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From enzyme kinetics to dynamic models of metabolism

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'What I cannot create, I do not understand'

(Richard Feynman, Nobel laurate)

Prologue

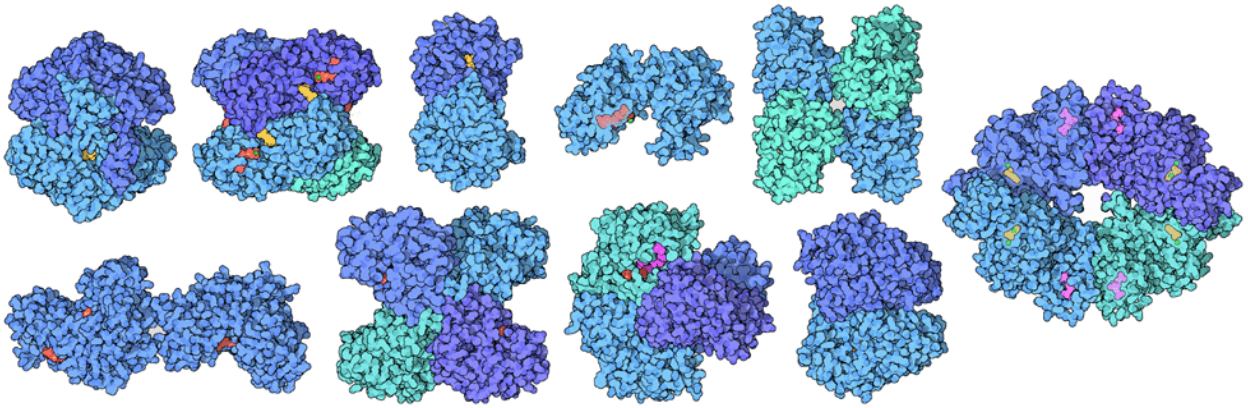


Figure 1: **All the protein structures of the enzymes occurring in glycolysis (from: `https://pdb101.rcsb.org/motm/50`, consult this page to warm up).** What is striking is that glycolytic enzymes have multiple subunits, catalyse reactions with multiple substrates and products, have allosteric as well as catalytic and have greatly varying equilibrium constants. In other words, glycolysis, which is omnipresent across species, is a remarkably complex metabolic system.

All life on our planet depends on the activity of enzymes. All enzymes are proteins and encoded on genes. (The ribosome is the odd one out, as it partially consists of RNA and protein.) Enzymes are generally active inside cells: either as motor proteins walking over, for instance, actin or DNA (see `https://pdb101.rcsb.org/motm/176`); as transporters, carrying (macro)molecules over membranes (see `https://pdb101.rcsb.org/motm/118`); or as metabolic enzymes (Fig. 1), taking care of chemical conversions. Some have, however, extracellular functions such as breaking down sugar polymers or scavenging nutrients.

Enzymes are catalysts, they are recycled unaltered after the completion of a single catalytic event, ready for the next. Accordingly, all catalytic events correspond to cyclic mechanisms (Fig. 2), composed of sequences of transitions between enzyme states, starting and ending at the same state. The rate of such transitions can be described in terms of mass-action kinetics. Together with conservation of the total enzyme concentration and assuming steady state, this leads to an universal description of steady-state enzyme kinetics, such as, in the

The rate equation of reaction 1 in figure 2 expressed in mass-action kinetics equals $v_1 = k_1^+ \cdot e \cdot s - k_1^- \cdot es$

simplest case, the rate equation known as the reversible Michaelis-Menten equation (or Briggs-Haldane relationship).

Deriving rate equations of enzymes in the general case – incorporating multiple substrates, products, effectors and subunits – for later use in mathematical models of the dynamics and regulation of metabolism is one of the aims of this course. Two methods exist for derivation of enzyme kinetics. The rapid-equilibrium approximation, which is fast and efficient, but more approximative than the steady-state method, which is more realistic but more complex. We will focus most on the rapid-equilibrium approximation. An associated aim is to understand the role of thermodynamics and kinetics in such descriptions.

The rate equation for the enzyme catalysing the reaction $E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$ (shown in figure 2), was derived first by Briggs & Haldane: $v = \frac{V_M^+ \frac{s}{K_S} - V_M^- \frac{p}{K_P}}{1 + \frac{s}{K_S} + \frac{p}{K_P}}$ in 1925 (`https://doi.org/10.1042/bj0190338`).

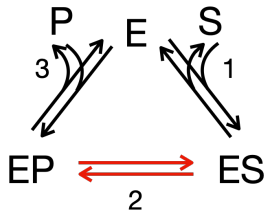


Figure 2: **Example of a cyclic enzyme mechanism.** The enzyme E converts substrate S into prodict P. The cycle starts with E and after a single catalytic event $E + S \rightarrow E + P$, E is returned. So, E is a catalyst. An example of an enzyme with only a single substrate and product – which is a bit of minority enzyme in the cell – is triose phosphate isomerase (TPI; see `https://www.rcsb.org/structure/2ypi`)).

Enzymes are generally active in networks, exchanging and converting reactants. Enzyme rates are dependent on concentrations of reactants, effectors and the catalysing enzyme. The reactants and effectors are generally also reactants of other enzymes in the cell, together forming a network.

Effector regulation may involve regulatory effects of distant chemical compounds in the network, via feedforward or feedback allosteric loops (Fig. 5). Enzymes that are regulated in this manner are often composed out of several cooperative subunits (Fig. 3). Subunit 'cooperation' sensitises them for regulation, while unregulated enzymes are generally monomeric and less sensitive (Fig. 4). Surprisingly, many enzymes in the cell are multimeric and regulated by effector molecules. Consider, for example, the metabolic regulation of the central metabolism of *E. coli* in figure 5.

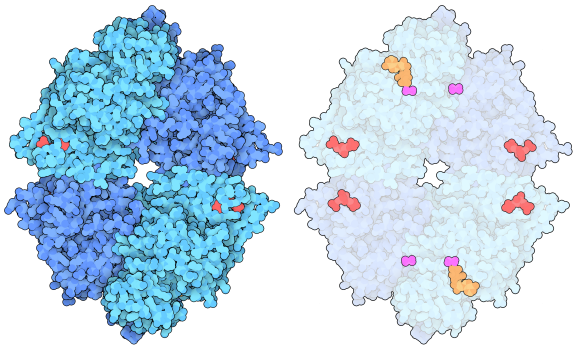


Figure 3: **Pyruvate kinase is an example of a multimeric enzyme, composed out for identical subunits (valid across speciesl; here the human complex is shown).** Pyruvate kinase catalyses the conversion $PEP + ADP \rightleftharpoons PYR + ATP$ in glycolysis. Phosphoenlopyruvate (PEP), ADP, pyruvate (PYR) and ATP (orange) bind to the catalytic site together with the competitive inhibitor oxaloacetate (purple). Fructose 1,6-bisphosphate is shown in red and binds to an allosteric distant from the catalytic site and change the conformation of the protein complex. This pictures was taken from `http://doi.org/10.2210/rcsb_pdb/mom_2022_6`. For more structures and the influence of effectors, investigate the weblinks in the legend of "Exploring the structure" on the bottom of the page of `http://doi.org/10.2210/rcsb_pdb/mom_2022_6`.

The dynamics of metabolic networks is dictated by chemical element conservation and enzyme kinetics, while steady states are also characterised, in addition, by balanced net rates of synthesis and degradation for each chemical compound.

One of the main aims of this course is also to make and analyse mathematical models of metabolism, given enzyme kinetic descrip-

tions.

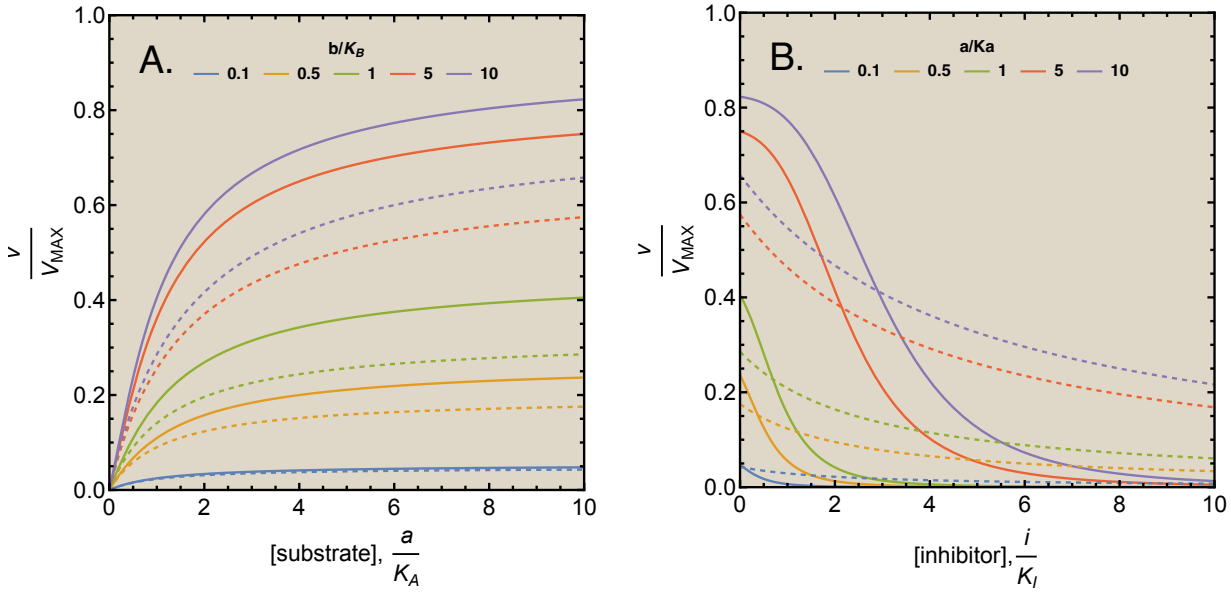


Figure 4: **Multiple subunits sensitises an enzyme to concentrations of reactants and, predominantly, of regulators.** We are considering an enzyme with either 1 subunit (dashed lines) or one with 4 (full lines). The enzyme catalyses the conversion $A + B \rightleftharpoons P + Q$. The kinetics of the enzyme is described by a reversible Monod-Wyman-Changeux rate equation, an equation we derive later in this text. A. Influence of multiple subunits and cooperativity on the rate of an enzyme as function of a substrate concentration. B. Influence of multiple subunits and cooperativity on the rate of an enzyme as function of an inhibitor concentration.

For all species on our planet the quantitative descriptions of enzyme kinetics, dynamics and steady states of metabolic networks is the same, because all species obey the same fundamental physicochemical laws. The theory of these course notes are therefore broadly applicable, across species.

An enormous scientific challenge is to understand the regulation of metabolic pathways in the context of the metabolic demands and selection pressures on a cell (or in case of a multicellular species, a 'coordination or coherence' pressure from the organ or tissue). Genotypes have been tinkered by evolution into ever better adapted genetic variants of ancestors they have outcompeted or coexist with. Part of this evolutionary process is the creation of novel genetic variations by mutations and the selection of fitter genetic variants. The fittest make most offspring and therefore become more frequent and fix – this is we call natural selection.

What fitter implies in terms of phenotypic characteristics one can debate about, but that this in some cases involves metabolism is beyond question. This also implies that the regulation of metabolism, via regulation of gene expression and feedback and feedforward circuitry, has been subject to evolutionary tuning.

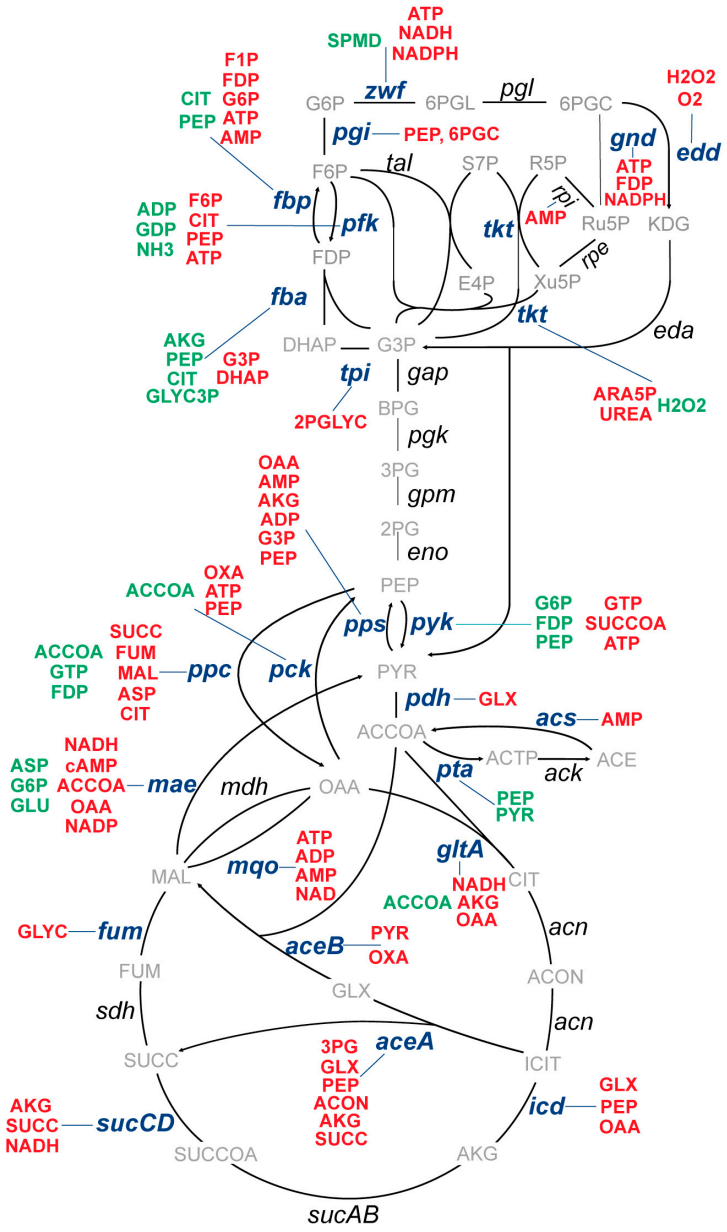


Figure 5: **Overview of the enzymes of central metabolism in *E. coli* and their regulators (green: activation, red: inhibitions).** From `https://doi.org/10.1016/j.celrep.2017.08.066`.

Yet, we lack a whole-cell understanding of the coordination of metabolism by local regulation of metabolic pathways. We do have some understanding of how molecular-mechanistically enzymes in metabolism are regulated but generally lack understanding of the cellular benefit of that regulation in terms of an improved fitness.

This is a huge scientific problem. Its solution will have enormous impact of medicine, biotechnology and our fundamental understanding of life.

Considering the complexity of the scientific challenges that we nowadays face, quantitative biochemistry and molecular biology are much needed disciplines. They remain nevertheless worryingly underrepresented in biology and chemical curricula.

This text should be informative for biotechnologists, biochemists, bioinformaticians and biophysicists alike. In particular for those who do not have a quantitative biological or biochemical background, I consider any text as this one mandatory.

In these course notes, I outline the basics from enzyme kinetics to dynamics of metabolic networks, sufficient to start thinking quantitatively about metabolism and making mathematical models. Whenever possible I give references to more advanced texts or papers. I hope that you enjoy studying this text and its exercises. On a first read you can skip the sections with a title that ends with the symbol "‡".

Suggestions for further reading

1. Milo, Ron, and Rob Phillips. Cell biology by the numbers. Garland Science, 2015.

2. Harold, F. M. (2003). The way of the cell: molecules, organisms, and the order of life. Oxford University Press, USA.

Part I

Models of Enzymes

Enzymes

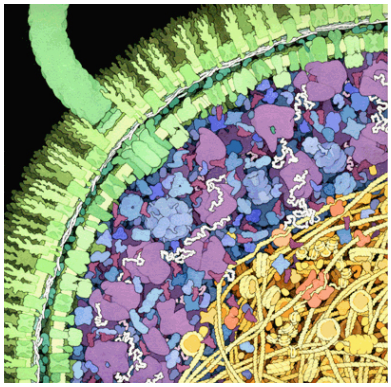


Figure 6: **An artist impression by David Goodsell of the interior of an *E. coli* cell.** Proteins, mRNA, plasma membrane, DNA, etc. are shown. See also this movie of the intracellular conditions in an *E. coli* cell at `https://youtu.be/8DOYCXWCqQg` and the publication about *Escherichia coli* by D. Goodsell who drew this picture (Goodsell, *Escherichia coli*, Biochemistry and Molecular Biology Education, 37(6), 325-332, 2009)

Proteins

The proteins or the subunits of multimeric proteins that we find inside cells (Fig. 6) are generally composed out of a few hundred amino acids (300-400 $aa/protein$) and are about 5 nm in radius. The protein-protein distance in a cell is about 1 diameter (so ~ 10 nm), so the cell is very crowded (Fig. 6).

