset? Which reaction is rate limiting during the lag phase? After the lag phase? Test your hypothesis by varying the concentration of B.

6.3. What happens if we vary the rate of the second reaction? Try k_{+2} = 1 or 10 s^{-1}. What happens to the equilibrium concentrations of reactants and products? What happens if we change the values of the rate constants for the second reaction while maintaining their ratio at 2:1? For example, try k_{+2} = 10 s^{-1}, k_{-2} = 5 s^{-1}.

6.4. In the example in which you varied k_{+2}, what happened to the equilibrium concentrations of AB and AB′? From the equilibrium concentrations of A, B, and AB′ calculate the overall equilibrium constants under the two conditions (k_{+2} = 10 or 100 s^{-1}). Also use the rate constants to calculate the equilibrium constants for both of the reactions under the two conditions. What is the relationship between the equilibrium constants for each of the pair of the reactions and the overall reaction? Verify your hypothesis with a third set of rate constants (k_{+1} = 1 $\mu M^{-1}s^{-1}$, k_{-1} = 1 s^{-1}, k_{+2} = 10 s^{-1}, k_{-2} = 1 s^{-1}) and the resulting equilibrium concentrations of reactants and products.

6.5. With the set of rate constants from 6.1, experiment with varying B = 4 through 5000 μM, doubling it each time. You will need to change the time constants over several orders of magnitude to get the data and measure accurate half-times. Estimate the values of the rate constant for the approximately first-order reaction after the lag phase. (To estimate the half-times for these very fast reactions, it may be helpful to save the output data. Use the PREFERENCES item in the FILE menu and look at the data in a graphing program. Look at the data in your graphing program and estimate k_{obs} from the half-time.) Plot k_{obs} vs. B. Where on this plot can you find k_{+1} and k_{-1}? What is the relationship of the rate on the plateau to the rate constants? (See Appendix 1.) Knowing the rate on the plateau, k_{+1} and k_{-1} (from the slope and intercept) and the overall equilibrium constant (from the equilibrium concentrations of reactants and products), how can you calculate the values of k_{+2} and k_{-2}?

6.6. Now load Datafile6. In this experiment A binds to B to form complex AB that undergoes a conformational change to AB′. A = 0.1 μM and B = 1, 2, 4, 8, 16, and 32 μM. What are the four rate constants? First note equilibrium concentration of AB′ and then try plotting k_{obs} vs. B to estimate the values. Then simulate the curves with KINSIM to determine them more accurately.

Problem 7: A two-hit reaction—dissociation of the actomyosin complex

This is a more complex set of paired reactions, three in total. Two have the same rate constants, making things relatively simple. This is a real example from biochemistry. The motor enzyme myosin (M) binds to an actin filament (A) by each of its two heads. Both heads must bind ATP (T) for the complex to dissociate. The ATP binding reactions are independent and have the same rate constants. Exactly the same mechanism explains the development of retinoblastoma in children. Two tumor suppressor genes need to be inactivated before the tumor develops. You may be able to think of other two hit reactions like these examples.

Reaction:	AM + T == AMT	k_{+1}/k_{-1}
	AMT + T == AMTT	k_{+2}/k_{-2}
	AMTT == A + MTT	k_{+3}/k_{-3}

Tasks

7.1. Load and run the reaction with the following parameters: AM = 1 μM, other protein species = 0 μM, T = 10 μM, k_{+1} = k_{+2} = 0.3 $\mu M^{-1}s^{-1}$, k_{-1} = k_{-2} = 0.001 s^{-1}, k_{+3} = 500 s^{-1}, k_{-3} = 10 $\mu M^{-1}s^{-1}$. Follow the concentrations of AM, AMT, AMTT, and MTT.

7.2. Why is there a lag? What happens to the lag as T is varied?

7.3. If faced with these rate constants, how could you design the experiment to observe directly the dissociation reaction and evaluate its rate constants? Simulate your experiment and confirm that you can evaluate the constants.

7.4. *Unknown:* Now load Dataset 7.1. This in Knudsen's (1971) classic analysis of the development of retinoblastoma. Patients with a family history of retinoblastoma develop their cancer earlier than those with no family history. We now know that mutations in both of the retinoblastoma genes are required for a tumor to develop. Postulate a mechanism that might explain the difference in the kinetics of tumor development in these two patient populations. Write out and compile your mechanism in KINSIM. In this case, you will need to define the time interval as a month rather than a second as we have done with our other examples. You will also need to define the concentration units arbitrarily, such as 100 patient units. Other than that, the same reasoning is involved. Do you think that you are dealing with first-order or second-order reactions? Are any of the reactions reversible? Use KINSIM to find rate constants that can account for the time course of the onset of the tumor.

Problem 8: A simple enzyme reaction

Here we will see how to evaluate a simple enzyme mechanism in which the enzyme (E) binds the substrate (S), converts substrate to product (P), and the product dissociates. This is a minimal mechanism, since most enzymatic reactions involve at least one additional conformational change of the enzyme-substrate or enzyme-product complex.

Nevertheless, this mechanism is more realistic than the classic Michaelis-Menten mechanism in which the EP intermediate is omitted to simplify the analysis. Although the mechanism has three steps, you will see that it can be dissected rather easily with a few transient experiments. Although this is an imaginary enzyme, the biochemical literature is full of examples of enzymes with mechanisms similar to this.

$$\text{Reaction:} \quad E + S == ES == EP == E + P$$

Note that we now must deal with six rate constants: k_{+1} and k_{-3} are second-order rate constants with units of $\mu M^{-1}s^{-1}$; the other four rate constants k_{-1}, k_{+2}, k_{-2}, and k_{+3} are first order with units of s^{-1}. Also note that our task may seem complex at first, but you should be able to appreciate its simplicity when you see the reaction broken down into individual steps that can be analyzed using the principles that you have learned to dissect first- and second-order reactions. This mechanism is nothing more than first- and second-order reactions linked together. The experiments will isolate the individual reactions for your analysis.

