Chemistry 5.07SC Biological Chemistry I

Fall Semester, 2013

Lectures 7 and 8 Enzyme Kinetics (I) and Enzyme Inhibition (II)

Go back and review chemical kinetics that you were introduced to in Freshman Chemistry. Also read Chapter 12 in the course textbook.

I. How do enzymatic reactions and chemically catalyzed reactions differ from uncatalyzed chemical reactions?

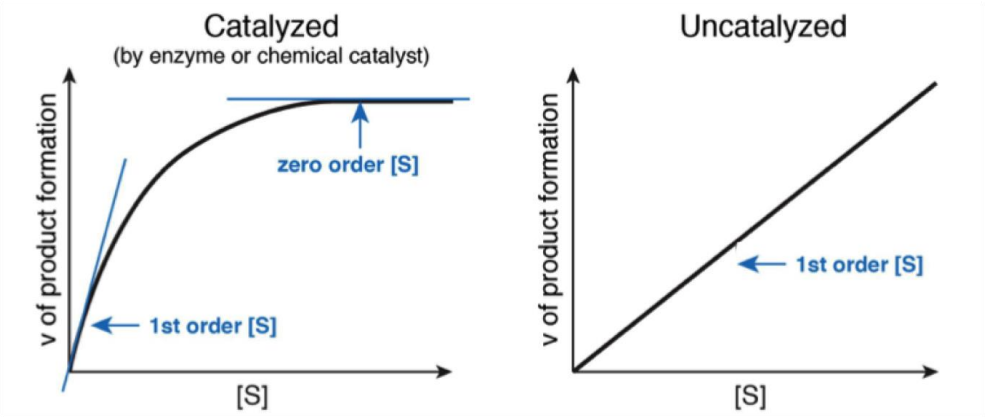


Figure by O'Reilly Science Art for MIT OpenCourseWare.

Figure 1. Experimental observations for a typical enzyme catalyzed reaction (left) and a typical uncatalyzed chemical reaction (right). On the left, the reaction becomes zero order in substrate as the enzyme active site is saturated. On the right, no saturation is observed and the rate continues to be proportional to the concentration of substrate ([S]).

For enzymatic reactions (**or any catalytic reactions** in general), the initial rate of the reaction is proportional to [S], as it is for the uncatalyzed reaction (Figure 1). However, at high [S], the reaction becomes zero order in [S], that is the rate of product formation is independent of the [S]. The active site of the enzyme is 100% saturated with S, thus increasing [S] has no effect on the rate of product formation.

How can we mathematically describe the experimental observations? The following is the

$$E + S \underset{}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

simplest description of an enzymatic reaction where a single substrate is converted to product. Most enzymatic reactions involve two or three substrates (products). The analysis described below, however, is the same regardless of the number of substrates/products. The algebra is more complex.

Digression about Enzyme Assays.

Conditions in vitro for assays:

1. In a typical experiment the [S] is >> [E]; the [S] is mM to μM and the [E] is μM to nM.
2. The reaction in the laboratory is often run under conditions where only a few % of S is converted to P (initial velocity conditions). However, the power of computers has made it possible to monitor the entire reaction course.
3. Most enzymes are found in the μM concentration within the cell. Some enzymes, for example, those in the glycolysis pathway are found in the 100 μM to mM range in the cell. With these high concentrations, the rates are **so fast,** that one cannot measure the kinetics using "hand" manipulations. We now have instrumentation (stopped flow spectrometer and rapid chemical quench) that allows us to mix samples (E + S) on 50 μsec to msec time scale. Of course one needs a way to monitor the reaction on that time scale. Recombinant methods and purification using affinity chromatograph with tags, has provided sufficient amounts of enzymes to carry out these types of experiment.

End of digression

Assumptions:

1. Substrate conservation Equation: $[S_T] = [S] + [ES] \sim [S]$ The $[S_T]$ can be experimentally measured (by UV/visible spectroscopy if colored, by scintillation counting if radioactive. What about measuring the concentration of the substrate by weight? In general the [S] is = $[S_T]$ as reactions in the steady state are carried out with $[S_T] >> [E_T]$.

2. Enzyme conservation Equation: $[E_T] = [E] + [ES]$ The $[E_T]$ is experimentally measurable. Use the A_{280nm} associated with Y, W; see the Expasy website, one can calculate the extinction coefficient of a protein whose sequence is known. In general the [E] and the [ES] **cannot** be measured. Only the concentration of E_T is measureable using Beer's law.

3. One now needs a description of the overall rate of the reaction, that is, the amount of product produced as a function of time. The rate of product produced can be described as the net flux through any step in the pathway. For the above Equation,

$$d[P]/dT = v = k_2[ES] = k_1[E][S] - k_{-1}[ES]$$

In general, if a step is irreversible, then choosing this step to describe the rate of product formation simplifies the algebra when trying to reorganize the equation in terms of experimentally measurable parameters. Regardless of which expression is used to describe the rate of product formation, you should convince yourself that you arrive at the same answer. As noted above, the [ES] is not experimentally measurable in general. One therefore needs to define [ES] in terms of experimentally measurable parameters: $[E_T]$ and [S].