Proteins continuously diffuse randomly throughout the cell, this diffusion precedes all chemical reactions. The diffusion rates provide an upper bound for rates of chemical reactions: the complex formation of two proteins A and B can of course not proceed faster than their collision rate.

A ribosome elongates amino-acid peptides, eventually leading to a protein, at a rate of about 20 aa/s. It takes a ribosome therefore about $350/20 = 17.5$ s to make a protein, making the corresponding mRNA from the gene is only just a tiny bit slower. So RNA polymerase and ribosomes are almost in physical contact when coupled transcription and translation occurs – which is possible in bacteria.

For a calculation of the transcription and translation times see `http://book.bionumbers.org/what-is-faster-transcription-or-translation/`.

Protein life times vary greatly. Most metabolic proteins are however stable and are only reduced in concentration due to cell volume growth, others can be subject to (regulated) degradation by proteases and have shorter life times.

Protein concentrations vary a lot too, from a few copies per cell (e.g. transcription factors) to tens of thousands (metabolic enzymes).

Exercise

1. An *E. coli* cell is approximately 1 μm^3 (a yeast cell is about 8 μm in radius) and the radius of a protein is about 5 nm.

   a. Calculate the concentration of 1 molecule in an *E. coli* cell.

   b. How many proteins fit in a *E. coli* cell when their protein-protein distance is roughly equal to their diameter? Assume that proteins are perfect spheres. (note: volume V of a sphere

with radius r equals $V = \frac{4}{3}\pi r^3$)

c. Say an *E. coli* cell doubles itself within 1 hour. How many ribosomes are needed per cell to make all the required proteins?

d. The ribosome of *E. coli* is a protein complex composed out of 7459 amino acids. The fast growth rate of an *E. coli* correspond to the shortest doubling time. What would this doubling time and growth rate be if an *E. coli* cell only consists out of ribosomes? Plot the growth rate and doubling time as function of the ribosomal protein fraction, ranging from 0.05 to 1.

d. The diffusion D of a protein is about $D = 5\ \mu m^2/s$. Calculate the time that it takes a protein to diffuse the length $L = 2\ \mu m$ of a *E. coli* cell, using the relation $L^2 = 6Dt$.

e. Calculate the time τ that it takes for two proteins A and B to collide with each other, given

$$\tau = \frac{V_{cell}}{4\pi(D_A + D_B)(r_A + r_B)}$$

with D_A and D_B as their diffusion coefficients and r_A and r_B as their radii.

f. When N_A copies of protein A and N_B copies of protein B occur the collision time reduces to $\tau/(N_A N_B)$. What is time for two proteins to collide that occur at 100 copies per cell?

g. The diffusion coefficient D can be calculated from $kT/(6\pi\eta r)$ with k as the Boltzmann constant $1.38 \times 10^{-23}\ JK^{-1}$, η as the viscosity (water: 1 $mPa \times s$) and r as the radius. Compare the diffusion of a protein ($\sim 5\ \mu m^2/s$) and an *E. coli* cell in water.

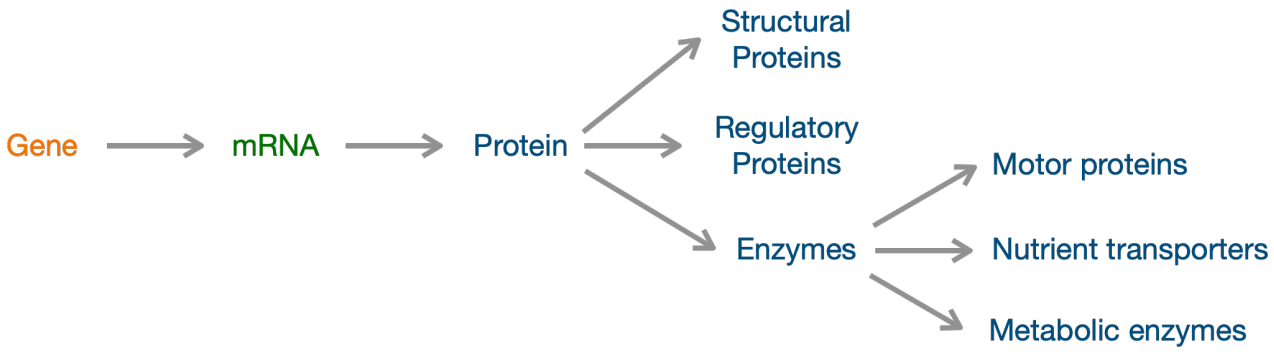


Figure 7: **Proteins have different cellular functions.** Only some of the proteins in a cell act as enzymes, catalysing reactions.

Structural proteins, regulatory proteins and enzymes

Genes code for proteins. Not all proteins are enzymes (Fig. 7), some have structural (such as actin `https://pdb101.rcsb.org/motm/19`) or

regulatory roles (such as Ras (https://pdb101.rcsb.org/motm/148) or Sox (https://pdb101.rcsb.org/motm/112)).

Examples of structural proteins in *E. coli* are H-NS (a DNA-binding nucleosome-like protein), MreB (a protein polymer underneath the plasma membrane supporting cell shape) or FtZ (a protein needed to form the septum in middle of a dividing mother cell).

Examples of regulatory proteins, without a catalytic function, are transcription factors that change in conformation and DNA affinity upon covalent modification or small-molecule binding (e.g. a metabolite). For instance, the Lac repressor is removed from the Lac operon by binding of allolactose, the metabolic product of the enzymes that are encoded Lac operon. Other examples are membrane-embedded G-protein coupled receptors that regulate intracellular processes upon extracellular ligand bind via a conformational change in their intracellular domain.

For a model of the Lac operon and its regulation see https://doi.org/10.1016/S0006-3495(03)70013-7.

Enzymes are proteins that catalyse events: such as vesicular transport along an actin polymer, template copying such as DNA replication, membrane transport or chemical reactions.

As we will see regulatory proteins and enzymes can all be described within the same kinetic framework, called the rapid-equilibrium approximation pioneered by Monod, Wyman and Changeux (MWC) in 1965.

For a recent review about the MWC model, see https://doi.org/10.1146/annurev-biophys-050511-102222, the original reference is https://doi.org/10.1016/S0022-2836(65)80285-6.

Enzyme types

Three types of enzymes are generally distinguished:

MOTOR PROTEINS Dynein (https://pdb101.rcsb.org/motm/176) is an example of a multimeric motor protein. It carries cellular cargo such as vesicles along microtubules in a eukaryotic cell. A single catalytic event is then a single step made on the microtubule molecule by the cargo-carrying dynein. F_1F_0 ATPase (https://pdb101.rcsb.org/motm/72 and Fig. 8) is also a motor protein, with each of its turns it transports protons over a membrane and uses the liberated free energy to catalyse the reaction $ADP + Pi \rightarrow ATP$.

Thus, motor proteins can catalyse reactions as metabolic enzymes do. Other examples of motor proteins are the ribosome, DNA polymerase, and RNA polymerase, although we also think of those as metabolic enzymes since they make a product from substrates.

Another example of a pure motor protein like dynein is the flagellar motor, which can cause bacteria to move in liquids or on surfaces by propelling them.

Since motor proteins have a net direction of motion, for which they require free energy, they generally hydrolyse ATP and exploit the

harvested free energy to induce conformational changes.

TRANSPORTERS Transporters transport solutes and proteins over organellar or cellular membranes (e.g. `https://pdb101.rcsb.org/motm/95`), in particular molecules that cannot freely pass over membranes such as charged or bulky molecules. Small-molecule, protonated, weak acids such as acetate, pyruvate or succinate can generally freely pass over membranes, but this does not mean that cells do not have (dedicated) transporters for them. Without a constant free energy input, transporters cannot cause the accumulation of chemical compounds in- or outside cells at concentrations higher than the out- or inside concentration. So also transporters are often reliant on a free energy source such as ATP which hydrolyse into ADP and Pi to induce the conformational changes associated with transport of a molecule over a membrane.

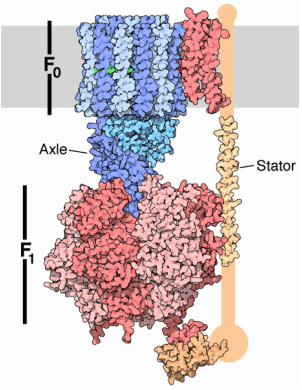


Figure 8: **ATP synthase which turns in the plasma membranes of cells (or mitochondria) making ATP from ADP and Pi, using the free energy harvest by the import of protons along their downwards gradient.**

METABOLIC ENZYMES Metabolic enzymes (`https://pdb101.rcsb.org/motm/50`) catalyse reactions in the cytosolic or intraorganellar environments of the cell. An example is hexokinase which catalyzes $glucose + ATP \rightarrow ADP + glucose - 6 - phosphate$. One of the features of enzymes is, in addition to speeding up reactions, that they can couple an energy-demanding reaction A to a energy-liberating reaction B to run A in the opposite direction of its natural 'tendency'. This makes enzymes so effective in making complex macromolecules such as lipids, RNA, DNA and protein which cannot form spontaneously without enzyme activity and free energy consumption.

MONOMERIC VERSUS MULTIMERIC ENZYMES Feedback-regulated metabolic enzymes or ligand-binding activated transcription factors are often composed out of multiple, interacting subunits. Cooperation of subunits sensitises them to concentration changes of effector molecules via changes in conformations with different reactant and effector affinities (Fig. 4, 10 and 9) – giving rise to switch-like changes in activity–, which we will explore later.

An example is the activation of pyruvate kinase (Fig. 3) – a multimeric enzyme – in *E. coli*'s and *S. cerevisiae*'s glycolysis by fructose-1,6-bisphosphate. Phosphofructokinase is another well known multimeric en feedback-regulated enzyme of glycolysis.

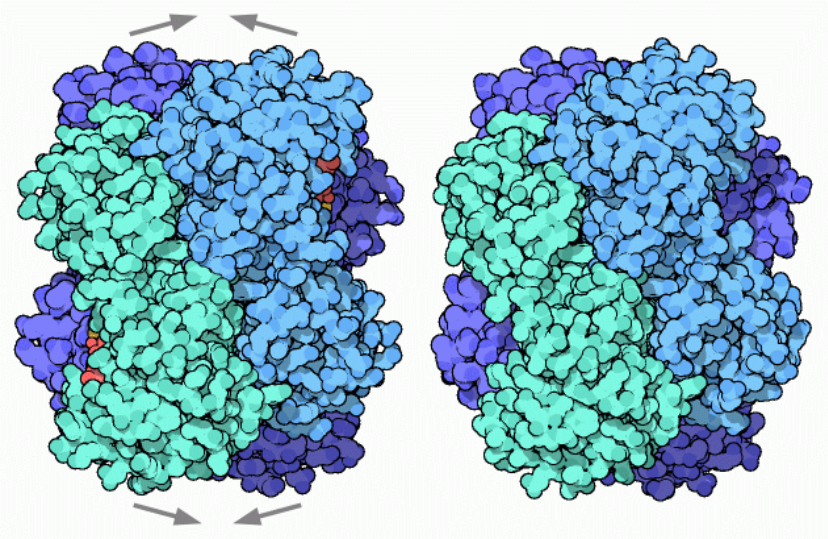


Figure 9: **The active (left) and inactive (right) conformations of the multimeric protein phosphfructokinase (`https://pdb101.rcsb.org/motm/50`).**

Enzyme catalysis can be described within one enzyme-kinetics formalism

All enzymes are catalysts and are therefore recycled after use, to be reused for the same purpose again. This means that their mechanism – the order of enzyme-state transitions associated with a single catalytic event – is always cyclic. Enzymes therefore start and end at the same starting state, regardless of their function. Also since all enzyme-state transitions can be described by mass-action kinetics, all mathematical models of enzyme cycles are based on the same kinetics, thermodynamics and conservation principles. This will enable us to derive one generally-applicable rate equation for enzymes.

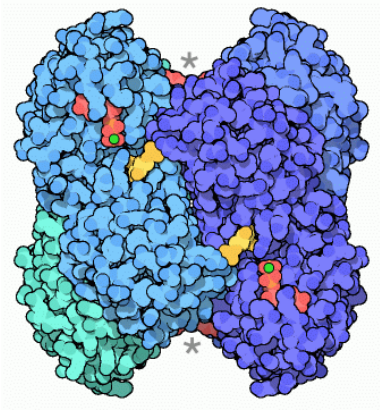


Figure 10: **Phosphofructokinase bound to its reactants (fructose 6-phosphate in orange, ADP in red and the cofactor mangesium in green).** From: `https://pdb101.rcsb.org/motm/50`. Note that the those different chemical compounds do not bind to the same sites on the protein complex and therefore can only influence each other their effects on the enzyme via changes in the structure of the protein complex.

Mass-action-kinetics models of the catalytic cycle of enzymes

Mass-action kinetics

Before we start with deriving models of the catalysis rate and regulation of enzymes, we need to briefly revisit mass-action kinetics to describe the rate of chemical reactions in terms of rate constants and the concentrations of reactants.

The rate of uncatalysed reaction is universally described by mass-action kinetics. Here are some examples:

I assume that you have seen mass-action kinetics before.
We use the following convention for names and concentration of chemical compound. We write their name in capitol font, e.g. X, and their concentration in normal font, i.e. x.

1. $S \xrightarrow{1} P$, $v_1 = k_1 \cdot s$, for reaction 1 with rate v_1 in $\frac{\text{concentration}}{\text{time}}$, the (elementary) rate constant k in time^{-1} and s as the concentration of (substrate) S.

2. $S \overset{1}{\rightleftharpoons} P$, $v_1 = k_1^+ \cdot s - k_1^- \cdot p$, for reaction 1 with rate v_1 in $\frac{\text{concentration}}{\text{time}}$, the forward (elementary) rate constant k_1^+ in time^{-1} and the backward (elementary) rate constant k_1^- in time^{-1} and s as the concentration of (substrate) S and p of the product P. The forward rate equals $v_1^+ = k_1^+ \cdot s$ and the backward rate $v_1^- = k_1^- \cdot p$. Thus, $v = v^+ - v^-$.

3. $S_1 + S_2 \overset{1}{\rightleftharpoons} P$, $v_1 = k_1^+ \cdot s_1 \cdot s_2 - k_1^- \cdot p$, for reaction 1 with rate v_1 in $\frac{\text{concentration}}{\text{time}}$, the forward (elementary) rate constant k_1^+ in $\frac{1}{\text{conc} \times \text{time}}$ and the backward (elementary) rate constant k_1^- in time^{-1}. The forward rate equals $v_1^+ = k_1^+ \cdot s_1 \cdot s_2$ and the backward rate $v_1^- = k_1^- \cdot p$. Thus, $v = v^+ - v^-$.

4. $2S \overset{1}{\rightleftharpoons} SS$, $v_1 = k_1^+ \cdot s^2 - k_1^- \cdot ss$, for reaction 1 with rate v_1 in $\frac{\text{concentration}}{\text{time}}$, the forward (elementary) rate constant k_1^+ in $\frac{1}{\text{conc} \times \text{time}}$ and the backward (elementary) rate constant k_1^- in time^{-1}. The forward rate equals $v_1^+ = k_1^+ \cdot s^2$ and the backward rate $v_1^- = k_1^- \cdot ss$. Thus, $v = v^+ - v^-$.

From these examples we can conclude that for a chemical reaction such as,

$$m_1S_1 + m_2S_2 + ... + m_kS_k \rightleftharpoons n_1P_1 + n_2P_2 + ... + n_lP_l,$$

the rate is decribed in terms of mass-action kinetics as,

$$v = k^+ \prod_{i=1}^{k} s_i^{m_i} - k^- \prod_{j=1}^{l} p_j^{n_j}.$$

Note that chemical reactions rarely have more than 2 substrates and 2 products, since the spontaneous collision of three molecules and their conversion into products is extremely unlikely.

Exercise

1. Chemical reaction exercises

   a. Why is this reaction $s_1 + s_2 + s_3 \rightarrow p$ effectively impossible and is this process better described by a set of reactions such as this?:

$$\begin{aligned} s_1 + s_2 &\rightarrow s_1s_2 \\ s_2 + s_3 &\rightarrow s_2s_3 \\ s_1 + s_3 &\rightarrow s_1s_3 \\ s_1s_2 + s_3 &\rightarrow p \\ s_1 + s_2s_3 &\rightarrow p \\ s_2 + s_1s_3 &\rightarrow p \end{aligned}$$

   b. Say you investigate the dimerisation process $2S \overset{1}{\rightleftharpoons} S_2$ in a test tube and you start with 1 mM of S. Then which relation between s and s_2 exists that is at each time obeyed? Show that this relationship implies that any concentration change Δs equals $2\Delta s_2$. Say, $k_1^+ = 10\ s^{-1}mM^{-1}$ and $k_1^- = 100\ s^{-1}$, what are the concentrations of S and the dimer S_2 at thermodynamic equilibrium when $v = 0$?