Tasks

8.1. Write out the mechanism and compile it in KINSIM.

8.2. If you were provided with assays for P, ES, and EP as well as a supply of E, S, and P, consider what experiments might reveal the rate constants for the various steps. Where would you begin? Once you have designed a strategy, you will be able to call up data for the most common experiments.

8.3. Choose from the following list the data file that you find most useful. Use analysis of exponentials or KINSIM to learn about the reaction. Then choose additional data files and continue until you have solved the mechanism. You should be able to determine all of the rate constants with only three experiments. The additional experiments will allow you to confirm your results.

a. Steady-state rate of conversion of S to P as a function of S.

b. Time course of conversion of S to P upon mixing S with E.

c. Time course of S binding to E as a function of S.

d. Time course of P binding to E as a function of P.

e. Interrupt steady-state production of radioactive P from radioactive S by adding excess cold S; observe the time course of the release of radioactive P from E.

The details of each of these sorts of experiments follow; you may wish to think about how to do them and how to get the rate constants out of the results before looking ahead.

a. This is the classic steady-state experiment. We mix a large amount of S with a tiny concentration of E (0.1 μM), observe the accumulation of P over short period of time, and repeat this experiment for many different concentrations of S. The data from such an experiment is in Datafile8a. It may help to look at the data in your graphing program rather than loading it into HopKINSIM. The plot displays free product, P, vs. time for S = 0.3, 0.5, 0.7, 1, 2, 3, 6, 9, 12 μM. Note that there is a short lag before the slope reaches its steady-state value. Where does the lag come from? For this steady-state experiment, we ignore the lag. Calculate the rates (the slopes) and plot the rates as a function of S. Note the hyperbolic shape of the curve. Then plot the 1/rate vs. 1/S. This is the Lineweaver-Burk transformation of the data. The y intercept is called the V_{max} and the x intercept the Michaelis constant, K_m. As you will recall from biochemistry, given certain assumptions (rapid binding, slow turnover, no reverse reactions) the K_m is related to the affinity of the enzyme for its substrate. We will see whether these values really tell us anything useful about the actual mechanism.

b. This is a burst experiment that allows us to follow the time course of the first two steps in the reaction. It is accomplished by mixing E and S and then stopping the reaction at intervals with acid in a quench flow machine. We measure and display the total P, that is EP + P as a function of time. Note that P does not need to dissociate from E to be detected. However, P must dissociate from E before another S can bind and initiate another round of the reaction. For the experiment in Datafile8b, we mixed 100 μM S with 1 μM E. We observe that almost 1 mol of P is formed rapidly before the reaction rate slows down considerably. What does the fast phase represent? (Hint: this fast phase corresponds to the lag in experiment (a) above, when we only looked at free P). What rate constants control this phase? Given the concentrations of E and S, which reaction is rate limiting? What can you conclude about the rate constants for the rate limiting reaction? Why is there less than one mole of P released at the end of the fast phase? What does the slow phase represent? What rate constants control the slow phase? What can you conclude about the rate constants for the rate limiting reactions for this phase?

c. This is a simple bimolecular binding reaction, just like those we have examine above. It might be done with fluorescent analogue of S which gives a signal when complexed with the enzyme or if one is lucky, the fluorescence of the enzyme itself may change when S binds. In our experiments in Datafile8c, we have mixed 0.1 μM E with several concentrations of S (= 1, 2, 4, 8, 12 μM) and followed the concentration of E with S bound. This is equivalent to following ES + EP, since the two can rapidly interconvert. From the raw data estimate the values of k_{+1} and k_{-1} by analysis of exponentials. These will only be estimates in the absence of information about the second and third reactions.

d. This is a simple bimolecular binding reaction, just like reaction (c), except that we look at the the binding of the product, P, to the enzyme. As before, we follow the total ES + EP, but this time start with free P and no free S. All chemical reactions are reversible at some rate, and we can take advantage of this to "run the enzyme backwards." This simple experiment can be very revealing as we shall see. Again, the experiment might be done with fluorescent analogue of P, which gives a signal when complexed with the enzyme or if one is lucky, the fluorescence of the enzyme itself may change when P binds. In our experiments in

Datafile8d, we have mixed 0.1 μM E with several concentrations of P (= 1, 2, 4, 8, 12 μM) and followed the concentration of E with P bound. From the raw data calculate the values of k_{+3} and k_{-3} by analysis of exponentials or by KINSIM. Note that since we read the reaction from left to right, k_{-3} is the association reaction, and k_{+3} is the dissociation reaction.

e. This is a chase experiment that reveals directly the rate of product dissociation. We start by mixing 1 μM E with 20 μM radioactive S and wait just long enough for the reaction to achieve a steady-state rate. Then we add 1000 μM cold S. Thereafter, essentially all of the S binding to E will be cold, so all radioactive P that we observe from this time point will have come from radioactive S or P bound to E at the time of the addition of the cold S. In Datafile8e we see the time course of the dissociation of radioactive P. Which reactions are being observed in this experiment? What can this data tell you about the rate constants of these reactions? To simulate this sort of experiment, start with zero concentrations of everything except 1 μM EP, to simulate the fact that the enzyme is fully saturated with substrate at the beginning of the chase part of the experiment and all the free radioactive substrate has been competed away. Assuming EP = 1 μM is not really valid. Why not? Nevertheless, to get started set the reassociation rate for the product (k_{-3}) to 0, to simulate the fact that the radioactive product will not bind the enzyme again (why not?). Set the association rate for the substrate (k_{+1}) to 0, to simulate the fact that new radioactive substrate will not bind (why not?). Then follow the concentration of free P. You will learn that it is necessary to modify your initial assumptions to get a reasonable fit to the data.

8.4. Once you have estimates of all six rate constants, go back to the key experiments and simulate the time courses with a full set of rate constants. You should be able to fit the data exactly after making a few modifications of your initial assumptions. Now that you know all six rate constants, do you understand intuitively why the various experiments produced the observed data? Think about which reactions are rate limiting in each experiment.

8.5. Calculate the equilibrium constants for each of the reactions and compare these values with the K_m. In this case, is the K_m a reliable indicator of the affinity of the enzyme for its substrate?