4. The steady state assumption is in general valid under the conditions outlined above and using this assumption facilitates the solution to the problem.

$$d[ES]/dT = 0$$

After a brief time called the pre-steady state (millisec, see lag phase in the Figure 2), the rate of formation of ES or E is equal to the rate of its disappearance

$$k_1[E][S] = k_{-1}[ES] + k_2[ES]$$

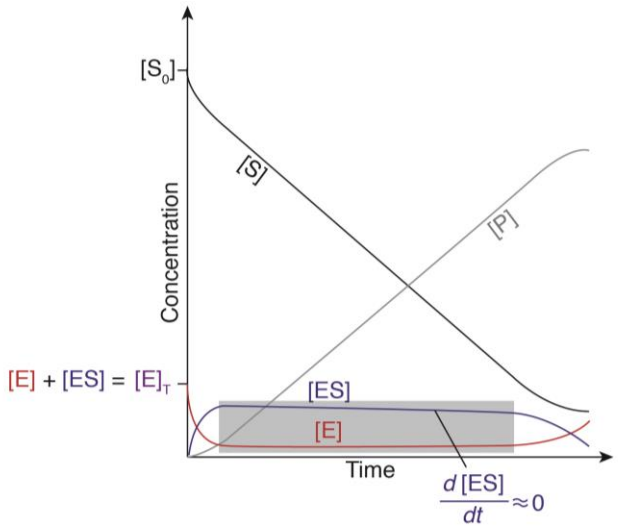


Figure by O'Reilly Science Art for MIT OpenCourseWare.

Figure 2. The fate of substrate, product, and intermediates in a simple enzyme catalyzed reaction. Once steady state is reached (gray box), the steady state assumption holds. The region preceding the gray box is the pre-steady state.

Now rearrange the expression for [ES] in terms of experimentally measurable parameters.

$$[ES] = k_1[E_T][S]/ (k_{-1} + k_2 + k_1[S])$$

Using the expression $v = k_2[ES]$ then gives

$$v = k_1k_2[E_T][S]/ (k_{-1} + k_2 + k_1[S])$$

where v (the rate of substrate disappearance or product formation) is experimentally measurable, $[E_T]$ and $[S_T]$ are known and [S] can be varied. We thus have two variables (v and S) and three unknowns k_{-1}, k_1, k_2. We cannot solve for the individual rate constants. We need to regroup the rate constants into new constants that in the limit, have intuitive and informative meaning.

Grouping the rate constants:

Enzymologists group the rate constants as follows: $V_{max} = k_2[E_T]$. In the case of the reaction above, we are monitoring $k_2 = k_{cat}$, the turnover number of the enzyme. The turnover number is the number of reaction processes that each active site catalyzes per unit time. Units are given in $time^{-1}$. k_{cat} is in general composed of a large number of first order rate constants that cannot be

evaluated individually in the steady state. V_{max} implies all the catalyst is tied up with substrate and therefore the reaction cannot go any faster unless additional enzyme (catalyst) is added.

$K_m = (k_{-1} + k_2)/k_1$ and it can formally be described as the [S] required to reach 1/2 V_{max}. Thus, K_m is not a fundamental constant of interest.

If $k_{-1} >> k_2$, then $K_m = k_{-1}/k_1 = K_d$; K_d is the thermodynamic dissociation constant. In most cases, however, the K_m is NOT the measured thermodynamic affinity of the E for the S, but as we saw with k_{cat}, it is also composed of a large number of first order rate constants.

Substituting these new constants into the above Equation gives the familiar Michaelis Menton (MM) Equation:

$$v = V_{max}[S]/(K_m + [S])$$

k_{cat} and k_{cat}/K_m are the two important kinetic parameters that you need to think about:

k_{cat} the turnover number ($time^{-1}$), tells you how good a catalyst you have.

k_{cat}/K_m is the specificity constant ($M^{-1}s^{-1}$, catalytic proficiency or catalytic efficiency). (see below).

Why are these the important kinetic parameters?