Chemical reactions occur in the direction of a loss of free energy‡

Reactions such as

$$\sum_i m_iS_i \rightleftharpoons \sum_j n_jP_j,$$

e.g. $A + B \rightleftharpoons P + Q$ proceed either in the forward direction, making P and Q from A and B, or backwards. In the first case, the forward rate v^+ of the reaction exceeds the backward rate v^-, i.e.

$$v = v^+ - v^- > 0 \quad \Rightarrow \quad v^+ > v^-.$$

What is the fundamental principle that dictates the direction of the reaction?

What follows is completely general and applies to mass-action and enzyme kinetics and works for any number of reactants.

The rate of the reaction shown above, and its forward and backward component, equal,

$$v = k^+ \cdot a \cdot b - k^- \cdot p \cdot, \;\; v^+ = k^+ \cdot a \cdot b, \;\; v^- = k^- \cdot p \cdot q$$

and depend on the concentrations of reactants, which are dependent on time. So, we can write,

$$v = v^+ \left(1 - \frac{v^-}{v^+}\right) = v^+ \left(1 - \frac{k^- \cdot p \cdot q}{k^+ \cdot a \cdot b}\right),$$

In the case of an enzyme-catalysed reaction we would also obtain

$$v = v^+ \left(1 - \frac{v^-}{v^+}\right)$$

with $v^+ = V_M^+ k_{cat}^+ f$ with f as a positive function of reactant and effector concentrations.

and conclude that

$$\begin{aligned} v &> 0 \;\Rightarrow\; \frac{p \cdot q}{a \cdot b} < \frac{k^+}{k^-}, \\ v &= 0 \;\Rightarrow\; \frac{p_e \cdot q_e}{a_e \cdot b_e} = \frac{k^+}{k^-}, \quad \text{(Thermodynamic equilibrium)} \\ v &< 0 \;\Rightarrow\; \frac{p \cdot q}{a \cdot b} > \frac{k^+}{k^-}, \end{aligned}$$

Thus when the concentration ratio p/s is less than k^+/k^- the rate is positive and otherwise it is negative. When $p/s = k^+/k^-$ the rate is zero. This state we call thermodynamic equilibrium. Then we denote the concentration of the reactants with a subscript 'e'. We also define the equilibrium constant K_{eq} of the reaction as,

$$K_{eq} = \frac{p_e q_e}{a_e b_e} = \frac{k^+}{k^-}, \; v = v^+ \left(1 - \frac{v^-}{v^+}\right) = v^+ \left(1 - \frac{p \cdot q}{a \cdot b \cdot K_{eq}}\right),$$

A central concept in thermodynamic is the formation Gibbs free energy μ of a chemical compound, defined under particular conditions (e.g. 1 M concentrations, particular T, particular osmotic strength, etc.). We start from the concept of Gibbs free energy of formation, and ask the question how much energy did it cost, or was obtained, when the compound was made from a set of starting compounds? Say, this cost or gain equals $\mu_S^{0'}$ for compound S and $\mu_P^{0'}$ for P, with the superscripts denoting the precise standard conditions. When we have a concentration s of S and p and P then the actual formation free energies of S and P also depend on their concentrations – as this determines how much we have to make of each –,

$$\mu_S = \mu_S^{0'} + RT \ln \frac{s}{s_0}, \;\; \mu_P = \mu_P^{0'} + RT \ln \frac{p}{p_0}$$

with s_0 and p_0 as the concentration of S and P under standard conditions, say 1 M; so, $s_0 = 1$ and $p_0 = 1$ and they drop out of the equation (we omit them therefore from now on).

Note that you cannot take a logarithm of a variable with a unit, e.g. since $dx/x = \ln x$ and since dx/x is dimensionless so is $\ln x$, hence what is meant always is that $\ln x = \ln x/x_0$ with x_0 as some reference value set to 1.

Now we can define the free energy difference, change or potential associated with the reaction $A + B \rightleftharpoons P + Q$ in such a way that it indicates the Gibbs free energy liberated, or consumed. This we do by subtracting the formation energy of the substrate from that of the product,

$$\begin{aligned} \Delta\mu_r &= \mu_P + \mu_Q - (\mu_A + \mu_B) \\ &= \underbrace{\mu_P^{0'} + \mu_Q^{0'} - (\mu_A^{0'} + \mu_B^{0'})}_{\Delta\mu_r^{0'}} + RT \ln \frac{p \cdot q}{a \cdot b} \end{aligned} \tag{1}$$

This difference $\Delta\mu_r$ indicates the difference in the free energy when you synthesise P and Q or A and B from reference compounds under standard conditions. It is also the free energy that is liberated ($\Delta\mu_r < 0$) or needed ($\Delta\mu_r > 0$) for the conversion of A and B into P and Q.

Now we define that in thermodynamic equilibrium:

$$\Delta\mu_r = 0 \;\Rightarrow\; -\Delta\mu_r^{0'} = RT \ln \frac{p_e \cdot q_e}{a_e \cdot b_e} = RT \ln K_{eq} \;\Rightarrow\; K_{eq} = e^{-\frac{\Delta\mu_r^{0'}}{RT}},$$

which implies that (valid for enzyme-catalysed and uncatalysed chemical reactions),

$$\Delta\mu_r = \Delta\mu_r^{0'} + RT \ln \frac{p \cdot q}{a \cdot b} = -RT \ln K_{eq} + RT \ln \frac{p \cdot q}{a \cdot b} = RT \ln \frac{p \cdot q}{a \cdot b \cdot K_{eq}},$$

and therefore, using a result from above,

$$\frac{v^-}{v^+} = \frac{p \cdot q}{a \cdot b \cdot K_{eq}} = e^{\frac{\Delta\mu_r}{RT}},$$

such that we obtain a relation, valid for enzyme-catalysed and uncatalysed chemical reactions,

$$v = v^+ \left(1 - \frac{v^-}{v^+}\right) = v^+ \left(1 - e^{\frac{\Delta\mu_r}{RT}}\right)$$

Thus last relation shows that

$$\begin{aligned} v &> 0 \;\Rightarrow\; \Delta\mu_r < 0, \\ v &= 0 \;\Rightarrow\; \Delta\mu_r = 0, \quad \text{(Thermodynamic equilibrium)} \\ v &< 0 \;\Rightarrow\; \Delta\mu_r > 0, \end{aligned}$$

Thus a reaction rate is positive when the Gibbs free energy change of the reaction is negative and vice versa: hence,

$$-v\Delta\mu_r = -\left(v^+ - v^-\right) \ln \frac{v^-}{v^+} \geq 0$$

This is in fact a version of the second law of thermodynamics and therefore in agreement with it – as it should.

Thus: all reactions – catalysed and uncatalysed – proceed in a direction of a Gibbs free energy loss.

The second law states that chemical reactions (when considered as isolated systems) proceed in a direction of entropy S production. This means that $dS/dt \geq 0$ and $dS/dt = -v\frac{\Delta\mu_r}{T} \geq 0$.

The association and dissociation constant of complex formation reaction

When we consider the 'rapid-equilibrium binding approximation' and 'binding polynomials' below for the derivation of rate equations of enzymes we will use the concept of a 'dissociation constant' all the time. So this is an important section.

Dissociation constants are the equilibrium constants of complex formation reactions. Consider the reversible formation of the molecular complex AB from A and B,

$$A + B \rightleftharpoons AB.$$

Complex formation, or association, corresponds to

$$A + B \rightarrow AB,$$

while dissociation corresponds to,

$$AB \rightarrow A + B.$$

The net rate of association equals,

$$v = k^+ \cdot a \cdot b - k^- \cdot ab.$$

The net rate of dissociation equals,

$$-v.$$

The concentration of A, B and AB are constant with time when $v = 0$ then

$$\frac{a_e \cdot b_e}{ab_e} = \frac{k^-}{k^+} = K_D,$$

we label the concentrations at this thermodynamic equilibrium state with the subscript 'e'. The ratio of k^+ and k^- is an example of an equilibrium constant, in this case a dissociation constant K_D. (The association constant K_A equals $K_A = 1/K_D$.)

The unit of the dissociation constant is concentration, which agrees with the units of the rate constants in the above expression. We will need this dissociation constant later.

Rates of change in concentrations due to the occurrences of reactions

Consider the concentration of molecule X defined as

$$x = \frac{n_X}{V}$$

with n_X as the number of molecules X and V as the volume in which X resides. Let's now consider that the number of molecules of X is dependent in time, due to the occurrence of reactions, and the volume remains constant, so

$$x(t) = \frac{n_X(t)}{V}.$$

The change in the concentration of X at time t is then given by the rate of change

$$\frac{dx}{dt} = \frac{1}{V}\frac{dn_X}{dt}.$$

Note the units of dx/dt are $\frac{\text{concentration}}{\text{time}}$

This concentration change is either due to:

1. the in- or export of molecules X from the volume V,
2. the synthesis, degradation or conversion of X molecules.

When we denote the rate of such processes by v_j and express them in units $\frac{\text{number of reaction events}}{\text{volume}\times\text{time}}$ then it logically follows that

Note that $\frac{\text{number of reaction events}}{\text{volume}\times\text{time}} = \frac{\text{concentration events}}{\text{time}}$

$$\frac{dx_i}{dt} = \sum_{j=1}^{R} n_{ij} v_j,$$

with n_{ij} as the stoichiometric coefficient of X_i in reaction j, in units $\frac{\text{concentration}}{\text{concentration events}}$. Thus the stoichiometric coefficient gives the number of molecules X produced (when $n_{ij} > 0$) or consumed (when $n_{ij} < 0$) per single event of reaction j.

Exercises

1. Consider the following reaction network (underlined reactant denote those with fixed concentrations)

$$\begin{aligned} \underline{A} + 2X &\xrightarrow{1} 3X \\ X &\to 2\ \underline{B} \end{aligned} \tag{2}$$

   and the rate equations

$$\begin{aligned} v_1 &= k_1^+ a x^2 - k_1^- x^3 \\ v_2 &= k_2^+ x - k_2^- b \end{aligned}$$

   and parameters: $k_1^+ = 10,\ k_1^- = 1,\ k_2^+ = 10,\ k_2^- = 1,\ a = 1,\ b = 1.$

   a. Give the differential equation for the variable concentrations.
   b. Plot v_1 and v_2 and dx/dt as function x.
   c. Show that different initial conditions the concentration of x (at time 0) can lead to different eventual constant concentrations of x.

d. Show that when $dx/dt = 0$ that $v_1 = v_2 \neq 0$. This is called a steady state. When $v_1 = v_2 = 0$ then we call this state an equilibrium, we will come back to this later.

e. Consider the concentrations of A and B not as constant any more. Calculate the concentrations of A, X and B at thermodynamic equilibrium when their starting concentrations are each 1 mM.

2. Consider the following model of a transcription factor T binding to a promoter P with two binding sites for T.

$$\begin{aligned} T + P &\overset{1}{\rightleftharpoons} TP \\ T + P &\overset{2}{\rightleftharpoons} PT \\ T + TP &\overset{3}{\rightleftharpoons} TPT \\ T + PT &\overset{4}{\rightleftharpoons} TPT \end{aligned}$$

a. Make a drawing of the reaction network.

b. Assume that this experiment is done with a constant promoter (or DNA) concentration p_T. What is the conservation relationship that bounds the concentrations of all the promoter-state concentrations? The promoter states are P, TP, PT and TPT.

c. Determine the four dissociation constants in terms of their associated concentrations and rate constants.

d. Use the promoter conservation relationship and the four dissociation constant relation to express the concentration of the free promoter state in terms of the total promoter concentration, the dissociation constants, and the concentration of T.

e. Determine the concentration fractions of all the promoter states.

f. Note: in your equations, you will see the appearance of the term

$$1 + \frac{t}{K_1} + \frac{t}{K_2} + \frac{t^2}{K_1 K_3}, \text{ or } 1 + \frac{t}{K_1} + \frac{t}{K_2} + \frac{t^2}{K_2 K_4}$$

such equations return later and are called 'binding polynomials'. They play a central role in rapid-equilibrium kinetics.

g. Show that the following relation holds:

$$K_1 K_2^{-1} K_3 K_4^{-1} = 1.$$

and therefore $K_1 K_3 = K_2 K_4$!

Enzymes accelerate chemical reactions by offering favourable conditions in their catalytic site

Chemical reactions that occur uncatalysed may run very slowly in a watery environment such as the cytosol. Enzymes offer favourable conditions in their catalytic site such that those reactions occur faster. For instance, adenylate kinase accelerates its reaction by several orders of magnitude (Kerns, S., Agafonov, R., Cho, YJ. et al. The energy landscape of adenylate kinase during catalysis. Nat Struct Mol Biol 22, 124–131 (2015)). We will later shortly return to this issue.

This is a very good paper for explaining this topic, using trosephosphate enomerase as an example: `https://doi.org/10.1021/acs.biochem.1c00211`.

A mass-action kinetic model of a simple catalytic cycle

Since all enzymes are catalysts, all their catalytic mechanisms are cyclic, starting and ending at the same enzyme state – generally taken as the free, unbound enzyme state E.

An example of such a cyclic mechanism is shown in Figure 11. It is composed of three reactions,

$$\begin{aligned} E + S &\overset{1}{\rightleftharpoons} ES \\ ES &\overset{2}{\rightleftharpoons} EP \\ EP &\overset{3}{\rightleftharpoons} E + P. \end{aligned}$$

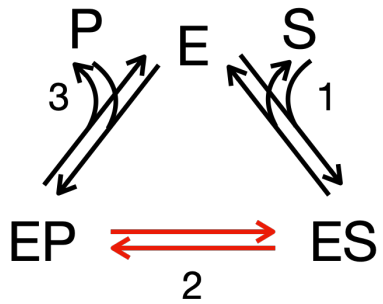


Figure 11: **The simplest cyclic mechanism of enzyme catalysis.** The catalytic event, or reaction, is shown in red. The other two reactions are either an association or dissociation event.

The first and the third reactions are associated and dissociation events, while the second is the catalytic event. These events or reactions are sometimes also called enzyme-state transitions, making figure 11 a enzyme-state transition diagram.

The rates of the three reactions are given by the following rate equations of mass-action kinetics:

$$\begin{aligned} v_1 &= k_1^+ \cdot e \cdot s - k_1^- \cdot es \\ v_2 &= k_2^+ \cdot es - k_2^- \cdot ep \\ v_3 &= k_3^+ \cdot ep - k_3^- \cdot e \cdot p. \end{aligned}$$

The rates of change in the concentration are given by the following

differential equations,

$$\begin{aligned}
\frac{ds}{dt} &= -v_1 \\
\frac{de}{dt} &= -v_1 + v_3 \\
\frac{des}{dt} &= v_1 - v_2 \\
\frac{dep}{dt} &= v_2 - v_3 \\
\frac{dp}{dt} &= v_3.
\end{aligned}$$

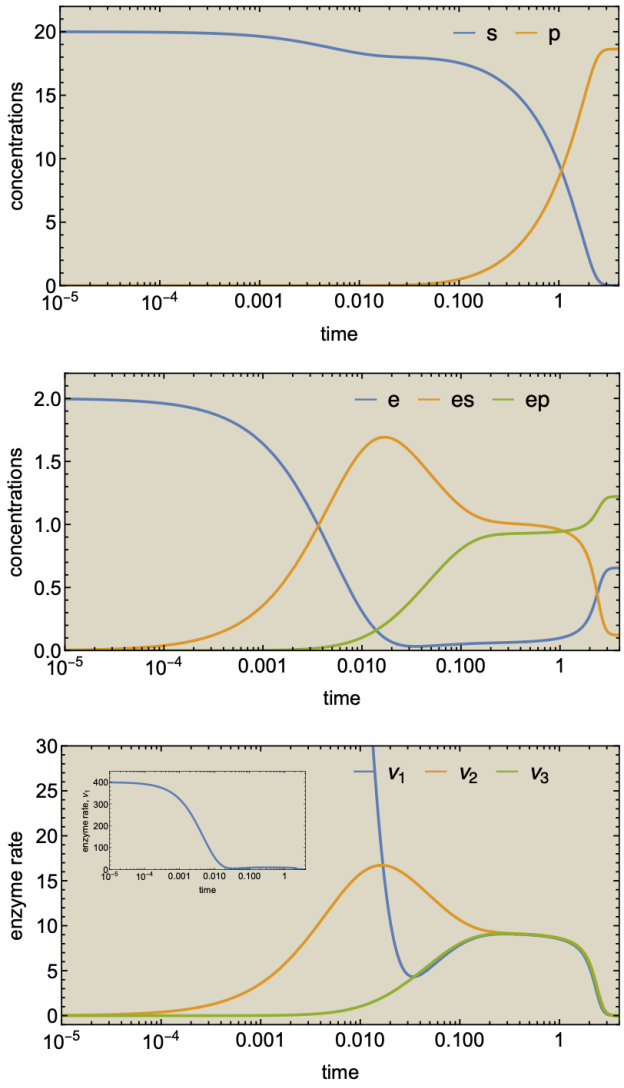


Figure 12: **The dynamics of the simplest cyclic mechanism of enzyme catalysis shown in fig. 11.** All the forward rate constants were chosen equal to 10 and the backward rate to 1, the starting concentrations were $s(0) = 20$, $e(0) = 2$ and all others 0. The time axis is logarithmic to make sure all the dynamics is visible. I advice you to reproduce these plots.