8.6. Compare the steady-state V_{max} with the rate constants for the individual steps. What does the V_{max} tell us about the mechanism? Does it correspond to any particular step in the reaction?

Problem 9: Multiple interacting components: actin, ATP, and profilin

For your final exam, you can try a reaction that involves multiple interacting components. As far as we know, mechanisms of this complexity cannot be solved analytically. Kinetic simulation is the only available approach. Fortunately, by building up our knowledge of the reactions one step at a time, we will be able to understand the whole process in detail. We will look at the exchange of nucleotide bound to actin and how the actin binding protein profilin promotes this reaction (Goldschmidt-Clermont et al., 1991). The guanine nucleotide exchange proteins that promote exchange of GTP for GDP on small G-proteins probably work the same way, but the decisive experiments have not yet been done.

Here is the background. Actin (A) is the major subunit of microfilaments. The actin monomer is stabilized by ATP (T) bound in a deep cleft in the middle of the molecule. This bound nucleotide can exchange slowly with free ATP in the medium. The actin monomer binding protein, profilin (P), accelerates the exchange reaction and may be used in the cell to recycle ADP-actin to ATP-actin. We will measure exchange by adding the fluorescent ATP analogue, etheno-ATP (eT), to actin. eT has the convenient property of being much more fluorescent when it binds to actin than when it is free, so we can easily measure the concentration of AeT and PAeT (profilin binding does not affect the eT fluorescence).

Reactions:

$$A + T == AT \quad (1)$$
$$A + eT == AeT \quad (2)$$
$$P + AT == PAT \quad (3)$$
$$P + AeT == PAeT \quad (4)$$
$$PA + T == PAT \quad (5)$$
$$PA + eT == PAeT \quad (6)$$
$$P + A == PA \quad (7)$$

Even the most enthusiastic kinetic simulator would have trouble with all of these reactions without some simplifying assumptions and your computer would get bogged down as well. Therefore:

I. We will eliminate Reaction 7, since the concentration of free A is vanishingly small. (Actually one should leave this reaction in the mechanism to make the mechanism thermodynamically legitimate, but it will have little effect on the simulation.)

II. We will assume that the association rate constants for nucleotide binding to actin (Reactions 1, 2, 5, and 6) are the same with a value of 1 $\mu M^{-1}s^{-1}$. (This approximate value is known from the literature and its absolute value $\pm$ 10$\times$ will not affect the time course of the reaction significantly.)

III. To keep things simple, we will assume that $k_{+3} = k_{+4}$; $k_{-3} = k_{-4}$; and $k_{-5} = k_{-6}$.

Tasks

9.1. First we note that profilin is involved in all but the first two reactions, so when the profilin concentration is 0, we can look at the first two reactions alone. Write out and compile Reactions 1 and 2. Display the data in Datafile 9.1. We mix 2 μM AT with three different concentrations of eT (2, 20, or 200 μM). We follow the time course of the change in the fluorescence as eT replaces T bound to the actin by a fluorescence change.

9.2. Search for values of k_{-1} and k_{-2} that fit the data. Assume that the initial concentration of A is 0. Think about which experiment is most sensitive to the relative values of

these rate constants. Remember that the rate constants determine both the rate of the reaction and the equilibrium concentrations of the products. First look for a ratio of rate constants that gives the observed equilibrium concentrations. Start with the curve most sensitive to the values of k_{-1} and k_{-2}. Then confirm the value of the equilibrium constant with other curves. Finally, maintaining the ratio of the rate constants, vary there absolute values to achieve a fit of the curves. Now you can proceed to the experiments with profilin, using the four rate constants from P = 0 as givens.

9.3. In the next series of experiments, we mix 200 μM eT with 2 μM AT in the presence of various concentrations of profilin (P = 0, 0.02, 0.2, or 2.0 μM). Display Datafile 9.2 showing the concentration of AeT + PAeT (determined by fluorescence) as a function of time.

9.4. Search for values of $k_{+3} = k_{+4}$, $k_{-3} = k_{-4}$, and $k_{-5} = k_{-6}$ that simulate the experimental data. Hint: Start with diffusion limited association rate constants and vary the dissociation rate constants to bring the curves into approximate agreement with the data. You may be suspicious that the data will not sufficiently constrain the simulations for you to solve for so many unknowns. Never fear, only one set of these numbers will actually work. Let us know if you find a solution significantly different from ours. How tightly constrained are the values of the unknown rate constants?

9.5. Examine the rate constants. Why does profilin increase the rate of nucleotide exchange? Note that low concentrations of profilin can effect the nucleotide exchange of all of the actin even if the actin is present at a much higher concentration. What features of the rates of these reactions accounts for this "catalytic" effect of profilin?

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APPENDIX 1: RATE CONSTANTS AND EQUILIBRIUM CONSTANTS

The concepts in this section form the basis for understanding all of the molecular interactions in chemistry and biology. Most molecular interactions are driven by the diffusion of the reactants that simply collide with each other on a random basis. Similarly, the dissociation of molecular complexes is a random process with a probability determined by the affinity of the molecules for each other. Many other reactions occur within molecules or molecular complexes. Together these classes of molecular interactions are responsible for all life processes.

We will review the physical basis for reaction rate constants and their relation to the thermodynamic parameter, the equilibrium constant. These simple but powerful principles are essential to appreciate the molecular interactions in cells. In many cases in biology, rate constants are even more important the equilibrium constants, since the rates of reactions govern the dynamics of the cell.

Definitions

Rate constants, designated by lower case k's, are constants that relate the concentrations of reactants to the rate of a reaction.

Equilibrium constants are given as upper case K's. A very important and useful concept is that the equilibrium constant for a reaction is related directly to the rate constants for the forward and reverse reactions as well as the equilibrium concentrations of reactants and products.

The rate of a reaction is the rate of change of concentration of a reactant (R) or product (P) with time. As reactants disappear, products are formed so that the rate of reactant loss is directly related to the rate of product formation in a manner determined by the stoichiometry of the mechanism. The arrows in all of the reaction mechanisms in this tutorial indicate the direction of the reaction.