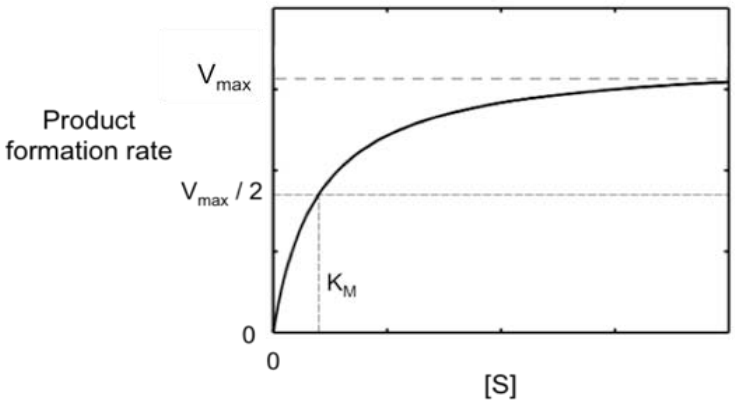
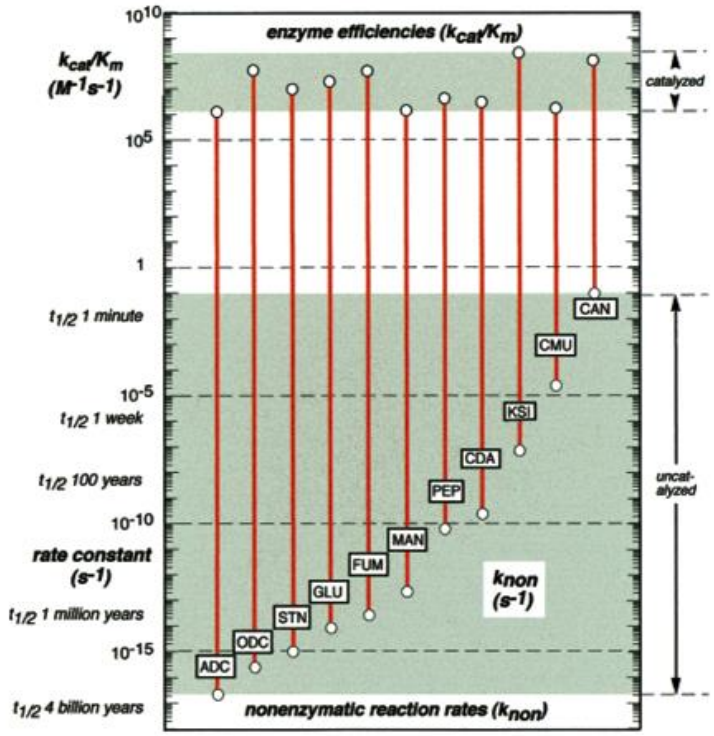


Figure 3. Plot of rate of product formation versus the concentration of substrate ([S]). Once the enzyme is saturated with substrate (see Figure 1), the reaction is zero order in substrate and the rate of product formation is maximized (V_{max}). From the Michaelis Menton equation, the concentration of substrate required to reach half V_{max} is the K_m.

Let us look at the two limiting cases:

1. $[S] \rightarrow \infty$, then $v \rightarrow V_{max}$ The reaction becomes zero order in S. V_{max} tells you how good your catalyst is.

2. $[S] \rightarrow 0$, then $v \rightarrow (k_2/K_m)[E_T][S]$ Under these conditions, the reaction is first order in S and first order in E. k_2/K_m is therefore in this limit, a second order rate constant and is called the **specificity constant or proficiency constant**. This constant is limited by diffusion, that is, the catalyst and substrate need to find each other in solution before any reaction can happen. The rate constant for diffusion control in chemical reactions is between $10^8\ M^{-1}s^{-1}$ to $10^{10}\ M^{-1}s^{-1}$. However, with enzymatic systems, as noted above, this rate constant varies between $10^5\ M^{-1}s^{-1}$ to $10^9\ M^{-1}s^{-1}$. In the case where the reaction is diffusion controlled, the physical step is rate-limiting and the enzyme has reached catalytic perfection. No matter what is done to increase the actual rate of the chemical transformation, the reaction is still controlled by rate at which the catalyst finds the substrate(s) in solution, a physical step.



Source: Wolfenden, Richard, and Mark J. Snider. "The depth of chemical time and the power of enzymes as catalysts." Accounts of chemical research 34, no. 12 (2001): 938-945.

Figure 4. Logarithmic scale of k_{cat}/K_m and k_{non} values for some representative reactions at 25°C. The length of the vertical bar represents transition-state affinity or catalytic proficiency.

Evaluation of Kinetic Parameters Experimentally

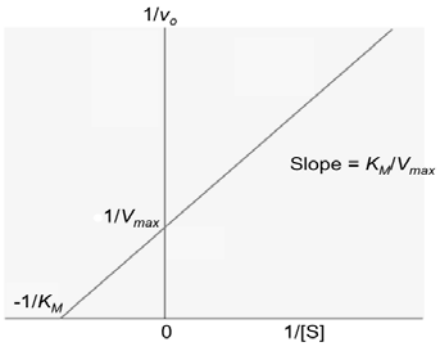


Figure 5. Lineweaver Burke plot of a reaction carried out by a typical enzyme.

Lineweaver Burke plots (Figure 5) provide a method, often used in kinetics, to linearize your data and quickly assess the quality of your experiments. Typically the concentrations of substrate in your assay should be in the range of 0.2 to 5 x K_m):

$$1/v = K_m/V_{max}(1/[S]) + 1/V_{max} \quad y = mx + b$$

In general, analysis of data is obtained by goodness of fit to the MM Equation.

SUMMARY: There exist two important kinetic parameters: k_{cat} the turnover number and an indicator of how effective your catalyst is and k_{cat}/K_m the proficiency constant (how perfect your enzyme is) and the specificity constant (how good is one substrate relative to another). Perfection in catalysis is achieved when the reaction is diffusion controlled, physically limited.