When we consider this system as a function of time then eventually all concentrations will become constant (Fig. 12)because all the

rates have become equal to zero (we will consider this explicitly in the next section). When this is the case, the following relationship holds,

$$\frac{k_1^+ s k_2^+ k_3^+}{k_1^- k_2^- k_3^- p} = 1.$$

This is called the detailed balance relationship, we will study it more in depth later when we relate it to its thermodynamic origins and explain it. This relationship indicates that you cannot assign values to rate constants randomly they have to agree with this relationship. In fact, when you have multiple cycles in a diagram it applies to all of them.

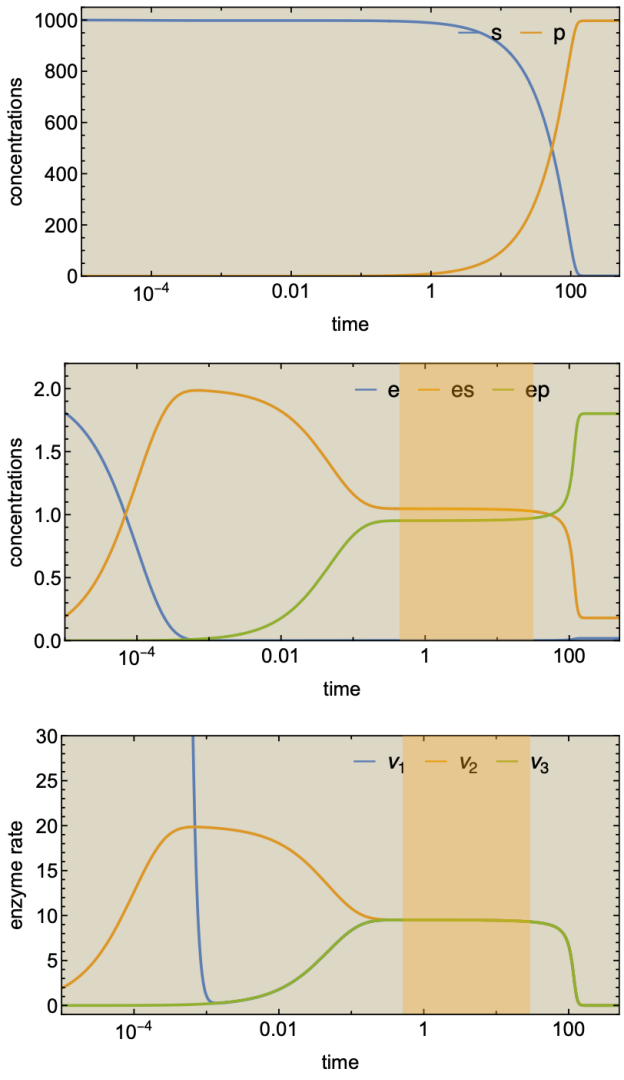


Figure 13: **The dynamics of the simplest cyclic mechanism of enzyme catalysis shown in fig. 11 and the illustration of the quasi-steady state regime.** The same simulation as in Fig. 12, only now the starting concentration of S was set to 1000. The quasi-state regime is shown in yellow, during this time period the rates of the enzyme are constant, because the concentrations of the enzyme-reactant species have attained a *quasi*-steady state. I advice you to reproduce these plots.

Exercise

1. Consider the catalytic mechanism of the previous section.
   (a) Why are all the rates zero when the concentrations have all become constant?
   (b) Derive the detailed balance relationship.
   (c) (Warning: complicated and derived step-by-step later) Consider the case that s and p are considered constant. Then the concentrations of the enzyme states can become constant when $v_1 = v_2 = v_3 \neq 0$. The free energy of the first reaction is now given by $\Delta\mu_1 = RT \ln \frac{es}{e \cdot s \cdot K_1}$, that of the second reaction by $\Delta\mu_2 = RT \ln \frac{ep}{es \cdot K_2}$, and of the third reaction by $\Delta\mu_2 = RT \ln \frac{e \cdot p}{es \cdot K_3}$. Show that when you sum those free energies, you obtain the free energy $\Delta\mu$ of the net reaction $S \rightleftharpoons P$, that now the following relationship holds,

$$\frac{k_1^+ s k_2^+ k_3^+}{k_1^- k_2^- k_3^- p} = e^{\frac{-\Delta\mu}{RT}}$$

   and that the equilibrium constant of the reaction $S \rightleftharpoons P$ has to equal $K_1 K_2 K_3$. This indicates that indeed the detailed balance relationship holds in thermodynamic equilibrium when $\Delta\mu = 0$ and $v_1 = v_2 = v_3 = 0$.

2. The sarcoendoplasmic reticulum contains an ATP-dependent calcium pump (SERCA) that exploits the free energy liberated from ATP hydrolysis for calcium import. In figure 14, the catalytic mechanism of SERCA is shown together with the rate constants of the enzyme-state transitions. Make a mathematical model of this enzyme and investigate how the steady-state calcium-import flux depends on the free energy potential of ATP hydrolysis, given that total adenosine equals 5 mM and phosphate equals 1 mM.

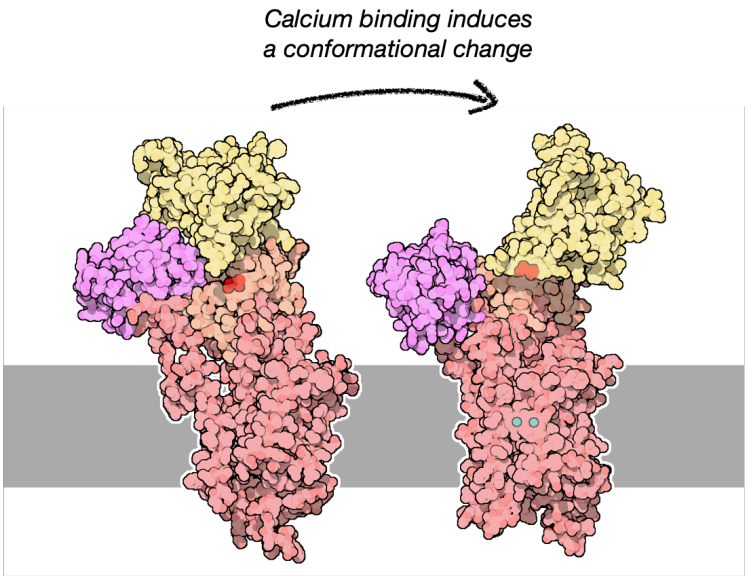
The free energy of formation μ_X of a chemical compound X at concentration x under particular conditions equals $\mu_X = \mu_X^{0'} + RT \ln x/x_0$ with $x_0 = 1$, the concentration of X under standard conditions, and $\mu_X^{0'}$ as the standard free energy of formation of X. The free energy difference of a reaction $X \rightleftharpoons Y$ equals $\Delta\mu = \mu_Y - \mu_X = \mu_Y^{0'} - \mu_X^{0'} + RT \ln \frac{y}{x}$. At thermodynamic equilibrium: $\frac{y_e}{x_e} = K_{eq}$ and $\Delta\mu = 0$ such that $\mu_Y^{0'} - \mu_X^{0'} = -RT \ln K_{eq}$. Thus: $\Delta\mu = RT \ln \frac{y}{xK_{eq}}$. I fear that some of you have forgotten this, if so, then do not attempt question c. on the left. It will be explained once more later.

Quasi-steady state regimes of catalytic cycles of enzymes

Clearly inside the cell enzymes are operating at a rate that is unequal to zero, since life is associated with a net flux of material through the cell's metabolic network. During cell growth at a fixed cellular growth, metabolism operates at steady state and the rate of metabolism are all constant.

This must mean that the catalytic event in the enzyme mechanism shown in figure 11 operates at a rate that is unequal to zero. When we consider the net synthesis of P from S then

$$v_2 = k_2^+ \cdot es - k_2^- \cdot ep > 0.$$



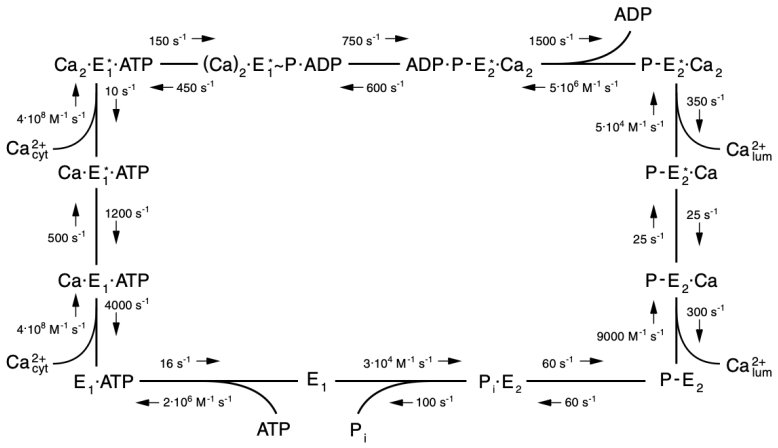


Figure 14: **The ATP-driven calcium pump (SERCA) and its catalytic cycle.** The sarcoendoplasmic reticulum contains a pump that imports calcium from the cytosol into the reticulum's lumen at the expense of the hydrolysis of ATP. Two calcium atoms are imported per ATP. The binding of calcium causes a conformational change of the protein that is associated with calcium import. The upper figure comes form `https://pdb101.rcsb.org/motm/51`, where you can also find more information about the pump. The lower figure comes from the book by Christopher P. Fall, Eric S. Marland, John M. Wagner, & John J. Tyson (Computational Cell Biology, Springer New York, NY, 2002) who discuss such models. The conformational change is indicated by the "*" in the enzyme's mechanism.

This rate can only be constant when the concentration of the enzyme states ES and EP are constant. This implies that $v_1 = v_2 = v_3$. This can either occur at constant concentrations of S and P, so during a metabolic steady state, but also when the concentrations of S and P are not constant, as long as they are in great excess relative to the total amount of enzyme. This is illustrated in Figure 13. This shows that steady-state enzyme kinetics can still apply when the concentrations of the reactants are time varying. This is, of course, a requirement when we want to exploit steady-state kinetics, e.g. the Briggs Haldane equation mentioned in the prologue, in dynamic models of metabolism when reactant concentrations are varying with time.

Thus, our next task is to figure how we derive the rate equation of enzyme under quasi-steady state conditions, when the enzyme states are constant in time while the reactants are time varying. We can even make a more stringent assumption on the speed of enzyme-state transitions associated with non-catalytic events and assume that those reactions are operating close enough to thermodynamic equilibrium to be able to assume that their rates are zero. This is of course a severe assumption and how good, or how bad, it is is shown in Figure 15.

The rate equation for the enzyme catalysed reaction $E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$, derived first by Briggs & Haldane: $v = \frac{V_M^+ \frac{s}{K_S} - V_M^- \frac{p}{K_P}}{1 + \frac{s}{K_S} + \frac{p}{K_P}}$.

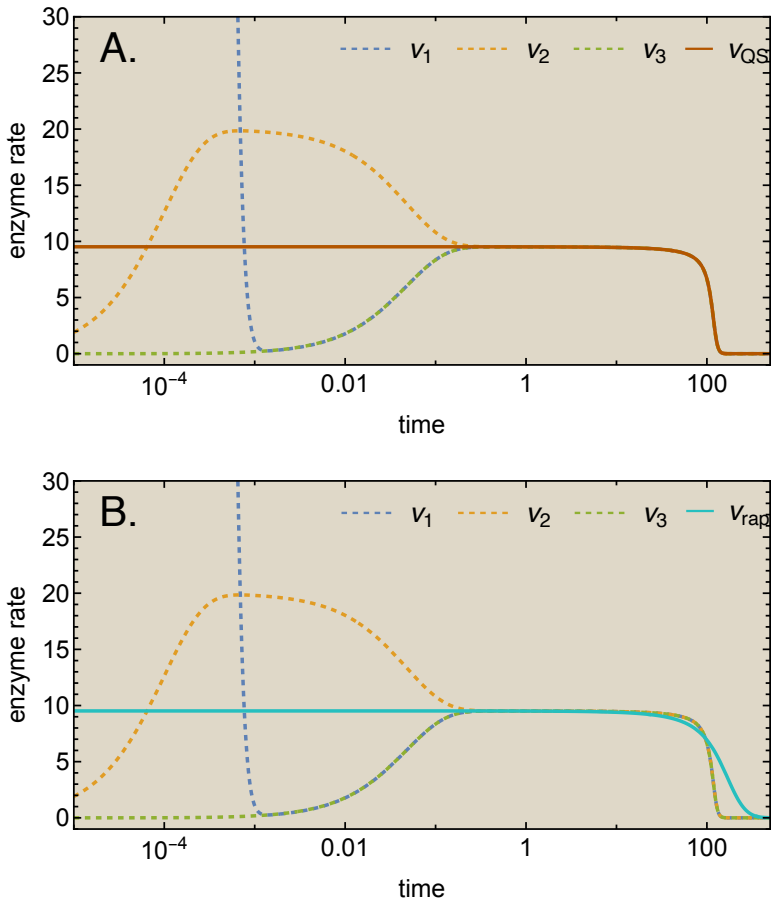


Figure 15: **The dynamics of the simplest cyclic mechanism of enzyme catalysis shown in fig. 11 and the illustration of the quasi-steady state regime.** The same simulation as in Fig. 13. Now the quasi-steady state approximation (A) and rapid-equilibrium approximation (B) of the enzyme rate – valid only in the quasi-steady state region – are shown and compared to the exact simulation (dashed lines).

The rapid-equilibrium approximation for enzyme kinetics

Rapid equilibrium approximation is fast and sufficient for most applications

The problem with the steady-state method for derivation of rate equations of enzymes is that it is a lot of work to derive and leads to equations with a lot of parameters, many of which cannot be reliably determined from experiments. We treat this method in a later chapter. The mathematical form of those equations is, however, very similar to that of rate equations derived using a more harsh approximation, the so called rapid-equilibrium approximation. This method is really simple to use as it can be done by hand in a series of simple steps. For applications such as mathematical modelling of metabolic networks, the rapid-equilibrium approximation gives rise to rate equations that are realistic enough and in agreement with thermodynamics (but may not always agree with results from enzyme kinetics assays in cell-free extract, for that the steady-state method is better).

The binding polynomials of an enzyme its catalytic sites and allosteric sites

A central concept in the rapid-equilibrium approximation is the binding polynomial of a binding site on a (macro)molecule such as DNA or a protein. You already encountered one in the exercise on the transcription factor binding to a promoter of a gene. For enzymes, the key binding sites are the catalytic site – where reactants and effectors bind – and the allosteric site(-s) – where effectors bind.