As a preview, here are the the relationships in the general case:

Reaction mechanism R <==> P

Reaction rates

Forward rate = $k_+[R]$

Reverse rate = $k_-[P]$

Net rate = $k_+[R] - k_-[P]$

At equilibrium the net rate is 0, so $k_+[R_{eq}] = k_-[P_{eq}]$

The equilibrium constant K is defined as $K_{eq} = \frac{k_+}{k_-} = \frac{[P_{eq}]}{[R_{eq}]}$

In specific cases, these relationships depend on the reaction mechanism, particularly on whether one or more than one chemical species constitutes the reactants and products. We will derive the equilibrium constant from a consideration of the reaction rates. We will begin with the simplest case where there is one reactant.

First-order reactions

First-order reactions have one reactant. The general case is simply

R --> P

A common example of a first-order reaction is a conformational change in a protein, where A and A′ are the two conformations

A --> A*

Another example is the dissociation of a molecular complex such as

AB --> A + B

where A and B could be two proteins or an enzyme and a product.

The rate of a first-order reaction is directly proportional to the concentration of the reactant (R, A, or AB in our examples). The numerical values of the rate of a first-order reaction expressed as a differential equation (rate of change of reactant or product as a function of time, t) is simply the concentration of the reactant times a constant, the rate constant k, with units of s^{-1}.

$$\text{Rate} = \frac{-dR}{dt} = \frac{dP}{dt} = kR$$

The rate of the reaction has units of M s^{-1} where M is moles per liter and s is seconds. This is verbalized as "molar per second." As the reactant is depleted, the rate slows.

A first-order rate constant can be viewed as a probability. For the conformational change, it is the probability per unit time than any A will change to A* in a unit of time. For the dissociation of complex AB, the first-order rate constant is determined by the strength of the bonds holding the complex together. This "dissociation rate constant" can be viewed as the probability that the complex will fall apart in a unit of time. Note also that the probability of the conformational change of each A to A* or of the dissociation of each AB is independent of its concentration. Each A or AB does its own thing. The concentrations of A and AB are only important in determining the rate of the reaction observed in a bulk sample.

When thinking about a first-order reaction, it is sometimes useful to refer to the "half-time" of the reaction. The half-time, $t_{1/2}$, is the time for one-half of the existing reactant to be converted to product. This time depends only on the rate constant and therefore is the same regardless of the starting

concentration of reactant. The relationship is derived as follows:

$$\frac{dR}{dt} = -kR \quad \text{so} \quad \frac{dR}{R} = -k\,dt.$$

Integrating,

$$\ln R_t - \ln R_o = -kt,$$

where R_o is the initial concentration and R_t is the concentration at time t. Rearranging,

$$\ln R_t = \ln R_o - kt \quad \text{or} \quad R_t = R_o e^{-kt}.$$

When the initial concentration R_o is reduced by half

$$0.5 = e^{-kt_{1/2}} \quad \text{or} \quad 2 = e^{kt_{1/2}} \quad \text{thus} \quad \ln 2 = kt_{1/2}$$

so, rearranging,

$$t_{1/2} = \frac{0.693}{k} \quad \text{or} \quad k = \frac{0.693}{t_{1/2}}.$$

Therefore you can estimate a first-order rate constant simply by dividing 0.7 by the half-time. Obviously, a similar calculation yields the half-time from a first order rate constant. This relationship is very handy, since one frequently can estimate the extent of a reaction without even knowing the absolute concentrations and since this relationship is independent of the extent of the reaction at the outset of the observations.

To review, the rate of a first-order reaction is simply the product of a constant (k) that is characteristic of the reaction and the concentration of the single reactant. The constant can be obtained from the half-time of a reaction.

Second-order reactions

Second-order reactions have two reactants. The general case is

$$R_1 + R_2 \text{ --> Product.}$$

A very common example is a bimolecular association reaction, such as

$$A + B \text{ --> } AB,$$

where A and B are two molecules that bind together. Such association reactions are very common in biology. Some examples are the binding of substrates to enzymes, the binding of ligands to receptors, and the binding of proteins to other proteins or nucleic acids.

The rate of a second-order reaction is the product of the concentrations of the two reactants, R_1 and R_2, and the second-order rate constant, k:

$$\text{Reaction rate} = \frac{dP}{dt} = kR_1R_2.$$

The units for reaction rate are M s^{-1}, just like a first-order reaction. The units for R_1 and R_2 are M, so the second-order rate constant, k, has units of $M^{-1}s^{-1}$. It cannot, therefore, be directly compared with a first-order rate constant.

Solving the differential equation for a second-order reaction is generally not so simple as that for a first-order reaction. It is not just a simple exponential function except under special conditions called "pseudo-first-order reactions." When one reactant, R_1, is greatly in excess, the total amount of product that can be formed is limited by the amount of the other reactant. Therefore, little R_1 will be consumed during the reaction and its concentration changes negligibly with time. If R_1 is constant, then the differential equation becomes

$$\frac{dP}{dt} = k\,R_1R_2 = (kR_1)R_2 = k_{obs}R_2$$

where k_{obs} is the effective, or observed, rate constant. This is just the first-order equation, so the result is an exponential with a half-time of $0.693/k_{obs}$.

The value of the association rate constant, k_+, is determined mainly by the rate that the molecules collide (Berg and von Hippel, 1985). This collision rate depends on the rate of diffusion of the molecules, which is determined by the size and shape of the molecule, the viscosity of the medium, and the temperature. These factors are summarized in a parameter called the diffusion coefficient, D, with units of cm^2s^{-1}. The rate constant for collisions is described by the Debeye-Smoluchowski equation, simple relationship that depends only on the diffusion coefficients and the area of interaction between the molecules:

$$k = 4\pi b(D_A + D_B)N_o10^{-3}$$

where b is the interaction radius of the two particles (in centimeters), the D's are the diffusion coefficients of the reactants, and N_o is Avogadro's number. The factor of 10^{-3} converts the value into units of $M^{-1}s^{-1}$.