II. Inhibitors of Enzyme Reactions

Usefulness of inhibitors:

1. Inhibitors have played and continue to play a key role in unraveling metabolic pathways. Inhibition of a step in a pathway allows build up of the metabolite that precedes the inhibited step and facilitates its characterization. It is the chemical equivalent to a gene knockout experiment.
2. Inhibitors play a key role in elucidation of the mechanisms of enzyme-catalyzed reactions. An example is tosylphenylchloroketone for the serine protease, chymotrypsin. Inhibitors are used in crystallography to limit the conformational flexibility of an enzyme. The inhibitor often stabilizes the protein in a singular conformation and facilitates crystal formation.
3. Inhibitors are important in the pharmaceutical industry (Table 1, 2): antibacterials (penicillin); fluorouracil, methotrexate, gemzar (anticancer agents). Roughly 30% of FDA-approved drugs are enzyme inhibitors.

Target	Inhibitor
ACE (Angiotensin converting enzyme)	Enalapril
HMG-CoA reductase	Lipitor
Prostaglandin cyclooxygenase	Aspirin
bacterial cell wall crosslinking enzymes	Penicillins
cGMP phosphodiesterase isoforms	Viagra

Table 1. Enzyme inhibitors that are FDA-approved drugs.

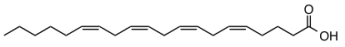
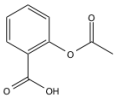
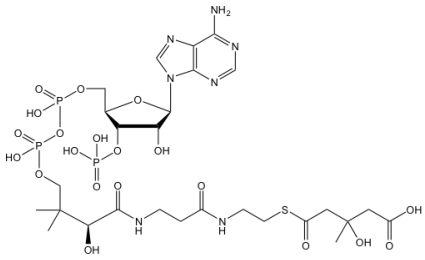
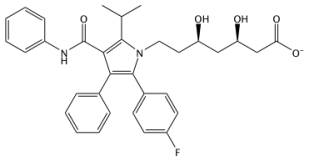
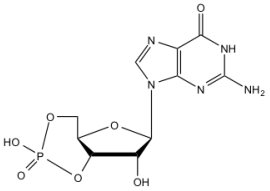
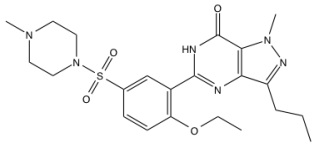
Mechanism of Inhibition	Substrate	Drug (Inhibitor)	Treatment
Noncompetitive; time-dependent	 Arachidonate	 Aspirin	Acute pain, fever, inflammatory diseases (e.g., rheumatoid arthritis)
Competitive	 HMG-CoA	 Atorvastatin (Lipitor, Atorva)	Lower blood cholesterol
Competitive	 cGMP	 Sildenafil (Viagra, Cialis, etc.)	Male erectile dysfunction

Table 2. Examples of drugs, their targets, and their therapeutic roles.

Inhibitors come in many flavors. They can be reversible (Figure 6) or they can be irreversible (time-dependent). In the former case there are three well characterized mechanisms of reversible inhibition: competitive (C); uncompetitive (UC); and noncompetitive (NC). In general the C and UC patterns of inhibition are mechanistically most informative. NC inhibitors are much less mechanistically informative. Irreversible inhibitors show time dependent behavior, but bind reversibly to the enzyme before the irreversible step occurs. The following mathematical

analysis provides a brief description of C and UC inhibition. Because of time constraints you will only be responsible for C inhibition.

Competitive inhibition is the easiest to understand. Using the same approach described above for the MM Equation,

$$E + S \leftrightarrows ES \rightarrow E + P \qquad K_{is} = [E][I]/[EI]$$

$$[I] \downarrow\uparrow$$

$$EI$$

the following equation can easy be derived:

$$v = V_{max}[S]/(K_m(1 + [I]/K_{is}) + [S])$$

The K_{is} is the thermodynamic dissociation constant of the inhibitor for the enzyme. For competitive inhibition, the effect of the inhibitor is ONLY on the slopes in Lineweaver Burke plot (Figure 6A), hence the name $K_{is.}$ (K_{is} on the slope (s)). The reciprocal plot gives a linearized form of the equation:

$$1/v = (K_m/V_{max})(1 + [I]/K_{is})(1/[S]) + 1/V_{max}$$

$$y = mx + b$$

In the case of competitive inhibition where frequently one is trying to inhibit the reaction catalyzed at the active site of an enzyme, I looks structurally like S. The hallmark of competitive inhibition is that if you increase the [S], you can always overcome the inhibition by I (Le Chatelier's principle), if your S is not limited by solubility. The inhibitor does not need to bind to the same form of the E as S, but it can also bind to a form of the enzyme (E′) that is reversibly connected to E. Binding of S can still pull the reaction to the right and overcome the inhibition.