We will introduce the binding polynomial for the catalytic site of a simple enzyme, catalysing the following reaction

$$E + S \overset{1}{\rightleftharpoons} ES \overset{2}{\rightleftharpoons} EP \overset{3}{\rightleftharpoons} E + P.$$

Thus, three enzyme states exist: E, ES and EP. The total concen-

tration of the catalytic site, equals the concentration of the enzyme (because only one such site occurs per enzyme). The concentrations of the enzyme states of the catalytic site therefore sum to the total enzyme concentration:

$$e_T = e + es + ep.$$

Part of the rapid-equilibrium assumption is to assume that the dissociation and association reactions in the catalytic cycle, i.e. in this case reaction 1 and 3, are in equilibrium while the catalytic reaction (number 2) is not. We can therefore relate the concentration of the enzyme-reactant complexes to dissociation constants, i.e.

$$es = \frac{e \cdot s}{K_S},\ ep = \frac{e \cdot p}{K_P},$$

and substitute those relations into the total enzyme conservation relation and derive the binding polynomial of the enzyme, which we denote by $\mathcal{B}$,

$$e_T = e + \frac{e \cdot s}{K_S} + \frac{e \cdot p}{K_P},\ \Rightarrow\ \mathcal{B} = \frac{e_T}{e} = \underbrace{1 + \frac{s}{K_S} + \frac{p}{K_P}}_{\text{binding polynomial}}.$$

If this enzyme would also have an allosteric site that can bind the inhibitor I then the following reactions would also exist, in addition to those above,

$$E + I \overset{4}{\rightleftharpoons} IE,\ ES + I \overset{5}{\rightleftharpoons} IES,\ EP + I \overset{6}{\rightleftharpoons} IEP.$$

Now, the enzyme conservation relation become,

$$e_T = e + ie + es + ies + ep + iep,$$

and we have the following additional relations,

$$ie = \frac{e \cdot i}{K_I},\ ies = \frac{es \cdot i}{K_I},\ iep = \frac{ep \cdot i}{K_I}.$$

(Note that we assumed in the previous equations that the affinity of the enzyme for I does not depend on the binding state of its catalytic site, i.e. whether it is empty or bound to S or P.) In this case, the binding polynomial becomes, first we substitute the dissociation relationships

$$e_T = e + \frac{e \cdot i}{K_I} + \frac{e \cdot s}{K_S} + \frac{es \cdot i}{K_I} + \frac{e \cdot p}{K_P} + \frac{ep \cdot i}{K_I}$$

and then we determine the binding polynomial of the enzyme

$$\mathcal{B} = \frac{e_T}{e} = 1 + \frac{i}{K_I} + \frac{s}{K_S} + \frac{i \cdot s}{K_I K_S} + \frac{p}{K_P} + \frac{p \cdot i}{K_I K_P} = \underbrace{\underbrace{\left(1 + \frac{i}{K_I}\right)}_{\substack{\text{binding polynomial} \\ \text{of the allosteric site, } \mathcal{A}}} \underbrace{\left(1 + \frac{s}{K_S} + \frac{p}{K_P}\right)}_{\substack{\text{binding polynomial} \\ \text{of the catalytic site, } \mathcal{C}}}}_{\text{binding polynomial of the enzyme, } \mathcal{B}}.$$

The term $1 + \frac{i}{K_I}$ is the binding polynomial $\mathcal{A}$ of the allosteric site! Thus, an enzyme with several binding sites that act independently of each other – so the affinity of one site for molecules does not depend on the binding state of any of the other sites – has a binding polynomial $\mathcal{B}$ that is a product of the binding polynomial of its sites, i.e. in this case $\mathcal{B} = \mathcal{C} \times \mathcal{A}$.

Consider, as another example, the enzyme mechanism shown in figure 16. The enzyme catalyses the following reaction $A + B \rightleftharpoons P + Q$. Its total enzyme conservation relation equals the sum of all the concentrations of the enzyme states,

$$e_T = e + ae + pe + eb + eq + aeb + peb + aeq + peq$$

and its binding polynomial equals (Note that we skipped a few steps here, do those on paper yourself!)

$$\begin{aligned} \mathcal{B} &= 1 + \frac{a}{K_A} + \frac{p}{K_P} + \frac{b}{K_B} + \frac{q}{K_Q} + \frac{a \cdot b}{K_A K_B} + \frac{p \cdot b}{K_P K_B} + \frac{a \cdot q}{K_A K_Q} + \frac{p \cdot q}{K_P K_Q} \\ &= \underbrace{\underbrace{\left(1 + \frac{a}{K_A} + \frac{p}{K_P}\right)}_{\substack{\text{binding polynomial of site} \\ \text{1 in the catalytic site, } \mathcal{C}_1}} \underbrace{\left(1 + \frac{b}{K_B} + \frac{q}{K_Q}\right)}_{\substack{\text{binding polynomial of site} \\ \text{2 in the catalytic site } \mathcal{C}_2}}}_{\substack{\text{binding polynomial of the catalytic site of the enzyme, } \mathcal{B}}} . \end{aligned} \tag{3}$$

From this last equation we conclude that the catalytic site of the enzyme consists of two independent binding sites: site 1 for A or P and site 2 for B or Q, since the binding polynomial of the enzyme factorizes. We also conclude that A and P compete for binding 1 and B and Q compete for binding site 2, because they do not occur together in any of the enzyme states.

Now consider figure 17, where we consider an enzyme that again converts $A + B \rightleftharpoons P + Q$ but using a mechanism that is different from figure 16. Now substrates and products cannot bind simultaneously. For instance, because the binding site of A covers the binding site of P and Q and the binding site of P covers the binding sites of A and B. This means that we cannot think of the catalytic site of the enzyme as consisting of two independent binding sites anymore. Therefore, the binding polynomial of the catalytic site of the enzyme does not factorise into a product of binding polynomials,

$$\mathcal{B} = \underbrace{1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{a \cdot b}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{p \cdot q}{K_P K_Q}}_{\substack{\text{binding polynomial of the catalytic site} \\ \text{of the enzyme (does not factorize)}}} .$$

Finally, consider the enzyme mechanism for $A + B \rightleftharpoons P + Q$ shown in figure 18. In this case, substrates and products compete for

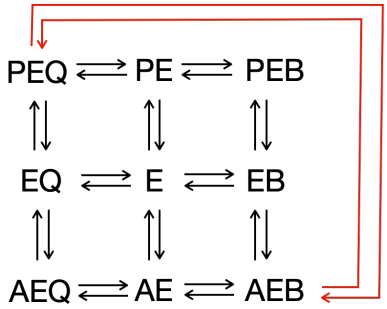


Figure 16: **An enzyme mechanism for** $A + B \rightleftharpoons P + Q$**.** The catalytic site can bind A, B, P and Q, but A occupies the same location in the catalytic site as P so they cannot simultaneously bind and B occupies the same location in the catalytic site as Q so they cannot simultaneously bind too. You can conclude this because enzymes state with both A and P or both B and Q bound do not occur. Thus the catalytic consists of two binding sites: one for A and P and another for B and Q. All the association and dissociation reactions are shown in black and the catalytic reaction in red.

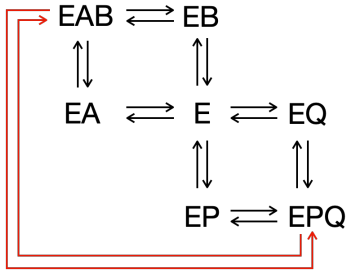


Figure 17: **An alternative enzyme mechanism for** $A + B \rightleftharpoons P + Q$**.** This mechanism can either bind substrates or products and not both. For instance, because the binding of A covers the binding of P and Q and the binding of P covers the binding sites of A and B. All the association and dissociation reactions are shown in black and the catalytic reaction in red. See Cleland, What Limits the Rate of an Enzyme-Catalyzed Reaction? (Accounts of chemical research, 8(5), 145-151, 1975) on page 150 in this paper for an explicit example of an enzyme with this mechanism and its rate constants parameters.

binding to the catalytic sites and they bind in an ordered manner: A before B and P before Q. In this case, the binding polynomial corresponds to

$$\mathcal{B} = \underbrace{1 + \frac{a}{K_A} + \frac{a \cdot b}{K_A K_B} + \frac{p}{K_P} + \frac{p \cdot q}{K_P K_Q}}_{\text{binding polynomial of the catalytic site of the enzyme (does not factorize)}}.$$

It is nearly identical to the binding polynomial of the previous example, but now two enzyme state no longer occur, i.e. EB and EQ.

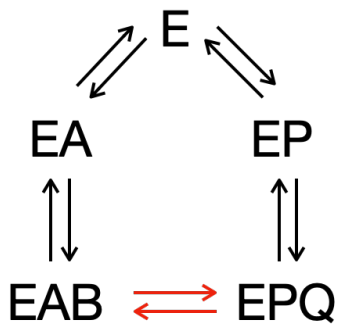


Figure 18: **An alternative enzyme mechanism for** $A + B \rightleftharpoons P + Q$**.** This mechanism can either binds substrates or products and not both. The binding of substrates and products is in an ordered sequence. All the association and dissociation reactions are shown in black and the catalytic reaction in red.

Rapid equilibrium approximation of the catalytic cycle of an enzyme

In this section, we will derive the rapid-equilibrium approximation for 4 different enzymes (Fig. 11, 16, 17, and 18), varying in the number of their reactants and catalytic mechanisms. You will see that if you do this stepwise then it is easy to derive rate equations of enzymes with this method. In fact, in the next section we will see that we can simplify this all to one step in case when we know the binding polynomial of the enzyme.

Let's start with deriving the rate equation for the reversible reaction (Fig. 11)

$$E + S \overset{1}{\rightleftharpoons} ES \overset{2}{\rightleftharpoons} EP \overset{3}{\rightleftharpoons} E + P.$$

The rapid equilibrium approximation method for the derivation of a rate equation of an enzyme can be broken down into the following steps,

1. write down the conservation equation of the total enzyme concentration, i.e.

$$e_T = e + es + ep$$

2. write down the rate of enzyme which equals the rate of the rate-limiting step in the mechanism, which is always assumed to be the catalytic reaction, which is reaction 2 in this case – all the other reactions are either association or dissociation reactions,

$$v = v_2 = k_2^+ es - k_2^- ep.$$

   Thus, we need to express the concentration of ES and EP into the kinetic parameters of the enzyme-state transitions in the mechanism to determine the rate equation of the enzyme.

3. assume that all the association and dissociation reaction are at thermodynamic equilibrium and express all the concentration of the enzyme-reactant complexes, occurring in the total enzyme

conservation equation, in terms of dissociation constants and concentrations of the free enzyme state E and the concentrations of the reactants, i.e.

$$es = \frac{e \cdot s}{K_S},\ ep = \frac{e \cdot p}{K_P}.$$

4. substitute the previous relation into the conservation equation of the total enzyme and solve for the free enzyme concentration (note that the binding polynomial appears),

Note that the binding polynomial $\mathcal{B} = e_T/e$ equals $1 + \frac{s}{K_S} + \frac{p}{K_P}$!

$$e_T = e + es + ep = e + \frac{e \cdot s}{K_S} + \frac{e \cdot p}{K_P} \ \Rightarrow\ e = \frac{e_T}{1 + \frac{s}{K_S} + \frac{p}{K_P}}$$

5. determine the concentration of the enzyme-reactant complexes using the dissociation constant equations and the expression for the concentration of E,

$$es = \frac{e \cdot s}{K_S} = \frac{e_T \frac{s}{K_S}}{1 + \frac{s}{K_S} + \frac{p}{K_P}},\ ep = \frac{e \cdot p}{K_P} = \frac{e_T \frac{p}{K_p}}{1 + \frac{s}{K_S} + \frac{p}{K_P}}$$

6. substitute the previous relations into the rate equation of the enzyme,

$$v = k_2^+ es - k_2^- ep = \frac{k_2^+ e_T \frac{s}{K_S} - k_2^- e_T \frac{p}{K_p}}{1 + \frac{s}{K_S} + \frac{p}{K_P}} \tag{4}$$

7. finally, identify the maximal forward and backward rate V_M^+ and V_M^-,

$$v = \frac{V_M^+ \frac{s}{K_S} - V_m^- \frac{p}{K_p}}{1 + \frac{s}{K_S} + \frac{p}{K_P}},\ V_M^+ = k_2^+ e_T,\ V_M^- = k_2^- e_T,$$

and now you are done.

Let's now consider a slightly more complex example (Fig. 17) by going through the same steps,

1. write down the conservation of the total enzyme concentration

$$e_T = e + ea + eb + eab + ep + eq + epq$$

2. identify the rate of the enzyme as the rate of the catalytic reaction

$$v = k_5^+ eab - k_5^- epq$$

3. assume that all the association and dissociation reactions are at equilibrium and relate the concentrations of the enzyme species and the reactants to the the dissociation constants

$$ea = \frac{e \cdot a}{K_A},\ eb = \frac{e \cdot b}{K_B},\ eab = \frac{e \cdot a \cdot b}{K_A K_B}\ ep = \frac{e \cdot p}{K_P},\ eq = \frac{e \cdot q}{K_Q},\ epq = \frac{e \cdot p \cdot q}{K_P K_Q}$$

4. solve for free enzyme concentration (note that the binding polynomial is e_T/e).

$$e = \frac{e_T}{1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{ab}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{pq}{K_P K_Q}}$$

Note that the binding polynomial equals $\mathcal{B} = 1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{ab}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{pq}{K_P K_Q}$!

5. determine the reactant concentration of the catalytic reaction

$$eab = \frac{e \cdot a \cdot b}{K_A K_B} = \frac{e_T \frac{a \cdot b}{K_A K_B}}{1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{ab}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{pq}{K_P K_Q}}$$

$$epq = \frac{e \cdot p \cdot q}{K_P K_Q} = \frac{e_T \frac{p \cdot q}{K_P K_Q}}{1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{ab}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{pq}{K_P K_Q}}$$

6. substitute the previous results into the mass-action rate equation of the catalytic reaction

$$v = \frac{k_5^+ e_T \frac{a \cdot b}{K_A K_B} - k_5^- e_T \frac{p \cdot q}{K_P K_Q}}{1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{ab}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{pq}{K_P K_Q}}$$

now you are done. Now compare your last equation to equation 4, only the denominator which equals the binding polynomial of the enzyme has changed! It therefore plays a central role in the rapid-equilibrium approximation.

Exercise

1. Consider the enzyme mechanism shown in figure 18
   a. Determine the rate equation.
   b. Determine the binding polynomial $\mathcal{B}$.
   c. Conclude that the enzyme mechanisms shown in figures 16, 17, and 18 all agree with

$$v = \frac{V_M^+ \frac{a \cdot b}{K_A K_B} - V_M^- \frac{p \cdot q}{K_P K_Q}}{\mathcal{B}}$$

   and have different binding polynomials.

2. Consider the enzyme triose phosphate isomerase and its k_{cat} and K_M values for its reactants as reported in `https://doi.org/10.1111/j.1432-1033.1987.tb13388.x` (Lambeir, et al. Eur J Biochem 168, 69, 1987).
   a. Decide on a possible binding polynomial and rate equation of this enzyme.

b. Calculate the equilibrium constant, using the Haldane relationship, for the four different species. What do you conclude?

c. Plot the rate of the enzyme as function of the substrate concentration, at different values of the product concentration for the four different species. What does this tell you about the possible concentration ranges of the reactants in cells of those four species?

Quick derivation of rapid-equilibrium enzyme kinetics using the binding polynomials of its catalytic and allosteric sites

The rate equation of a monomeric enzyme, according to the rapid-equilibrium method, is always equal to:

$$v = \frac{V_M^+ \prod_i \frac{s_i}{K_{S_i}} - V_M^- \prod_j \frac{p_j}{K_{P_j}}}{\mathcal{B}}. \tag{5}$$

The binding polynomial of the enzyme can be a product of the binding polynomials of its independent binding occurring in its catalytic site or as allosteric sites (Fig. 19),

$$\mathcal{B} = \prod_k \mathcal{C}_k \times \prod_l \mathcal{A}_l$$

So, you only need to determine the polynomial equations of the enzyme's binding site and then you can derive the rate equation of the enzyme using the rapid-equilibrium method. Note that the catalytic site only factorises when it can be viewed as having multiple independent sites.

When we consider thermodynamic equilibrium, i.e.

$$v = 0$$

then we conclude that

$$V_M^+ \prod_i \frac{s_{i,e}}{K_{S,i}} - V_M^- \prod_j \frac{p_{j,e}}{K_{P,j}}, \Rightarrow K_{eq} = \frac{\prod_j p_{j,e}}{\prod_i s_{i,e}} = \frac{V_M^+ \prod_j K_{P_j}}{V_M^- \prod_i K_{S_i}},$$

this relation is known as the Haldane relationship and relates enzyme-kinetic parameters to the equilibrium constant, indicating that enzyme-kinetic parameters cannot be freely chosen as they have to obey this relation. Since, $K_{eq} = e^{\frac{\Delta\mu_r^{0'}}{RT}}$ with the standard Gibbs free energy change of the reaction defined in the terms of the standard formation Gibbs free energies, i.e $\Delta\mu_r^{0'} = \sum_j \mu_{P_j}^{0'} - \sum_i \mu_{S_i}^{0'}$, which are *independent* of the nature of the enzyme catalysing the reaction. Thus, we can write

$$v = \frac{V_M^+ \prod_i \frac{s_i}{K_{S_i}} - V_M^- \prod_j \frac{p_j}{K_{P_j}}}{\mathcal{B}} = \frac{V_M^+ \prod_i \frac{s_i}{K_{S_i}} \left(1 - \frac{\prod_j p_j}{\prod_i s_i K_{eq}}\right)}{\mathcal{B}} \tag{6}$$

In fact, all the rate equations derived in the previous section agree with this equation, indicating that you only need to know the binding polynomial of the enzyme, which depends on the exact mechanism of reactant and effector binding.