For particles the size of proteins, D is $\sim 10^{-7}$ cm^2s^{-1} and $b \sim 2 \times 10^{-7}$ cm, so the rate constants for collisions of two proteins are in the range of 3×10^8 $M^{-1}s^{-1}$. For small molecules like sugars, D is $\sim 10^{-5}$ cm^2s^{-1} and b is $\sim 10^{-7}$ cm, so the rate constants for collisions of a protein and small molecules are ~20 times larger, in the range of 7×10^9 $M^{-1}s^{-1}$. On the other hand, the experimentally observed association rate constants are on the order of 10^6 to 10^7 $M^{-1}s^{-1}$. The difference of 20 to 1000 is attributed to a steric factor that accounts for the fact that macromolecules must be correctly oriented relative to each other to actually bind together. Thus the complementary binding sites are aligned correctly only 0.1–5% of the times that the molecules collide.

Many binding reactions between two proteins, between enzymes and substrates, and between proteins and larger molecules like DNA are "diffusion limited" in the sense that the rate of correctly oriented, diffusion-driven collisions determine the rate constant. Thus many association rate constants are in the range of 10^6 to 10^7 $M^{-1}s^{-1}$. (Interested readers can consult the article by Northrup and Erickson (1992) that explains how the precise orientation for macromolecular binding is achieved more readily than expected from random collisions.)

To review, the rate of a second-order reaction is simply the product of a constant that is characteristic of the reaction and the concentrations of the two reactants. In biology, the rate of many bimolecular association reactions depends simply on the rate of diffusion-limited collisions between the reactants.

Reversible reactions

Most reactions are reversible, so the net rate of a reaction will be equal to the difference of the forward and reverse reactions. The forward and reverse reactions can be any combination of first or second-order reactions. We will start with a pair of simple first-order reactions, such as the conformational change:

$$A \rightleftarrows A^*$$

The forward reaction rate is k_+ A with units of M s^{-1} and the reverse reaction rate is k_- A* with the same units. At equilibrium

$$k_+ A = k_- A^* \quad \text{and} \quad K_{eq} = \frac{k_+}{k_-} = \frac{A^*}{A}.$$

Note that this equilibrium constant is unitless, since the units of concentration and the rate constants cancel out.

The same reasoning with respect to the equilibrium constant applies to a simple bimolecular binding reaction:

$$A + B \rightleftarrows AB,$$

where A and B are any molecule including an enzyme, receptor, substrate, cofactor, drug, etc. The forward (binding reaction) is a second-order reaction, while the reverse (dissociation) reaction is first order. The opposing reactions are

Rate of association $= k_+[A][B]$ units: Ms^{-1}
Rate of dissociation $= k_-[AB]$ units: M s^{-1}

The overall rate of the reaction is the forward rate minus the reverse rate:

$$\text{Net rate} = \text{Association rate} - \text{Dissociation rate}$$

$$= k_+[A][B] - k_-[AB]$$

Note carefully at this point, that depending on the values of the rate constants and the concentrations of A, B, and AB the reaction can go forward, backward, or nowhere.

At equilibrium the forward and reverse rates are (by definition) the same. Thus

$$k_+[A][B] = k_-[AB]$$

so

$$\frac{k_-}{k_+} = \frac{[A][B]}{[AB]} = K_d, \quad \text{the dissociation equilibrium constant!}$$

These relationships summarize how reaction rate constants and equilibrium concentrations of reactants and products are related to each other.

The equilibrium constant for such a bimolecular reaction can be written in two ways:

Association equilibrium constant

$$K_a = \frac{[AB]}{[A][B]} = \frac{k_+}{k_-} \quad \text{units:} \quad M^{-1} = \frac{M}{M \times M}$$

Dissociation equilibrium constant

$$K_d = \frac{[A][B]}{[AB]} = \frac{k_-}{k_+} \quad \text{units:} \quad M = \frac{M \times M}{M}.$$

What is the half-time of a reversible reaction? For a simple reaction, $A \Leftrightarrow A^*$ that starts with a given concentration of $A = A_o$, the rate of the reaction is

$$\text{rate} = \text{forward rate} - \text{backward rate}$$

$$= \frac{dA^*}{dt} = k_+[A] - k_-[A^*].$$

But the total material is constant, so $[A] + [A^*] = A_o$, or $[A^*] = A_o - [A]$ and

$$\text{rate} = k_+[A] - k_-(A_o - [A])$$

$$= (k_+ + k_-)[A] - k_-A_o$$

$$= k_{obs}[A] - k_-A_o$$

This is just the first-order equation with an extra constant on the end, which does not affect the half-time (see your introductory differential equations textbook). What matters is the coefficient of the reactant concentration, so the half-time is $0.693/k_{obs} = 0.693/(k_+ + k_-)$. The effective rate constant is the sum of the rate constants. This may seem counterintuitive; why should the reverse rate constant have anything to do with the time for the forward reaction? The reason is that the time course of the reaction is not the time to convert all of the A into A*, but is the time to reach equilibrium. The larger the reverse rate constant, the less A* will be present at equilibrium, and the faster the reaction will go from 100% A to the equilibrium mixture.

For the bimolecular reaction $A + B \Leftrightarrow AB$, the differential equation is:

$$\text{rate} = k_+[A][B] - k_-[AB] \quad \text{as above.}$$

The general solution of this equation is complicated, which is why numerical integrating programs (like HopKINSIM) are so useful for real- life reactions. However, the pseudo-first-order approximation above can be useful in this case. If B is in great excess, then [AB] will be limited by the concentration of A and the concentration of B will be effectively constant, and

$$\text{rate} = k_+[A][B] - k_-[AB] = (k_+[B])[A] - k_-(A_o - [A])$$

$$= (k_+[B] + k_-)[A] + \text{a constant}$$

and the apparent rate constant is $k_+[B] + k_-$. Thus, plotting [B] vs. apparent rate constant (which can be estimated from the half-time of the reaction) gives a line with a y intercept of k_- and slope of k_+. Remember, the concentration of B must be much higher than that of A to use the pseudo-first-order approximation. Actually estimating all the rate constants by fitting to simulated data has the advantage of being applicable to all conditions.