$$E' \leftrightarrows E + S \leftrightarrows ES \rightarrow E + P \qquad K_{is} = [E'][I]/[EIS]$$

$$[I] \downarrow\uparrow$$

$$EI$$

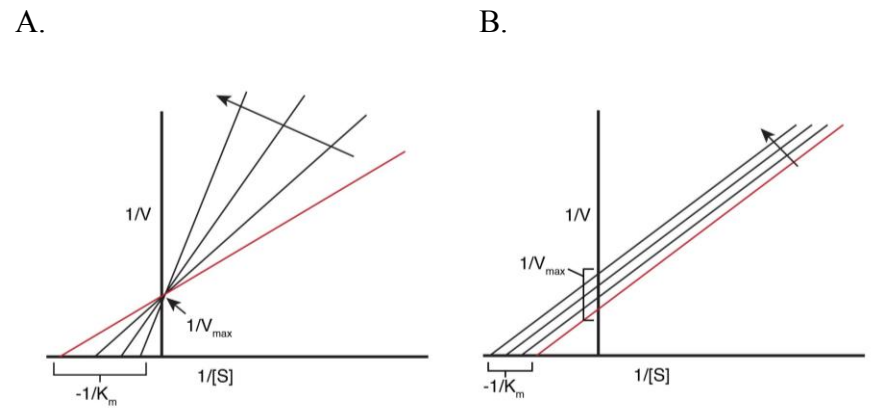


Figure by O'Reilly Science Art for MIT OpenCourseWare.

Figure 6. Lineweaver-Burke plots or a double reciprocal plot of the Michaelis Menton Equation. A describes expectations for a competitive inhibitor, a compound that looks like the normal S and binds in the same site. At high enough concentration of S, inhibition is always overcome, as the substrate outcompetes the inhibitor, [I]. Thus $1/V_{max}$ indicated is the same with every [I] when the substrate gets high enough. The observed effect is on the slopes of the lines and thus a competitive inhibitor is often written as K_{is}. On the other hand another less intuitive but equally informative inhibition pattern is seen in B, uncompetitive inhibition. In this case the lines are parallel in the reciprocal plot and the observed effects are on the intercepts of the plot, not the slopes. This inhibition is K_{ii}. Note in both cases the arrow at the top of each reciprocal plot is an indicator of increasing concentration of I.

As you see in Figure 6A above in the reciprocal plot shown, the effect of increasing the concentration of I is on the slopes and V_{max} (the y intercept) is always achieved at high concentrations of S.

This topic will not be covered due to time constraints, but is presented for your own education: The second informative inhibition pattern is **uncompetitive inhibition (UC).** This pattern is mechanistically very informative.

In this type of inhibition, I binds to a different form of the E than does S. Again one can derive this Equation, just like you did for the MM Equation above.

$$E + S \leftrightarrows ES \rightarrow E + P \qquad K_{ii} = [ES][I]/[EI]$$

$$[I] \downarrow\uparrow$$

$$ESI$$

$$v = V_{max}[S]/(K_m + (1 + [I]/K_{ii})[S]) \quad \text{The reciprocal}$$

$$\text{plot gives } 1/v = (K_m/V_{max}(1/[S]) + (1 + [I]/K_{ii})(1/V_{max})$$

$$y = mx + b$$

As you can see in the reciprocal plot, B above, the effect of increasing the concentration of I is on the **intercepts only**. In fact as you increase the concentration of S, you see more and more inhibition. **Since I binds to the ES form of the enzyme, it requires S for its binding!** This method is widely used when you have an enzyme that has two or more substrates and you want to understand the order of binding of your substrates.

Irreversible Inhibition: One observes time dependent inactivation with irreversible inhibitors. In general, a reversible EI complex is formed, prior to the time dependent component of the inhibition. Since inactivation occurs through the EI complex, a plot of log(% remaining activity) versus time gives a straight line (the inactivation is first order).

$$E + S \leftrightarrows ES \rightarrow E + P \qquad K_{is} = [E][I]/[EI]$$

$$[I] \downarrow\uparrow$$

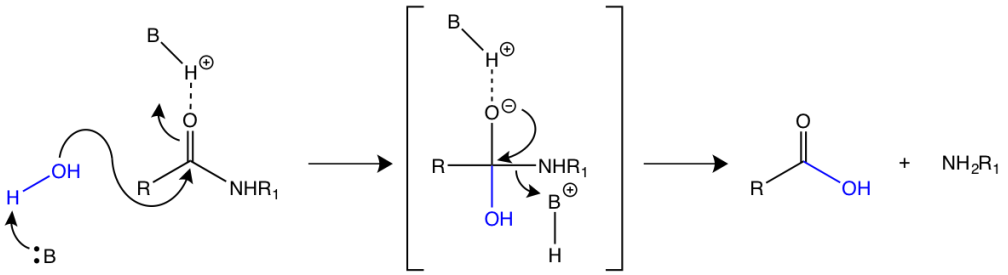
$$EI \rightarrow E\text{-}I \text{ (or } E\bullet I \text{ where I binds essentially irreversibly)}$$

E-I is a covalently modified active site where the inhibitor remains bound during the assay time. With time there is more and more of the active site of E that is taken out of commission until there is no more free E that can bind S. This can occur by covalent bond formation or by a very tight reversible interaction that behaves as if it is irreversible. An example would be a transition state analog.