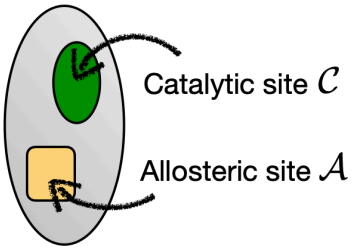


Figure 19: **A monomeric enzyme with a catalytic and allosteric site.** The idea is that the binding state of the allosteric site influences the affinity of the catalytic site for reactants, and possibly also vice versa, and that this happens via structural changes in the protein. This can happen at the level of a single protein or for a protein in a multimeric protein complex, where individual proteins then influence each other's conformations and therewith their reactant and effector affinities.

and

$$\frac{v^-}{v^+} = \frac{\prod_j p_j}{\prod_i s_i K_{eq}} = e^{\frac{\Delta\mu_r}{RT}}.$$

We remark that

$$0 < \frac{\prod_i \frac{s_i}{K_{S_i}}}{\mathcal{B}} < 1,$$

this term is often viewed as the saturation function of the enzyme with substrate and denoted by $f(\mathbf{x})$ with $\mathbf{x}$ as the vector of all molecule concentrations occurring in the cell, including the reactants and the effectors of the enzyme. Thus, we obtain the general relation

$$v = k_{cat}^{+} e_T f(\mathbf{x}) \left(1 - e^{\frac{\Delta\mu_r}{RT}}\right),$$

which, as we shall see later, also applies for multimeric enzymes and is, therefore, a generalised description of enzyme kinetics.

Inhibition

Enzyme inhibition by an effector I can occur in the catalytic site – then it typically competes for the same binding site as one or several of the reactants – or at an allosteric site – then the inhibition cannot be competitive.

In the case of binding to the catalytic site then the binding polynomial of the catalytic site gets in the simplest case an additional term, i.e. $+\frac{i}{K_I}$, e.g. for figure 17

$$\mathcal{B} = \mathcal{C} = 1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{ab}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{pq}{K_P K_Q} + \frac{i}{K_I}.$$

In this case, the inhibitor blocks the binding site of all the reactants (since no enzyme states occur with the inhibitor bound and a reactant bound). If the inhibitor only inhibits the binding of A and P then

$$\mathcal{B} = \mathcal{C} = 1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{ab}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{pq}{K_P K_Q} + \frac{i}{K_I} + \frac{q \cdot i}{K_Q K_I} + \frac{b \cdot i}{K_B K_I}.$$

Note B and Q can now still bind to the enzyme, because they are not competing for the same binding site as the inhibitor I.

In the case of the inhibitor binding to an allosteric site, with an affinity that is independent of the state of the enzyme, then, in the case of allosteric inhibition,

$$\mathcal{B} = \mathcal{C} \times \mathcal{A} = \left(1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{ab}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{pq}{K_P K_Q}\right)\left(1 + \frac{i}{K_I}\right).$$

If the inhibitor binds to the catalytic site and an allosteric site then

$$\mathcal{B} = \mathcal{C} \times \mathcal{A} = \left(1 + \frac{a}{K_A} + \frac{b}{K_B} + \frac{ab}{K_A K_B} + \frac{p}{K_P} + \frac{q}{K_Q} + \frac{pq}{K_P K_Q} + \frac{i}{K_{I,1}}\right)\left(1 + \frac{i}{K_{I,2}}\right).$$

Exercise

1. Consider the enzyme mechanism shown in figure 17
   a. Draw the enzyme mechanism that correspond to the four inhibition cases treated in the previous section.
   b. Determine the rate equation in all these cases.

Activation

An activator generally binds to an allosteric site on the enzyme, distant from the catalytic site. It can therefore only influence the catalytic site properties via a protein structural effect – a change in the protein's conformation. We will therefore treat the activation of a monomeric enzyme by an effector as a special case of the enzyme kinetics of cooperative enzymes that change conformation in response to binding events in the next chapter.

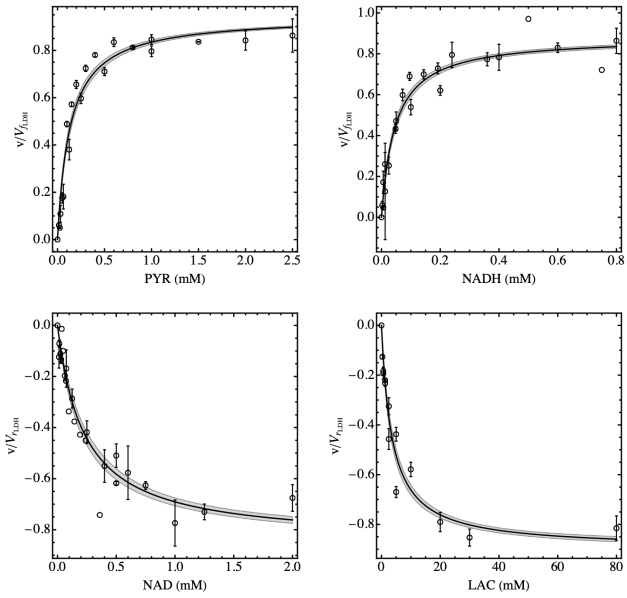


Figure 20: **Illustration of a fit of a rate equation to experimental of lactate dehydrogenase activity to obtains LDH's kinetic parameters for later use in a mathematical model.**

Exercise

1. FITTING ENZYME KINETIC PARAMETERS TO EXPERIMENTAL DATA GIVEN A RATE EQUATION. Go to `https://fairdomhub.org/investigations/56` where you find experimental data on enzyme kinetics, accompanying the paper by Penkler et al. `https://doi.`

org/10.1111/febs.13237. Download "LDH kinetic data" (an excel file) and click on kinetic model to see the rate equation of LDH which you will fit to the data. Your final fits should look like those shown in figure 20 (or use the fitted parameters shown on the site) and show that the fit becomes when you change parameters. Check whether the equilibrium constant agrees with literature data. Check in the literature that LDH in this organism is indeed a monomeric enzyme. Note that not all LDH are the same, some are composed of multiple subunits (see doi:10.1111/j.1742-4658.2007.06115.x).

2. ENZYME MECHANISM WITH PARAMETER VALUES FOR THE RATE CONSTANTS FOR THE ENZYME-STATE TRANSITION REACTIONS
‡In 'What limits the rate of an enzyme-catalysed reaction? by Cleland (in Acc of Chem Res, 8(5), 1975), you find an enzyme mechanism for hexokinase on page 150 (the units are mM and seconds). If you which you can code this model into a simulation package and investigate the rate of the enzyme as function of substrate and product concentrations to make, for instance, figures like figure 15 when using a rapid-equilibrium enzyme kinetic rate equation.

3. DERIVING RATE EQUATIONS FOR SEVERAL MONOMERIC ENZYMES OCCURRING IN GLYCOLYSIS.

   (a) Consider a glucose transporter that functions by facilitated diffusion. The reaction catalysed by this enzyme is $GLC_e \rightleftharpoons GLC_i$, with GLC_e and GLC_i as, respectively, the extra- and intracellular concentration of glucose.
      i. Draw a plausible enzyme mechanism.
      ii. Determine the binding polynomial for the catalytic site.
      iii. Give the entire rate equation.
      iv. Use the Haldane relationship to express the kinetic parameters in terms of the equilibrium constant.
      v. What is the most likely value of the equilibrium constant?
      vi. Note that facilitated-diffusion transporters (sometimes called permeases) often have an additional term appearing in the rate equation as you can see in equation 14 of https://doi.org/10.1111/febs.13237. If you want to understand why this occurs then read https://doi-org.vu-nl.idm.oclc.org/10.1016/0005-2736(67)90013-2 (Kotyk, A., Mobility of the free and of the loaded monosaccharide carrier in *Saccharomyces cerevisiae*, Biochimica et Biophysica Acta, 135, 112-119, 1967).

(b) Consider triose phosphate isomerase. In some microbes, e.g. *Plasmodium* (`https://doi.org/10.1111/febs.13237`), is inhibited by phosphoenol pyruvate (PEP).

i. Draw a plausible enzyme mechanism.

ii. Determine the binding polynomial for the catalytic site where the reactants bind and the inhibitor binds. PEP acts as a competitive inhibitor for both reactants. Does it bind in the catalytic site or in an allosteric site?

iii. Give the entire rate equation.

iv. Use the Haldane relationship to express the kinetic parameters in terms of the rate constant.

v. Calculate the equilibrium constant using the kinetic parameters of the enzyme shown in Table 5 of the paper `https://doi.org/10.1111/febs.13237`.

(c) Consider alcohol dehydrogenase. Assume that the catalytic site consists of two independent binding sites (binding pockets) where the different reactants bind. $NADH$ and NAD bind to one such pocket in the catalytic site while acetaldehyde and ethanol bind to another.

i. Draw a plausible enzyme mechanism.

ii. Determine the binding polynomial for the catalytic site. Why does it factorise?

iii. Give the entire rate equation.

(d) Consider aldolase.

i. Draw a plausible enzyme mechanism.

ii. Determine the binding polynomial for the catalytic site. Why does it not factorise?

iii. Give the entire rate equation.

(e) Consider glyceraldehyde 3-phosphate dehydrogenase.

i. Draw a plausible enzyme mechanism.

ii. Determine the binding polynomial for the catalytic site.

iii. Give the entire rate equation.

4. Consider an enzyme with two substrates and one product and an allosteric site where an inhibitor binds. Derive a plausible binding polynomial for its catalytic and allosteric site. Give the rate equation. Derive the Haldane relationship between the equilibrium constants and the kinetic parameters.

5. ‡Consider an enzyme with a single substrate and product. Draw a plausible catalytic cycle. Now assume that this enzyme is activated by an activator that binds in a allosteric site. Assume that the

enzyme is active with without the activator but more active when the activator is bound. What will be the resulting rate equation?

The enzyme kinetics of a multimeric enzyme with cooperative subunits

Reversible kinetics of a multi-subunit, cooperative enzyme according to the Monod Wyman Changeux formalism

In this section, we will derive the rate equation of a multimeric enzyme catalysing a reversible reaction. We will assume that the proteins works according to a mechanism that was first proposed by Monod, Wyman and Changeux (MWC) in 1965. Here we generalise their approach to enzymes that catalyse reversible reactions. This has so far only been done once by Popova and Selkov for a single-substrate and single-product reactions. By using the binding polynomial concept, the derivation, and in the particular the use, of the resulting rate equation is straightforward.

Here is the equation we are going to derive in the next section, which applies to enzyme composed out of n identical subunits,

$$v = \frac{V_M^+ \frac{ab}{K_A K_B} {\mathcal{C}_\mathcal{R}}^{n-1} \left(1 - \frac{pq}{abK_{eq}}\right)}{{\mathcal{C}_\mathcal{R}}^n + L \frac{{\mathcal{A}_\mathcal{T}}^n}{{\mathcal{A}_\mathcal{R}}^n} {\mathcal{C}_\mathcal{T}}^n}, \; \mathcal{B} = {\mathcal{C}_\mathcal{R}}^n + L \frac{{\mathcal{A}_\mathcal{T}}^n}{{\mathcal{A}_\mathcal{R}}^n} {\mathcal{C}_\mathcal{T}}^n. \quad (7)$$

The interpretation of the equation is explained in figure 21. MWC considered a multimeric enzyme. We limit ourselves to one with identical subunits. Each subunit can be either in an active, relaxed state called R or in a tensed, less-active (or inactive) state T – we assume that T is inactive. The subunits can change in conformation and when they do so, they all do this simultaneously: so, only R_4 and T_4 forms exist of a 4-mer enzyme – and not also R_1T_3, R_2T_2, etc.. This is assumption is called the 'concerted symmetry assumption'. (Other models such as the Adair-Pauling or the Koshland-Nemethy-Filmer model do not make this assumption.) T and R subunits have different affinities for reactants and effectors, which is what gives to heigthened sensitivity of the rate of the enzyme to their concentrations.

Each subunit has a binding polynomial for its catalytic site and

allosteric site(-s). The binding polynomial of a single subunit is then, as usual, given by their product. The binding polynomial of the multimeric enzyme is then the product of that of its subunits.

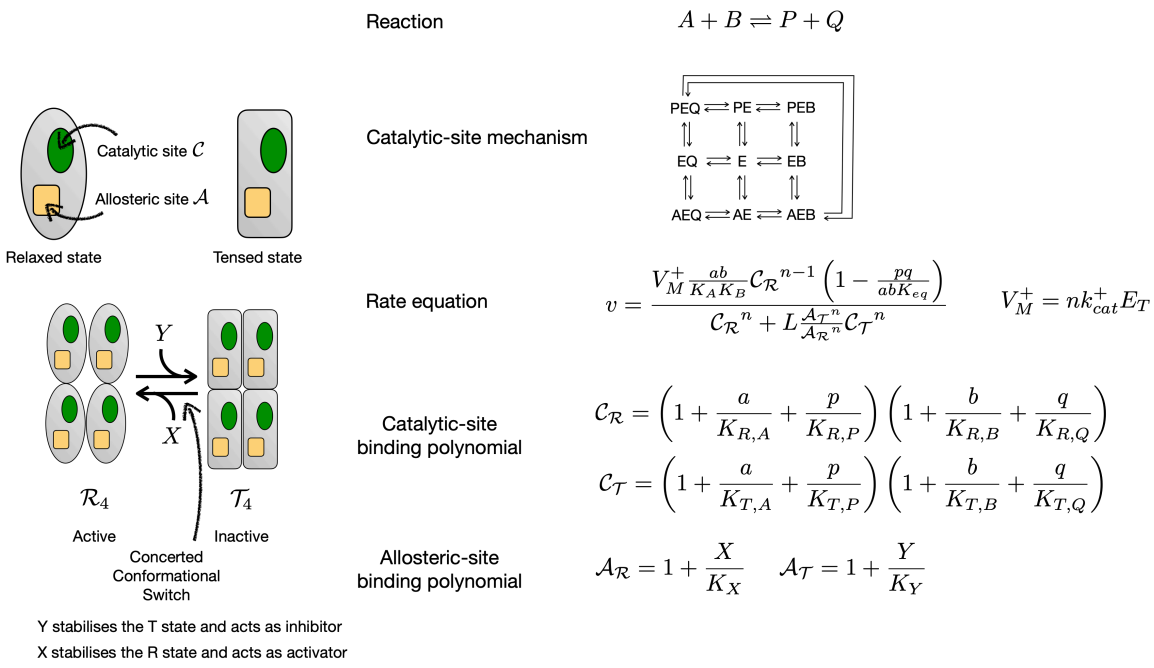


Figure 21: **Illustration of the key concepts of the reversible MWC rate equation for the reaction** $A + B \rightleftharpoons P + Q$. Here we illustrate it for an enzyme with 4 subunits (n=4) and a specific choice of its catalytic-site and allosteric-site binding polynomials. The mechanism is that each subunit can exist either in related R state, during which it is active, and in a tensed T, in which it either less or inactive, we assume it to be inactive. The enzyme has different affinity for reactants and effectors in its R and T state. The equilibrium constant for R and T concentrations in the absence of any reactants and effectors equals $L = t/r$. In this particular case we assume that molecule Y stabilises the T state, it can bind to the active site of the enzyme in the T state while X stabilises the R state. X is therefore an activator of the reaction and Y acts as an inhibitor.

Effectors can, for instance, bind exclusively to the R or the T state and stabilise those states, hereby shifting the conformational equilibrium either in the direction of R (active) or T (inactive) states. The ratio of t over r concentration in the absence of reactants and effectors is given by the conformation equilibrium constant L. The number of subunits is denoted by n. The binding polynomials have their usual symbols ($\mathcal{B}$, $\mathcal{C}$ and $\mathcal{A}$), except for the additional subscript that indicates whether the binding polynomial belongs to the R or the T state of the enzyme.