Linked reactions

Many important processes in the cell consist simply of a single reaction, but most of cellular biochemistry involves series of linked reactions. For example, when two macromolecules bind together the complex will often undergo some type of internal rearrangement or conformational change, linking a first-order reaction to a second-order reaction.

$$A + B \rightleftarrows AB$$

$$AB \rightleftarrows AB^*.$$

One of thousands of examples is GTP binding to a G-protein, causing it to undergo a conformational change from the inactive to the active state upon binding GTP.

Similarly, the simple enzyme reaction considered in most biochemistry books is simply a series of reversible second- and first-order reactions:

$$E + S \rightleftarrows ES$$

$$ES \rightleftarrows EP$$

$$EP \rightleftarrows E + P,$$

where E is enzyme, S is substrate, and P is products. These and much more complicated reactions can be described rigorously by a series of rate equations like those explained above. For example, simple enzyme reactions nearly always involve one or more additional first-order reactions where the molecules undergo conformational changes.

Linking reactions together is the secret of how the cell carries out unfavorable reactions. An unfavorable reaction can be driven forward by a favorable reaction up- or downstream. All that matters if the total free energy change for all of the coupled reactions is negative. For example, the unfavorable reaction producing ATP from ADP is driven by being coupled to an energy source in the form of a proton gradient across the mitochondrial membrane that is derived in turn from the oxidation of chemical bonds of nutrients.

APPENDIX 2: ANSWERS TO THE PROBLEMS

Problem 1

1.8. The half-time is 0.07 s.

1.9. k_{obs} is $0.693/0.07 = 10\ s^{-1}$.

1.10. The time to 0.75 μM is 0.14 s, so the time from 0.5 to 0.75 μM is 0.14–0.07 = 0.07 s, the same half-time as the first half of the reaction. From 0.75 to 0.875 μM it is also 0.07 s. It is always the same because the reaction is a simple exponential without a "memory." The reaction from any point onward is the same as the reaction from time zero, and the time to use up half of what is left is always the same.

1.12. Changing the concentration changes the rate by the same proportion (the rate is $k[A]$) so it takes just as long to use up half of the reactants.

Problem 2

2.2. Equilibrium concentration of A = 0.33 μM, A′ = 0.67 μM. $K_{eq} = k_+/k_- = 10/5 = 2 = A'/A = 0.67/0.33 = 2$.

2.3. 50% maximum at 0.05 s, 75% at 0.1 s, 87.5% at 0.15 s. Each half-time is 0.05 s.

2.4. $k_{obs} = 0.693/0.05 = 14\ s^{-1}$. (Quite close to $k_+ + k_- = 15\ s^{-1}$.) Part of the problem with numerical integration and estimating half-times from

discrete time points is that rounding errors occur. Dividing by a slightly erroneous number magnifies the error. The more accurate way to estimate k_{obs} is by running a simulation of an irreversible reaction with a final concentration equal to the equilibrium concentration in the unknown reaction and adjusting the first-order rate constant until the simulation matches the data.

2.5. Some values we tried:

k_-	half-time	k_{obs}	$k_+ + k_-$
10	0.035	19.8	20
2.5	0.055	12.6	12.5
1	0.065	10.7	11

Thus k_{obs} is the sum of the forward and reverse rate constants.

2.6.

k_-	A	A′	A′/A	k_+/k_-
10	0.5	0.5	1	1
2.5	0.67	.033	0.5	0.5
1	0.83	0.17	0.2	0.2

2.7. This is left as an exercise for the reader.

Problem 3

3.1.

pH	K
7	9
8	.01
9	.001

3.2. Dilution does not change the ratio; both are diluted equally.

3.5. Half-time is 0.07 s; k_{obs} is 10 s^{-1}.

3.6. We need $k_+/k_- = K = 9$ and $k_+ + k_- = k_{obs} = 10\ s^{-1}$, so $k_+ = 9\ s^{-1}$ and $k_- = 1\ s^{-1}$.

Problem 4

4.4. It is exponential. Half-time is 0.07 all along the curve, and k_{obs} is 10 s^{-1}.

4.5. k_{obs} is not obviously related to the rate constants until you do the next parts of the problem.

4.6.

k_+	0.5	0.75	1	2	4
k_{obs}	5	7.5	10	20	40

k_{obs} is proportional to k_+.

4.7.

B	10	20	30	40
k_{obs}	5	10	15	20

k_{obs} is proportional to B. $k_{obs} = k_+B$.

4.8.

k_-	1	3	5	8	10
k_{obs}	11	13	15	18	20
AB	0.09	0.8	0.67	0.56	0.50

4.9. $k_{obs} = k_+B + k_-$

4.10.

B	10	20	30	40
k_{obs}	20	30	40	50

Slope is 1 $\mu M^{-1}s^{-1}$ and y intercept is 10 s^{-1}.

4.11. k_+ is 2.5 $\mu M^{-1}s^{-1}$ and k_- is 1 s^{-1}.

Problem 5

5.2. It is not an exponential (although it is close). Successive half-times are 0.20, 0.28, and 0.30 s. It is faster at the beginning than at the end; it is actually a hyperbolic curve.

5.3. You can either solve the differential equation exactly (not impossible, but very difficult) or use the simulation program to generate theoretical curves until you find a single pair of rate constants that match the experimental data. This is straightforward, since the ratio of the rate constants is constrained to fit the equilibrium data from the long time scale part of the experiments, so there is only one variable. The higher the rate constants, the faster the reaction, so you can immediately tell if your values are too high or too low.

5.4. k_+ is 1 $\mu M^{-1}s^{-1}$ and k_- is 0.3 s^{-1}.

Problem 6

6.2. The apparent rate constant for the first reaction is $k_{+1}B + k_{-1}$, as in Problem 5, or 25 s^{-1}. The apparent rate constant for the second reaction is $k_{+2} + k_{-2}$, as in Problem 2. It is pseudo-first-order, because the first reaction is slower than the second, so it behaves like the one step reaction in the previous problem. The second reaction is invisible because it is so fast; AB is rapidly converted into AB′ and the time course of AB′ formation in this experiment is the same as that of AB in the previous experiment.