Below is an example of a reversible and an irreversible inhibitor of cell wall biosynthesis in bacteria (vancomycin and penicillin, respectively):

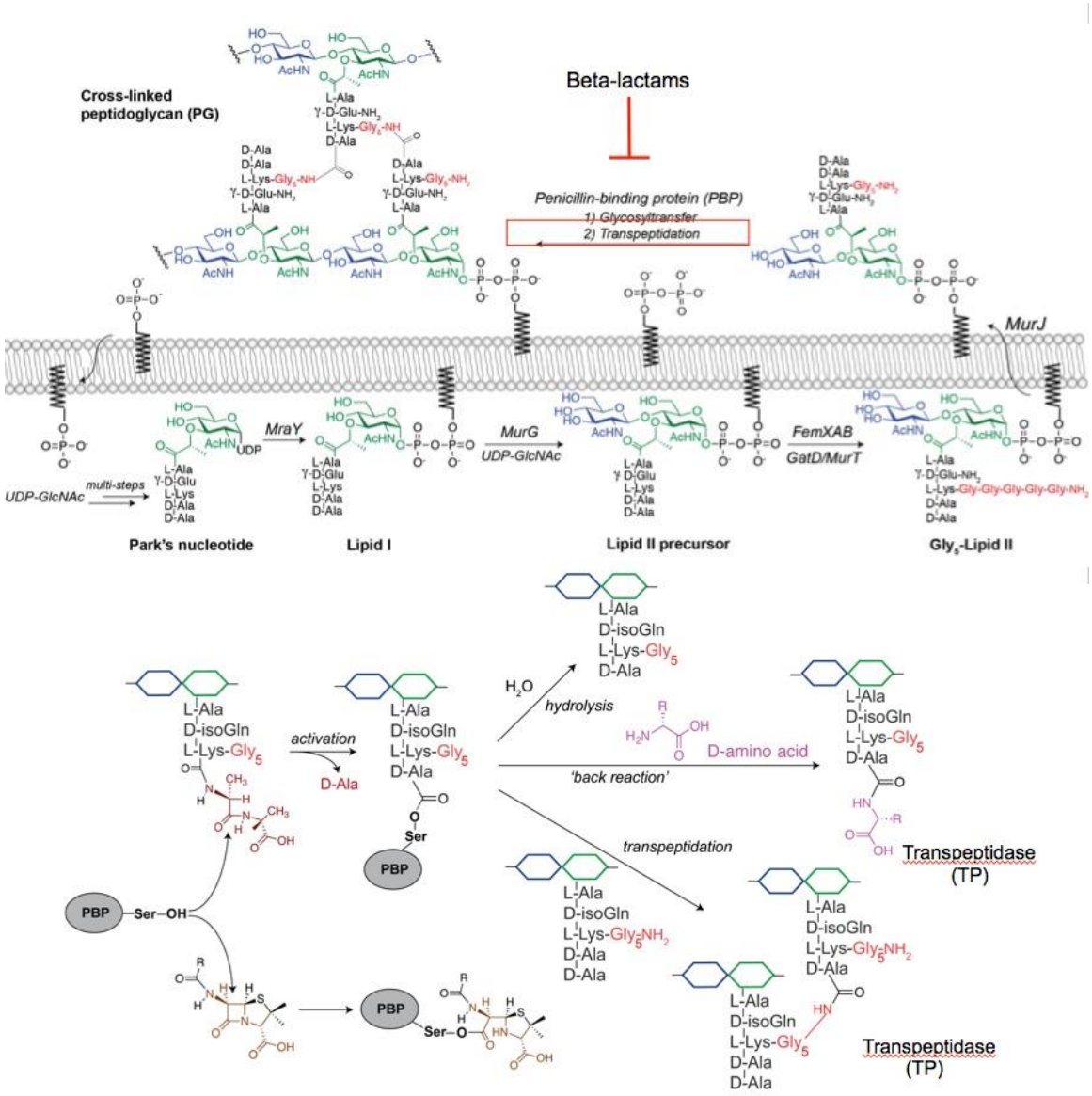
The cell wall is unique to bacteria (Figure 7), and is an excellent target for antibacterial therapeutics. It is composed of an alternating polymer made from two sugars: N-acetylglucoseamine and N-acetylmuramic acid. The N-acetylmuramic acid has attached to it a pentapeptide with two D-ala attached at its C-terminus. Note that within this peptide, the amino acids can be of the D or L configuration.

Digression on the mechanism of amide hydrolysis. The rate acceleration is 10^{10} by enzymes.



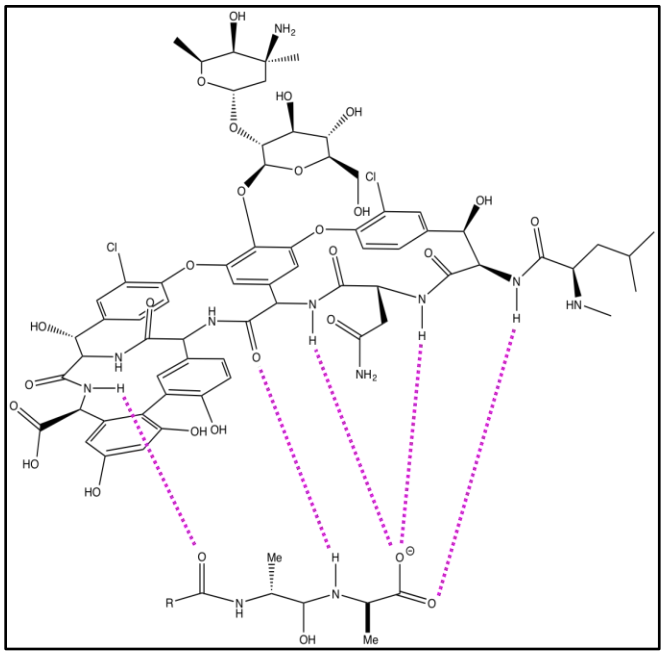
End digression.