Derivation of the reversible MWC equation‡

We start from the polynomial equation of the subunits in the R and T state, which are products of the polynomial equations of their allosteric sites and catalytic site,

$$\mathcal{B}_\mathcal{R} = \mathcal{A}_\mathcal{R} \times \mathcal{C}_\mathcal{R},\ \mathcal{B}_\mathcal{T} = \mathcal{A}_\mathcal{T} \times \mathcal{C}_\mathcal{T}.$$

MWC assumed that the subunits act independently, which means that the binding polynomial of a multimer composed out of n identical subunits, so R_n and T_n, equals,

$$\mathcal{B}_{\mathcal{R}n} = {\mathcal{B}_\mathcal{R}}^n,\ \mathcal{B}_{\mathcal{T}n} = {\mathcal{B}_\mathcal{T}}^n.$$

The ratio of the concentration of the unbound subunits in the T and R state, so in the absence of reactants and effectors, is defined by $L = t/r$. The polynomial equation of the multimeric enzyme now becomes

$$\mathcal{B} = \mathcal{B}_{\mathcal{R}}{}^{n} + L\mathcal{B}_{\mathcal{T}}{}^{n} = \mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{n} + L\mathcal{A}_{\mathcal{T}}{}^{n}\mathcal{C}_{\mathcal{T}}{}^{n}$$

The rate of the enzyme equals the summed rates of all the catalytic sites that have all the substrates bound. The exact value of the rate depends on the concentrations of the reactants and the effectors and the fraction of the catalytic sites bound to all the substrates. The total concentration of catalytic sites equals ne_T with e_T as the concentration of the multimers. The fraction of multimers with catalytic sites bound to all substrates equals,

$$\frac{\prod_i \frac{s_i}{K_i}\mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{n-1}}{\mathcal{B}}$$

Thus, one subunit has a catalytic site in the state with all substrates bound and its allosteric sites in an unknown state. This subunits contributes $\prod_i \frac{s_i}{K_i}\mathcal{A}_{\mathcal{R}}$ to $\prod_i \frac{s_i}{K_i}\mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{n-1}$ while the remaining $n-1$ subunits can be in any state and contribute $\mathcal{A}_{\mathcal{R}}{}^{n-1}\mathcal{C}_{\mathcal{R}}{}^{n-1}$.

this leads to a forward rate equal to

$$v^{+} = ne_T k_{cat}^{+} \frac{\prod_i \frac{s_i}{K_i}\mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{n-1}}{\mathcal{B}}.$$

The backward rate equals

$$v^{-} = ne_T k_{cat}^{-} \frac{\prod_j \frac{p_j}{K_j}\mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{n-1}}{\mathcal{B}}.$$

The net rate of the enzyme equals

$$v = v^{+} - v^{-} = \frac{ne_T k_{cat}^{+}\prod_i \frac{s_i}{K_i}\mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{n-1} - ne_T k_{cat}^{-}\prod_j \frac{p_j}{K_j}\mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{-1}}{\mathcal{B}}.$$

which we can rewrite using

$$v = v^{+}\left(1 - \frac{v^{+}}{v^{-}}\right)$$

yielding

$$v = \frac{V_{max}^{+}\prod_i \frac{s_i}{K_i}\mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{n-1}\left(1 - \frac{\prod_j \frac{p_j}{K_j}}{\prod_i \frac{s_i}{K_i}K_{eq}}\right)}{\mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{n} + L\mathcal{A}_{\mathcal{T}}{}^{n}\mathcal{C}_{\mathcal{T}}{}^{n}}.$$

with $V_{max}^{+} = ne_T k_{cat}^{+}$where we have used the previous result that $\mathcal{B} = \mathcal{A}_{\mathcal{R}}{}^{n}\mathcal{C}_{\mathcal{R}}{}^{n} + L\mathcal{A}_{\mathcal{T}}{}^{n}\mathcal{C}_{\mathcal{T}}{}^{n}$. Often this last equation is written as,

$$v = \frac{V_{max}^{+}\prod_i \frac{s_i}{K_i}\mathcal{C}_{\mathcal{R}}{}^{n-1}\left(1 - \frac{\prod_j \frac{p_j}{K_j}}{\prod_i \frac{s_i}{K_i}K_{eq}}\right)}{\mathcal{C}_{\mathcal{R}}{}^{n} + L\frac{\mathcal{A}_{\mathcal{T}}{}^{n}}{\mathcal{A}_{\mathcal{R}}{}^{n}}\mathcal{C}_{\mathcal{T}}{}^{n}}. \tag{8}$$

where $L\frac{\mathcal{A}_{\mathcal{T}}{}^{n}}{\mathcal{A}_{\mathcal{R}}{}^{n}}$ is sometimes referred to as L'.

From this equation all reversible monomeric and multimeric rate equations can be derived! So it is the only equation you need to know to make mathematical models of metabolic networks – provided you choose to accept the rapid-equilibrium assumption of enzyme kinetics and the MWC mechanism.

Simplifying conditions of the reversible MWC rate equation for a multimeric enzyme

The main equation (eq. 8) for a multimeric enzyme simplifies under particular conditions, as shown in figure 22, into that of a monomeric enzyme (eq. 5), which we derived in the previous chapter. Thus, knowing the multimeric equation suffices.

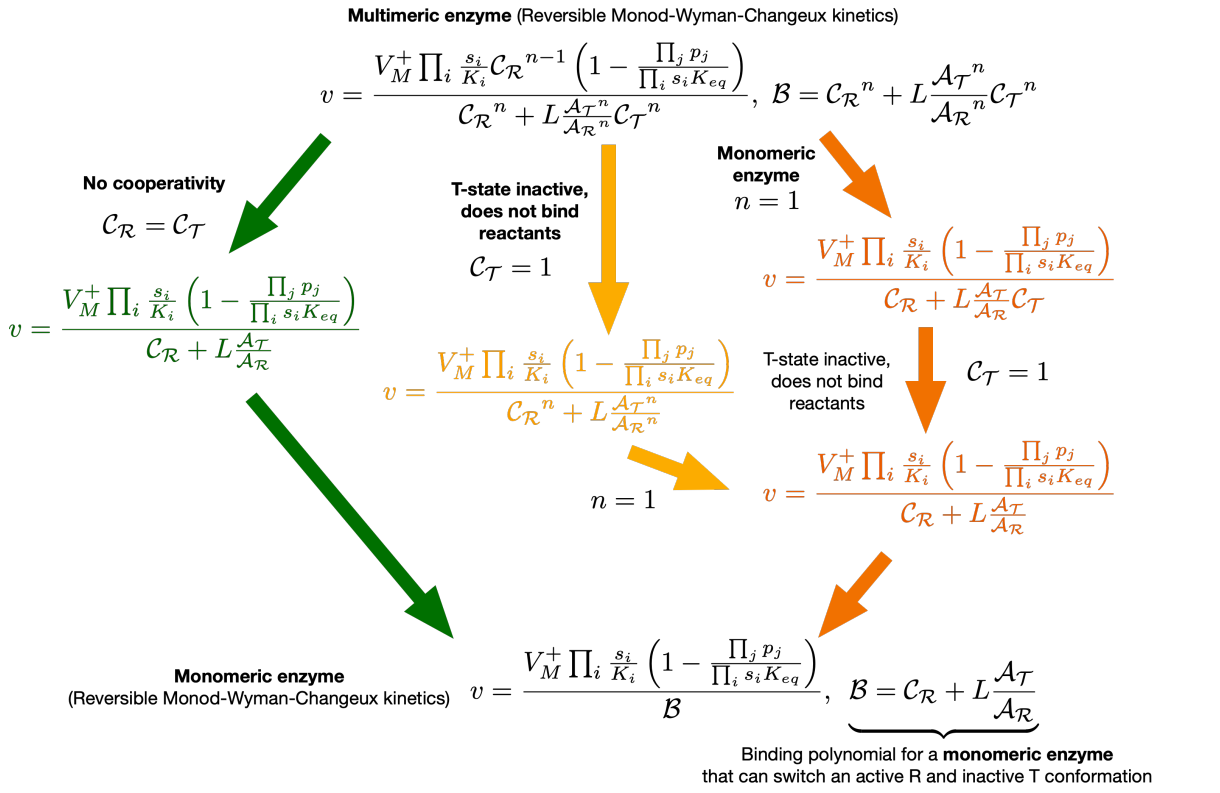


Figure 22: **The different ways by which the general reversible MWC rate equation for a multimeric protein simplifies to that of a monomeric enzyme with a conformation change.**

Example: phosphofructokinase in S. cerevisiae

Phosphofructokinase (PFK) catalyses the conversion: $F6P + ATP \rightleftharpoons F16BP + ADP$. It consists of two subunits (in yeast) and is a highly regulated enzyme across species (Fig. 9 and 10). Its kinetics has been intensively studied in yeast. Figure 23 shows the dependency of the

rate of PFK on reactants and effectors, which are in agreement with experimental data (see https://doi.org/10.1046/j.1432-1327.2000.01527.x). The rate equation of PFK, resulting from the analysis of experimental data, is given by a MWC model

$$
\begin{aligned}
v &= \frac{V_{max}\frac{\alpha\cdot atp\cdot f6p}{K_{atp}K_{f6p}}\mathcal{C}_{\mathcal{R}}}{{\mathcal{C}_{\mathcal{R}}}^2 + L\frac{{\mathcal{A}_{\mathcal{T}}}^2}{{\mathcal{A}_{\mathcal{R}}}^2}{\mathcal{C}_{\mathcal{T}}}^2} \\
\mathcal{C}_{\mathcal{R}} &= 1 + \frac{atp}{K_{atp}} + \frac{f6p}{K_{f6p}} + \frac{\alpha\cdot atp\cdot f6p}{K_{atp}K_{f6p}} \\
\mathcal{C}_{\mathcal{T}} &= 1 + \frac{\beta\cdot atp}{K_{atp}} \\
\mathcal{A}_{\mathcal{R}} &= \left(1 + \frac{atp}{K_{i,atp}}\right)\left(1 + \frac{amp}{K_{amp}}\right)\left(1 + \frac{f16bp}{K_{f16bp}} + \frac{f26bp}{K_{f26bp}}\right) \\
\mathcal{A}_{\mathcal{T}} &= \left(1 + \frac{\gamma_1\cdot atp}{K_{i,atp}}\right)\left(1 + \frac{\gamma_2\cdot amp}{K_{amp}}\right)\left(1 + \frac{\gamma_3\cdot f16bp}{K_{f16bp}} + \frac{\gamma_4\cdot f26bp}{K_{f26bp}}\right)
\end{aligned}
$$

with: $\alpha = 5.12$, $L = 0.66$, $K_{f6p} = 0.1$, $K_{atp} = 0.71$, $\beta = 3$, $K_{i,atp} = 0.65$, $\gamma_1 = 0.65$, $K_{amp} = 0.0995$, $\gamma_2 = 0.0845$, $K_{f26bp} = 0.000682$, $\gamma_4 = 0.0174$, $K_{f16bp} = 0.111, \gamma_3 = 0.397$ (all K's are in mM, the other parameters are dimensionless). This information was used to make the plots in figure 9.

Exercise

1. Analyse the rate equation of yeast's PFK and the dependency of its rate on reactants and effector concentrations as shown in figure 23.

   a. What are the effector metabolites of PFK? Which of those are activating and which ones are inhibiting?

   b. What's surprising about the binding polynomials of the catalytic site?

   c. Which metabolite is absent from the rate, which would you expect to occur?

   d. How would you call the influence of ATP on the rate of the enzyme?

   e. In https://doi.org/10.3390/ijms22031483 PFK of *Mycobacterium tuberculosis* is analysed? What are it's effectors and are those working similarly as in *S. cerevisiae*?

2. Pyruvate kinase is activated by fructose 1,6-bisphosphate as shown in figure 24. This enzyme has four subunits.

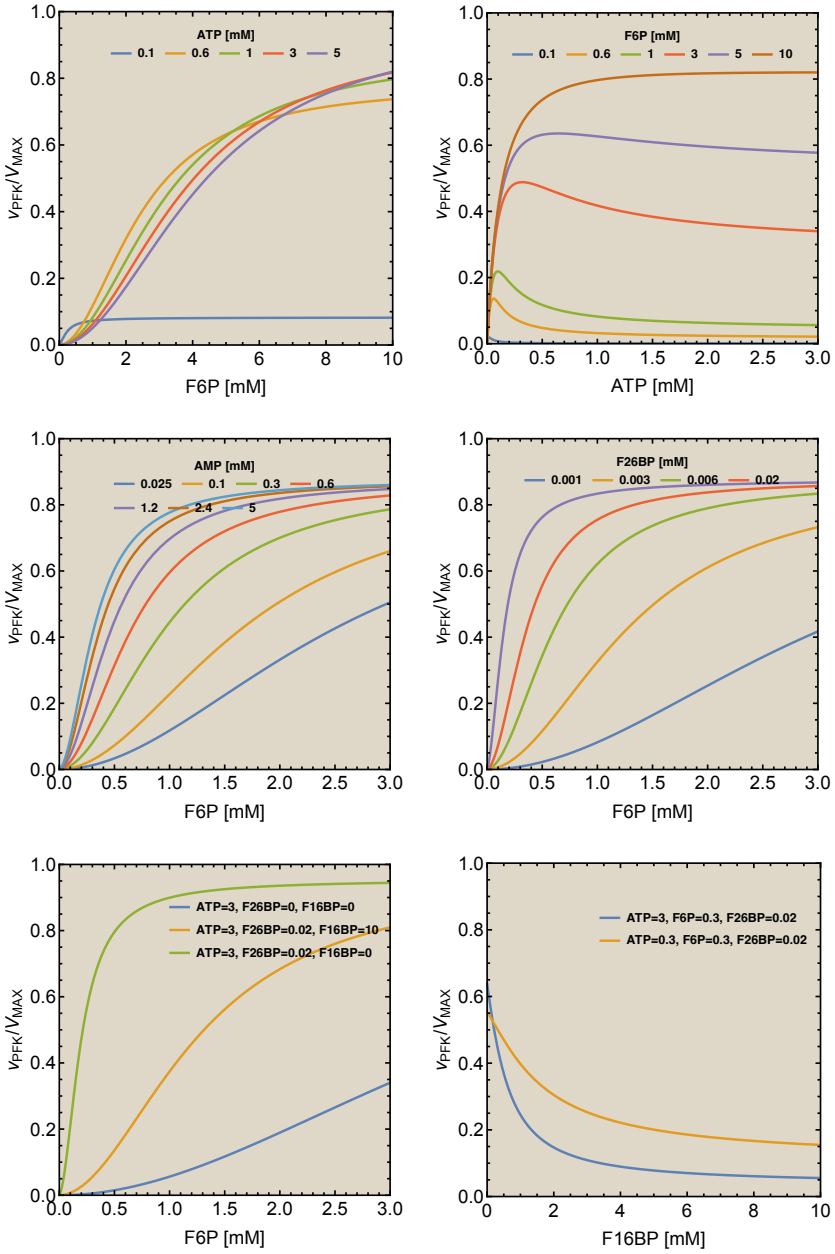


Figure 23: **Enzyme kinetics of phosphofructokinase (PFK) of *S. cerevisiaie*.** PFK catalyses the following conversion: $F6P + ATP \rightleftharpoons F16BP + ADP$. It is composed of 2 subunits in yeast and highly regulated by AMP and $F26BP$. The plots that are shown are the results of a MWC model of PFK constructed on the basis of experimental data (Teusink et al., `https://doi.org/10.1046/j.1432-1327.2000.01527.x`).

a. Suggest an enzyme kinetic model for it, using the reversible MWC equation for a multimeric enzyme.

b. In https://doi.org/10.1002/bit.10288 (Chassagnole, et al., Biotechnol Bioeng, 79(1), 53-73, 2002) you find a MWC-type rate equation of PYK. Rewrite it in a form that allows for the identification of the binding polynomials of the catalytic site and allosteric sites. Make plots of the rate of PYK as function of the reactants and effectors (as done for PFK in Fig. 9). Are FBP and AMP both activators or inhibitors?

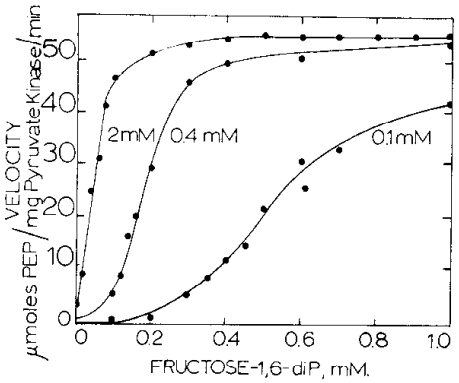


FIG. 10. Pyruvate kinase dependence upon fructose-1,6-P_2. Standard assays were carried out in the presence of 1.33 mM ADP. 10 mM $MgCl_2$ and concentrations of P-enolpyruvate as shown, The concentrations of fructose-1,6-P_2 which gave half-maximal velocity were identical with those obtained if 0.5 mM GDP replaced ADP in the assays.

Figure 24: **Pyruvate kinase is activated by fructose 1,6-bisphophatse.** Here an example from *E. coli* is shown from https://doi.org/10.1016/S0021-9258(19)43120-7.