6.3. The second reaction becomes slower, relative to the first, so the second reaction becomes rate limiting. Calculating the actual k_{obs} and comparing them to the predicted k_{obs} for each reaction is left as an exercise for the reader. Keeping the ratio the same leaves the equilibrium values the same but lengthens the lag, enough to be able to observe an overshoot in the value of [AB].

6.4.

k_{+2}	A	B	AB′	K_a = AB′/A*B	K_1	K_2	K = $K_1 * K_2$
1	0.2102	9.21	0.016	0.008	0.4	0.02	0.008
10	0.1849	9.185	0.136	0.08	0.4	0.2	0.08
100	0.0840	9.084	0.611	0.8	0.4	2	0.8

The equilibrium constant for the overall reaction A + B == AB′ is the product of the equilibrium constants for the two coupled reactions.

6.5.

B	half time	k_{obs}	B	half time	k_{obs}
4	.078	8.88	256	.00666	104.0
8	.046	15.1	512	.00579	119.7
16	.0284	24.4	1024	.00505	137.2
32	.018	38.5	2048	.00478	145.0
64	.01215	57.0	4096	.00465	149.0
128	.00875	79.2	5000	.00463	149.7

You could keep going, but you see that the plateau is at $k_{obs} = 150\ s^{-1}$ and it should be equal to $k_{+2} + k_{-2}$. It does not get quite linear at the low end, but taking the first three points as linear, the best fit is $k_{obs} = 4.23 + 1.3$ B, or you would estimate k_{+1} as 1.3 $\mu M^{-1}s^{-1}$ and k_{-1} as 4.23 s^{-1}. The overall equilibrium constant is 0.8 μM^{-1}, from the data in Problem 6.4, and this is equal to the product of the individual equilibrium constants,

$$\frac{k_{+1}k_{+2}}{k_{-1}k_{-2}}. \quad \text{or} \quad \frac{1.3k_{+2}}{4.23k_{-2}} = 0.8.$$

The plateau is $k_{+2} + k_{-2} = 150$, and you can solve for k_{+2} and k_{-2}. You would estimate k_{+2} as 108.1 s^{-1} and k_{-2} as 41.9 s^{-1}. This is not perfect, because we cannot get a clean line at the low end of the scale (see Problem 6.6) but it is certainly close. To get a more accurate answer, you would need to start with these estimates, simulate the whole set of reactions, and adjust the rate constants until the simulation fits the time courses over the whole range of reaction rates. This is somewhat tedious and has been automated in some systems.

6.7. The answer is: $k_{+1} = 15\ \mu M^{-1}s^{-1}$, $k_{-1} = 2\ s^{-1}$, $k_{+2} = 20\ s^{-1}$, $k_{-2} = 5\ s^{-1}$.

Problem 7

7.2. There is a lag because two T's need to bind before MTT, the final product, can be generated. One T binds with single exponential kinetics, but it may come off before the second one can bind, so the probability of getting both T's on simultaneously is low, and it takes some time before this happens. Once AMTT forms, it falls apart very quickly into A and MTT. Higher concentrations of T reduce the lag.

7.3. The key to this game is to reduce the number of unknowns as much as possible in a given experiment, then vary the unknowns that are left to best fit the experimental data. One idea: Based on the biology, it is reasonable to assume that the rate constants for the first two reactions are the same. This cuts the number of unknowns to four. Experimentally, you could mix the actin-myosin complex with ATP and observe the time course of the decrease in light scattering in a spotted-flow machine as the myosin dissociated from the actin filaments. By testing a range of ATP concentrations you would have enough data to find a unique solution of the kinetic mechanism by kinetic simulation with KINSIM.

7.4. One simple mechanism is the following: RB == RB'; RB' == RB"; where RB represents the pair of Rb tumor suppressor genes and the primes represent mutations in one or both of the genes. Patients with mutations in both genes (RB") develop cancer. Patients who inherit one mutation (initial concentration of RB' = 1.0) develop cancer with a first-order time course and a rate constant of 0.075 $month^{-1}$ for the second reaction. The fit to this curve is excellent. The reverse rate constants are 0 for both reactions. Patients born with two normal genes develop cancer with an initial lag. You will be able to get an approximate fit to the data with $k_{+1} = 0.05$ $month^{-1}$, $k_{-1} = 0$, $k_{+2} = 0.075$ $month^{-1}$, $k_{-2} = 0$. Considering that the data are very limited and based on referrals of patients to a cancer hospital, the fit is not so bad. In fact, things are much more complicated than this simple two-hit model, given that additional steps are required for the tumors to develop. We should also take into account that the retina contains about 2,000,000 cells, any one of which can develop cancer. In addition, we have not taken into account that the number of dividing neuroblasts decreases to 0 during the first few years of life, changing the number of cells in the retina that are susceptible to development of these tumors. Mutations that occur after the cells stop dividing presumably are silent. Let us know if you develop a more realistic model that gives a better fit to the data.

Problem 8

8.3. The answer is k_{+1} is 10 $\mu M^{-1}s^{-1}$ and k_{-1} is 1 s^{-1}. k_{+2} is 5 s^{-1} and k_{-2} is 1 s^{-1}. k_{+3} is 0.3 s^{-1} and k_{-} is 1 $\mu M^{-1}s^{-1}$.

8.3a. K_m is about 0.2 μM; V_{max} is 0.024 $\mu M\ s^{-1}$. It is very difficult to estimate because of the scatter in the points. The data are perfect. The scatter in the Lineweaver-Burk plot is the major disadvantage to all the classical methods of analyzing kinetic data. Before computers, the only kind of curve that was convenient to fit was a straight line. The classical approaches (Lineweaver-Burk, Scatchard, etc.) were designed to transform nonlinear data into straight lines. These manipulations (reciprocals, divisions) may exaggerate errors (in this case, rounding errors) to the point that a least-squares linear fit is inappropriate and misleading.