Cell wall of bacteria: the target of many antibacterial therapeutics (Figure 7). Vancomycin is the savior against methicillin-resistant Staph (MRSA) infections. **Vancomycin**: is a natural product that binds to D-ala-D-ala of the pentapeptide shown in Figure 7. The proposed interactions are shown in Figure 8.



Courtesy of Yuan Qiao, Harvard graduate student. CC license BY-NC-SA.

Figure 7. The peptidoglycan biosynthetic pathway showing sites of action of natural product inhibitors. The pathway involves three stages: (i) cytoplasmic steps, leading to the peptidoglycan precursor UDPMurNAc-pentapeptide; (ii) lipid-linked steps, involving lipid carrier, undecaprenyl phosphate; and (iii) polymerization and crosslinking of the cell wall on the cell surface. Abbreviations: GlcNAc, *N*-acetyl-glucosamine; MurNAc, *N*-acetyl-muramic acid.

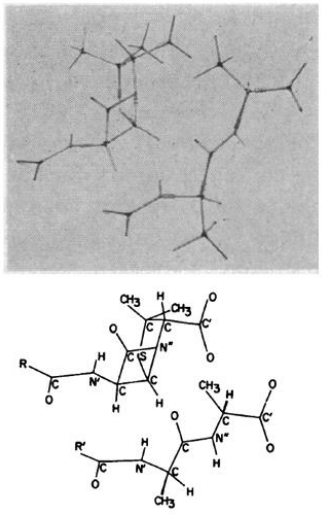


Source: Adapted from Figure 1c of Walsh, Christopher. "Molecular mechanisms that confer antibacterial drug resistance." *Nature* 406, no. 6797 (2000): 775-781.

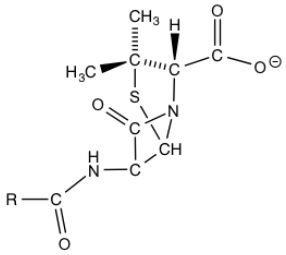
Figure 8. Structure of vancomycin and its binding to the terminal D-Ala-D-Ala of the pentapetide chain of peptidoglycan.

Penicillin: Penicillin (Figure 9) is an **irreversible** inhibitor of cell wall biosynthesis of bacteria (Figure 10). It inhibits the transpeptidase (an enzyme that cleaves one peptide bond and forms a new peptide bond). This enzyme is mechanistically similar to trypsin (chymotrypsin). A crosslink is formed between the peptides from the muramic acid sugars which gives the cell wall strength. The transpeptidase is essential for cell wall biosynthesis.

A.

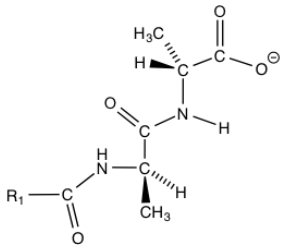


B.



penicillin

C.



Ac-D-Ala-D-Ala

Figure 9A courtesy of Tipper, Donald J., and Jack L. Strominger. Used with permission.
Source: Tipper, Donald J., and Jack L. Strominger. "Mechanisms of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine." *Proceedings of the National Academy of Sciences of the United States of America* 54, no. 4 (1965): 1133.

Figure 9. Structure of penicillin and its similarity to the terminal D-Ala-D-Ala of the pentapeptide side chain. A. Dreiding model (top) and chemical model of penicillin (bottom). B. Chemical drawing of penicillin. C. Chemical model of acyl-Dala-Dala.

The mechanism by which penicillin inactivates the transpeptidase is shown in Figure 10. This transpeptidase involves all three mechanisms of catalysis: binding energy, GAC/GBC and covalent catalysis.

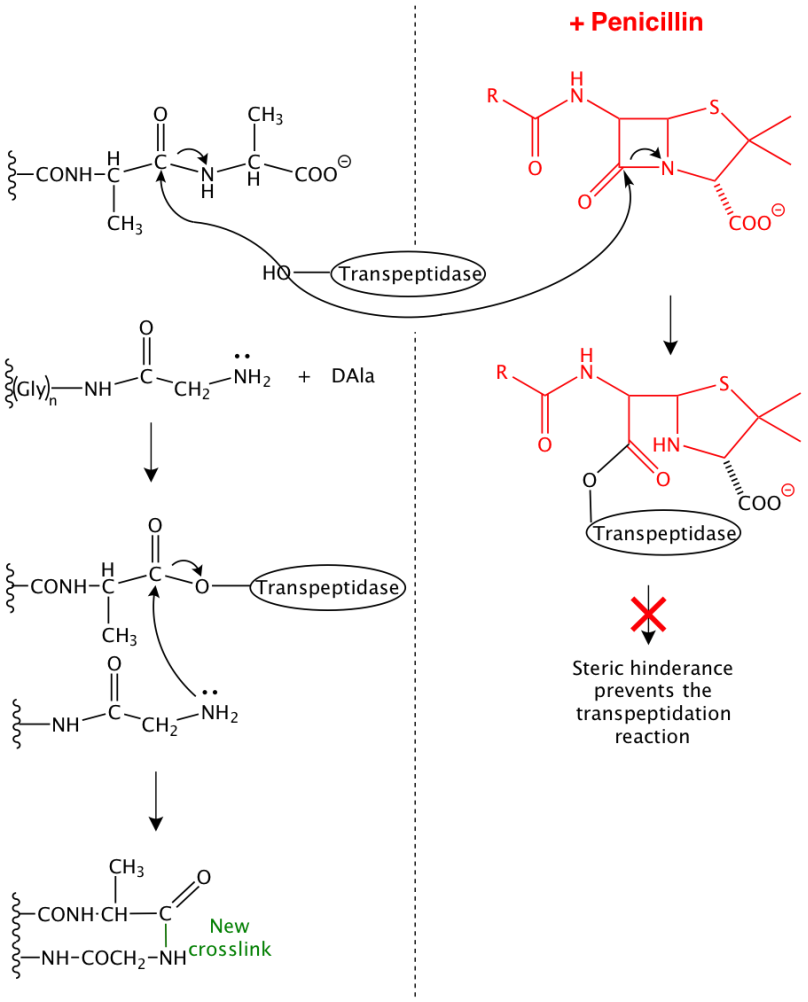


Figure 10. Mechanism of inhibition by penicillin.

In the case of the penicillin which acylates the active site serine, water cannot get into the active site and hydrolyze the ester to regenerate the active site serine. Thus the enzyme remains covalently modified. The proposal for the mechanism of action of penicillins came from a seminal paper in 1965: Tipper DJ, Strominger JL. Proc Natl Acad Sci U S A. 1965 54, 1133-41.

Figure 11 shows the mechanism for serine proteases (trypsin). Upon binding, the peptide substrate uses its binding energy to perturb the pKa of His57 from 6.8 to 11, which permits the His57 to deprotonate the active site serine at position 150.

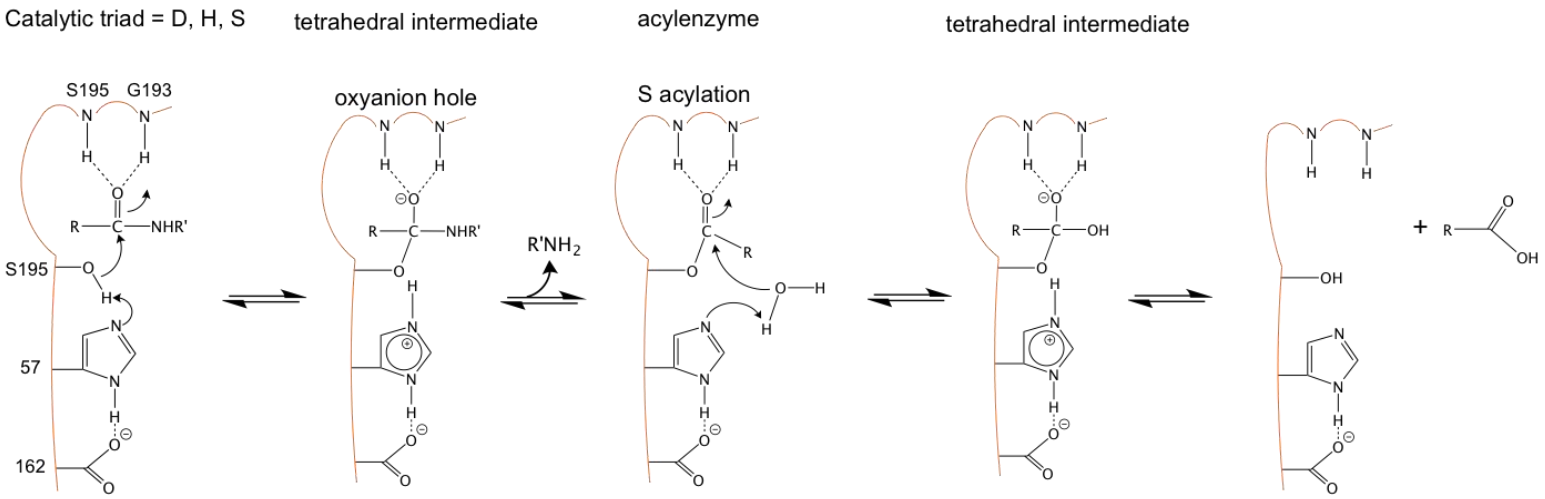


Figure 11. A generic mechanism of a serine protease such as chymotrypsin or trypsin. The serine involved in covalent catalysis and the histidine functions as a general base or general acid catalyst. The carboxylate is also critical although note it does not change protonation state during the reaction. This catalytic triad, described in lecture, provides an example of the use of binding energy in catalysis.

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