8.3b. The fast phase is the first turnover of S into P, as E binds its first S. This involves only the reactions E + S ⇔ ES ⇔ EP, and it is controlled by $k_{\pm 1}$ and $k_{\pm 2}$. With high S, the ES ⇔ EP reaction is rate-limiting. The fast phase has an initial rate of 4.2 $\mu M\ s^{-1}$, which will be approximately the rate of the reaction ES → EP, or k_{+2} ES. What is ES during the fast phase? During this phase, almost all of the E has bound S already (the second step is rate limiting because it is slower than the first step), but has not released it, so ES is approximately equal to the initial concentration of E, or 1 μM. Thus k_{+2} ES = 4.2 μMs^{-1} = k_{+2} 1 μM, or k_{+2} is ~4.2 s^{-1}, not far off the real answer of 5 s^{-1}.

The slow phase begins when EP starts dissociating into E and P, allowing E to react with another S and continue at a steady state. Thus the fast phase continues until all the E is saturated. Why is this not when EP is equal to the initial concentration of E? The answer is that EP is in equilibrium with ES, and this equilibrium determines the steady-state concentration of EP. From the graph, this is when EP is ~0.83 μM (the slow and fast phases do not form a sharp angle, so you have to extrapolate). Since the enzyme is saturated, ES = 1 μM − EP = 0.17 μM. This tells us the equilibrium constant for Reaction 2; k_{+2}/k_{-2} = 0.83/0.17 = 5. Using the estimate for k_{+2} above, we get k_{-2} = 0.84 s^{-1}.

The slow phase is the reaction EP ⇔ E + P, and its rate is approximately k_{+3} EP, where EP is the steady-state concentration of EP. This phase is what was seen in the steady-state experiment in part (a), and the lag in production of P there is the fast phase here (production of EP rather than P), which has told us so much. The slow rate from the graph is 0.27 $\mu M\ s^{-1}$ which should be k_{+3} EP = k_{+3} 0.8 μM, or k_{+3} = 0.3 s^{-1}.

8.3c. This is just the bimolecular binding reaction from Problem 4 above, and we have a vast excess of S, so the pseudo-first-order analysis applies. Alternatively, just use HopKINSIM. This is a two-unknown system ($k_{\pm 1}$) and we can measure the time course all the way to equilibrium, so it would be easy. Doing it the analytical way,

S	1	2	4	8	12
k_{obs}	9.24	19.8	34.65	69.3	99

Least squares fit gives k_{+1} = 8.2 $\mu M^{-1}s^{-1}$ and k_{-} = 2.4 s^{-1}. This is within an order of magnitude of the correct answer, but not accurate. The problem again is dividing by a number subject to rounding and other errors. This is the advantage of simulating the full time course of a reaction, which gives exactly the right answer and uses all the data. The general rule is fit the data, not the transform, whenever possible.

8.3d. Same problem as (8.3c), but starting with P rather than S. k's are given above.

8.3e. If we assume that the reverse reaction is negligible (a common assumption in this sort of problem), then this experiment is like Problem 1, a first-order, irreversible reaction, EP → E + P. Starting with all the enzyme as EP is wrong, because it will be in equilibrium with ES, but without doing the other experiments, we do not know what that equilibrium will be. If you knew the equilibrium constant from the burst experiment, then you could set ES to 0.17 μM and EP to 0.83 μM at the start. Still, running KINSIM would provide a reasonable estimate of k_{+3}.

Doing it analytically, assuming that the reaction EP → E + P is irreversible, gives a half-time of 1.7 s and a k_{+3} of 0.4 s^{-1}. This is close to the actual value of 0.3 s^{-1}, largely because we have been able to ignore the reverse reactions (EP → ES ⇔ E + S). The association reactions do not occur because of the presence of the large excess of nonradioactive substrate. Any free enzyme is immediately bound to a "black" molecule and thus will not be seen in our experimental results.

8.5. The equilibrium constants are K_1 = 10 μM, K_2 = 5, K_3 = 0.3 μM^{-1}. Do you understand the units involved? K_m (which we found above to be 0.2 μM) does not represent any of these equilibrium constants, so it is not really an affinity for anything. It represents the concentration of substrate needed to produce a half-maximal steady-state rate, so an enzyme with a lower K_m will require less substrate to saturate, so in that sense it has a higher affinity. It can be related to the k's if certain simplifying assumptions are made (ES to EP equilibrium is infinitely fast, irreversible product release) as you have learned in biochemistry, but it cannot be simply calculated for a more realistic model such as this one.

8.6. V_{max} is the maximum rate of production of P in the presence of infinite substrate. Under those conditions, the third step (product release) is limiting, so the rate of production of P is k_{+3} [EP], or 0.3 s^{-1} * 0.083 μM = 0.025 μMs^{-1}, close to the observed value of 0.02363 μMs^{-1}. (Where did the 0.083 μM come from?) It is an overestimate because the rate of production is really limited by two reactions, the second and the third. In our case, the third is much slower, so it dominated the calculation. For the mathematically inclined, the correct effective rate constant to use is

$$1 \Big/ \left(\frac{1}{k_{+2}} + \frac{1}{k_{+3}} \right).$$

This number gives some information about the rate constants in the mechanism, but only if we know the equilibrium concentration of EP, or assume that it is negligible and that the reaction is limited by the concentration of ES, as is assumed in the Michaelis-Menten analysis.

Problem 9

$k_{+1} = k_{+2} = 1\ \mu M^{-1}s^{-1}$; $k_{-1} = 0.01\ s^{-1}$; $k_{-2} = 0.03\ s^{-1}$; $k_{+3} = k_{+4} = 2\ \mu M^{-1}s^{-1}$; $k_{-3} = k_{-4} = 4\ s^{-1}$; $k_{+5} = k_{+6} = 1\ \mu M^{-1}s^{-1}$; $k_{-5} = k_{-6} = 6\ s^{-1}$. The profilin catalyzes the nucleotide exchange by hopping from one actin molecule to another on a subsecond time scale. The half-life of the complex of profilin with actin is 175 ms. During this time, the nucleotide highly is likely to dissociate from the PAT complex, since its half-life is 117 ms.

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