The complexity of the MWC model: effector-effector interactions

A remarkable property of the MWC model is that it is quite easy to make models that show unintuitive interactions between two effectors of an enzyme. For instance, an inhibitor can be turned into an activator by another effector that acts as an inhibitor when it is active alone. Two examples are shown in figure 25. They show that two effectors that are functional as inhibitors (or activators) in the absence of the other can be turned into an activator (or inhibitor) in the presence of the other, provided the concentrations of both are high enough. The model we choose was inspired by the reversible MWC equation at constant concentrations of the reactants, and all regulation by the effector is via two allosteric sites (one on the enzyme in the *R* state and other on the enzyme in the *T* state, each with

different affinities),

$$\frac{v}{V^+_{max}} = \frac{A}{A + L\left(\frac{1+x+y+c_{xy}xy}{1+c_x x+c_y y+c'_{xy}xy}\right)^4 C}.$$

One should think of A and C as dependent on the catalytic binding functions ($\mathcal{C}_\mathcal{R}$ and $\mathcal{C}_\mathcal{T}$) dependent on the concentrations of the reactants.

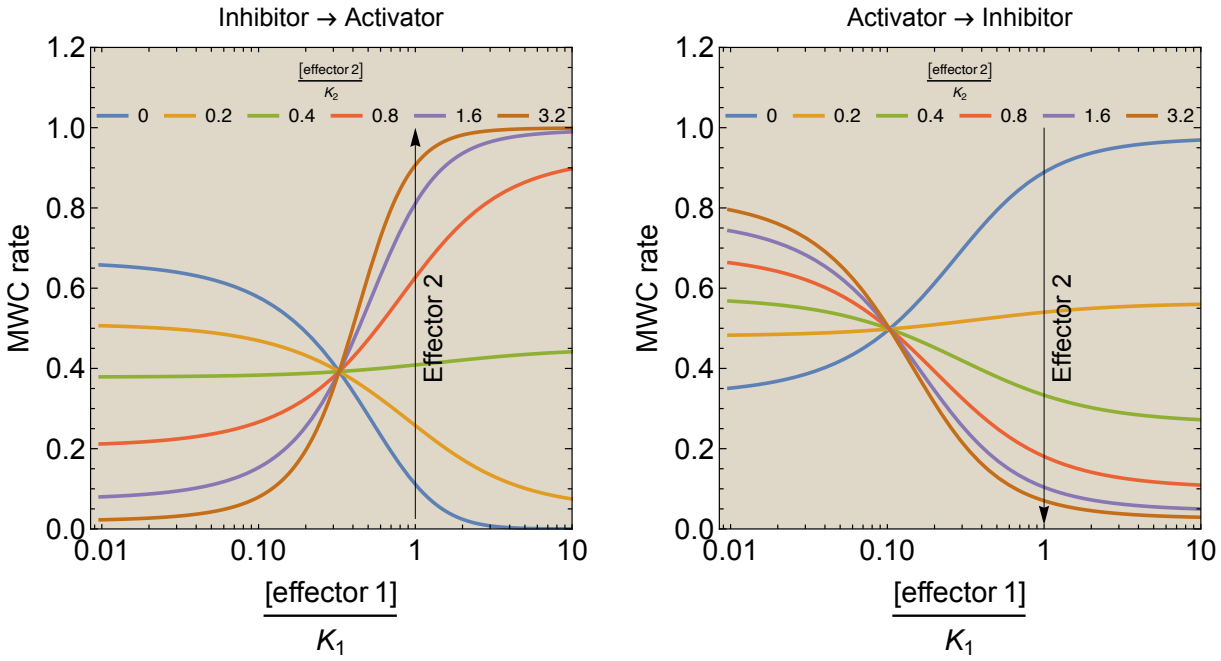


Figure 25: **Effector-effector interactions that turn an activator into inhibitor and vice versa.** A. An inhibitor (effector 1) is turned into an activator by effector 2. Effector 2 is also an inhibitor when effector 1 is absent. B. An activator (effector 1) is turned into an inhibitor by a second effector (effector 2) that also acts an activator in the absence of effector 1. Parameters: A.: $L = 1, c_x = 0,\ c_y = 0.1,\ c_{xy} = 0,\ c'_{xy} = 2,\ A = 2,\ C = 1.$ and B.: $L = 1, c_x = 3,\ c_y = 2,\ c_{xy} = 10,\ c'_{xy} = 4,\ A = 0.5,\ C = 1.$

When we make x and y dependent on each other, e.g. via $y = 10 - x$ or as $y = x/10$, mimicking respectively a conserved moiety or an equilibrium concentration relationship leads to even more exotic dependencies shown in figure 26.

Figures figure 25 and 26 illustrate that the study of enzyme with cooperative subunits that are regulated by multiple effectors can give rise to unexpected behaviours. Thus, when you encounter a cooperative protein – always a multimeric proteins – then beware of the complexities that it can give rise to.

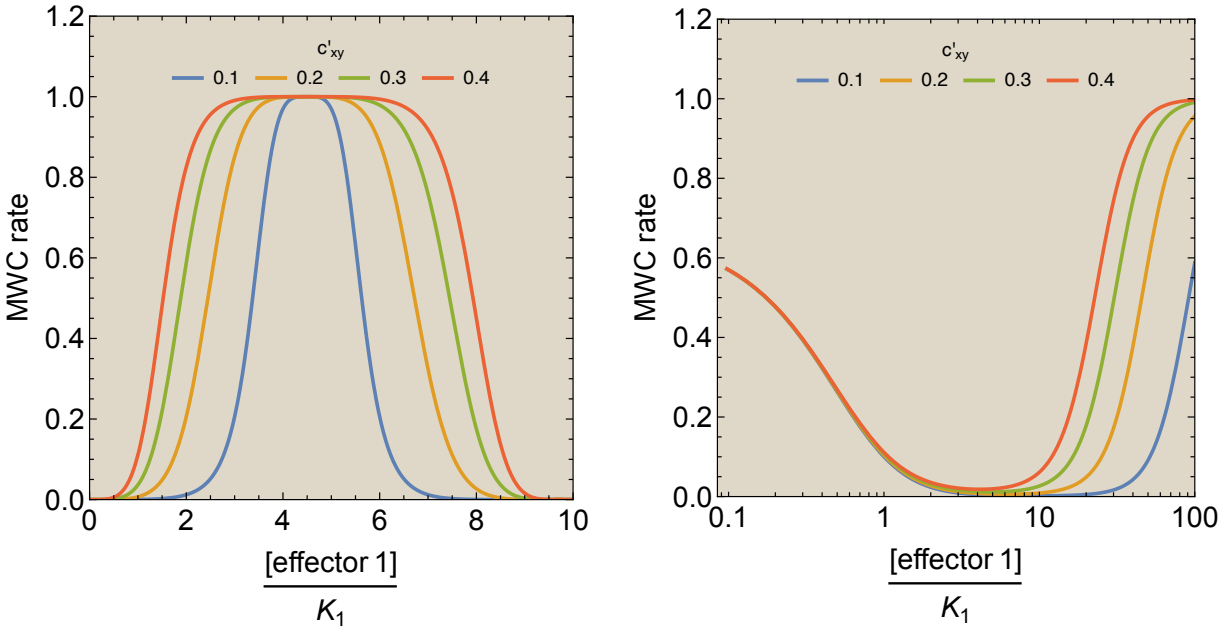


Figure 26: **Effector-effector interactions giving rise to peaked and vallyed activation or inhibiton.** A. two effectors are dependent and sum to 10. B. two effectors are dependent and effector 1/effector 2 equals 10. Parameters: A. & B.: $c_x = 0$, $c_y = 0.1$, $c_{xy} = 0$, $A = 2$, $C = 1$.

The steady-state method for enzyme kinetics

The quasi steady-state rate equation of enzymes

When enzymes are operating under quasi steady-state conditions (Figure 13), the differential equations describing the rate of change of the concentrations of the enzyme states are all zero,

Those of the reactants are now not equal to zero, unless the entire metabolic network is at steady state.

$$\begin{aligned} \frac{de}{dt} &= -v_1 + v_3 = 0 \\ \frac{des}{dt} &= v_1 - v_2 = 0 \\ \frac{dep}{dt} &= v_2 - v_3 = 0. \end{aligned}$$

This set of equations shows that at quasi-steady state all the rates of the catalytic cycle are equal: $v_1 = v_2 = v_3$. Since we are not considering enzyme synthesis or degradation, we also have to conclude that at all times the sum of the enzyme concentrations equals their sum at time 0, which we shall to refer to as the total enzyme concentration e_T,

$$e_T = e(0) + es(0) + ep(0) = e(t) + es(t) + ep(t).$$

That the previous conservation equation holds is also illustrated by the fact that

$$\frac{de}{dt} + \frac{des}{dt} + \frac{dep}{dt} = 0,$$

indicating that the sum of those concentrations does not change with time.

This last equation also indicates that the rate of change equations are linearly dependent. A consequence of that is that solving for the steady-state concentrations of the enzyme states is not possible from these three (since one contains exactly the same information then the other two such that we come one equation short). Thus, we need to take two rate of change equations and the conservation equation to arrive at the three equations needed to determine the three steady-

state concentration of the enzyme states, i.e.

$$\begin{aligned} k_1^+ \cdot e_s \cdot s - k_1^- \cdot es_s - (k_2^+ \cdot es_s - k_2^- \cdot ep_s) &= 0 \\ k_2^+ \cdot es_s - k_2^- \cdot ep_s - (k_3^+ \cdot ep_s - k_3^- \cdot e \cdot p) &= 0 \\ e + es + ep &= e_T. \end{aligned}$$

This is set of three equations are linear with respect to the concentrations we would like to determine. So, we can also write them in the following matrix format.

$$\begin{pmatrix} k_1^+ s & -k_1^- - k_2^+ & k_2^- \\ k_3^- p & k_2^+ & -k_2^- - k_3^+ \\ 1 & 1 & 1 \end{pmatrix} \begin{pmatrix} e_s \\ es_s \\ ep_s \end{pmatrix} = \begin{pmatrix} 0 \\ 0 \\ e_T \end{pmatrix} \tag{9}$$

Solving this set of equations – by hand, by linear algebra, or by making use of software (e.g. Mathematica) – leads to the following expression of the steady-state concentrations in terms of the kinetic parameters.

$$\begin{aligned} \begin{pmatrix} e_s \\ es_s \\ ep_s \end{pmatrix} &= \begin{pmatrix} k_1^+ s & -k_1^- - k_2^+ & k_2^- \\ k_3^- p & k_2^+ & -k_2^- - k_3^+ \\ 1 & 1 & 1 \end{pmatrix}^{-1} \begin{pmatrix} 0 \\ 0 \\ e_T \end{pmatrix} \\ &= e_T \begin{pmatrix} \frac{k_2^+ k_3^+ + k_1^-(k_2^- + k_3^+)}{k_1^- k_2^- + (k_1^- + k_2^+)k_3^+ + (k_1^- + k_2^- k_2^+)k_3^- p + k_1^+(k_2^- + k_2^+ + k_3^+)s} \\ \frac{k_2^- k_3^- p + k_1^+(k_2^- + k_3^+)s}{k_1^- k_2^- + (k_1^- + k_2^+)k_3^+ + (k_1^- + k_2^- k_2^+)k_3^- p + k_1^+(k_2^- + k_2^+ + k_3^+)s} \\ \frac{k_3^-(k_1^- + k_2^+)p + k_1^+ k_2^+ s}{k_1^- k_2^- + (k_1^- + k_2^+)k_3^+ + (k_1^- + k_2^- k_2^+)k_3^- p + k_1^+(k_2^- + k_2^+ + k_3^+)s} \end{pmatrix} \end{aligned}$$

The rate of the enzyme is generally associated with the catalytic reaction in the enzyme's mechanism. In this case this correspond to reaction 2. Thus, the (quasi) steady-state rate of the reaction, which we will denote by v, equals,

$$\begin{aligned} v &= v_2 = k_2^+ \cdot es_s - k_2^- \cdot ep_s \\ &= \frac{e_T k_1^+ k_2^+ k_3^+ s - e_T k_1^- k_2^- k_3^- p}{k_1^- k_2^- + (k_1^- + k_2^+)k_3^+ + (k_1^- + k_2^- k_2^+)k_3^- p + k_1^+(k_2^- + k_2^+ + k_3^+)s} \end{aligned}$$

It is customary to express rate equations of enzymes (derived under quasi steady-state or rapid-equilibrium conditions) in terms of enzyme kinetic parameters instead of the (elementary) rate constants of the enzyme mechanism. Accordingly, we define

The rules to identify enzyme kinetic parameters as function of elementary rate constants were developed by Cleland. Cleland, Wsd W. Biochimica et Biophysica Acta (BBA) 67 (1963): 104-137.; Cleland, W. W. Biochimica et Biophysica Acta (BBA) 67 (1963): 173-187.

$$
\begin{aligned}
k_{cat}^{+} &= \frac{k_1^+ k_2^+ k_3^+}{k_1^+(k_2^- + k_2^+ + k_3^+)} \\
V_{max}^{+} &= k_{cat}^{+} e_T \\
K_{M,S} &= \frac{k_1^- k_2^- + k_1^- k_3^+ + k_2^+ k_3^+}{k_1^+(k_2^- + k_2^+ + k_3^+)} \\
K_{M,P} &= \frac{k_1^- k_2^- + k_1^- k_3^+ + k_2^+ k_3^+}{k_3^-(k_1^- + k_2^- + k_2^+)} \\
k_{cat}^{-} &= \frac{k_1^- k_2^- k_3^-}{k_3^-(k_1^- + k_2^- + k_2^+)} \\
V_{max}^{-} &= k_{cat}^{-} e_T
\end{aligned}
$$

which leads to the following expression of the rate equation of the enzyme,

$$v = \frac{V_{max}^{+} \frac{s}{K_{M,S}} - V_{max}^{-} \frac{p}{K_{M,P}}}{1 + \frac{s}{K_{M,S}} + \frac{p}{K_{M,P}}}, \tag{10}$$

which is known as the reversible Michaelis Menten (MM) equation and was first derived by Briggs and Haldane in 1925.

Note that this equation is the same as the one obtained with the rapid-equilibrium approximation. Note also that relationships of the K's are now more complex functions than in the rapid-equilibrium approximation. Thus, for the same enzyme mechanism the two methods give rise to the same equations but different enzyme-kinetic parameters values. Consider once more figure 15 for a comparison of the performance of both methods.

Exercises

1. We will study some aspects of the reversible MM equation (eq. 10).

   (a) An important aspect of enzymes is that their rates are dependent on their substrate and product concentrations. Under which conditions occurs: i. $v \to V_{max}^{+}$, ii. $v \to -V_{max}^{-}$, iii. $v \to V_{max}^{+}/2$, or iv. $v \to -V_{max}^{-}/2$?

   (b) Now consider the enzyme as irreversible. The product concentration still influences the rate of the enzyme by so-called product inhibition. Convince yourself that this indeed happens and is a strong effect by plotting v/V_{max}^{+} as function of $s/K_{M,s}$ for different values of $p/K_{M,p}$.

   (c) Why will the rate of an enzyme inside a cell never attain its V_{max}^{+} value?

   (d) Determine the relationship between the concentration ratio p/s and the kinetic parameters of the enzyme when $v = 0$. How is this state of the enzyme called? Which other relationship holds for this ratio of p/s? So what do you conclude?

   (e) What is reaction that is catalysed by the enzyme?

2. Consider the enzyme with catalytic mechanism,

$$\begin{aligned} E + S &\overset{1}{\rightleftharpoons} ES \\ ES &\overset{2}{\rightarrow} E + P. \end{aligned}$$

(a) Draw the catalytic cycle diagram and confirm that it is cyclic (by only drawing each enzyme state only once in the diagram).

(b) Determine the rate equation of the enzyme under steady state conditions in terms of the elementary rate constants of the catalytic mechanism.

(c) Identify the kinetic parameters of the enzyme, V_{max} and $K_{M,S}$.

(d) What is the concentration of the S when $V_{max}/2$?

(e) What is reaction that is catalysed by the enzyme?

Thermodynamic driving force, thermodynamic equilibrium, and the Haldane relationship for the reversible MM equation‡

Consider the following reaction,

$$S \overset{1}{\rightleftharpoons} P,$$

which is catalysed by an enzyme according the reversible Michaelis-Menten rate equation (eq. 10). When the enzyme rate equals zero then

$$v = 0 \quad \Rightarrow \quad \frac{p_e}{s_e} = \frac{V^+_{max} K_{M,P}}{V^-_{max} K_{M,S}} = \frac{k^+_1 k^+_2 k^+_3}{k^-_1 k^-_2 k^-_3}.$$

The concentration of the product and the substrate have a subscript e under this condition because it is a thermodynamic equilibrium conditions (as opposed to a steady state). Thus, the concentrations of the reactants are constant now because all the rates are zero.

When rates of reactions are zero then the reaction does not have a net thermodynamic driving force,

$$\Delta\mu_r = \mu_P - \mu_S = \mu^{0'}_P + RT \ln p_e - \mu^{0'}_S - RT \ln s_e = \mu^{0'}_P - \mu^{0'}_s + RT \ln \frac{p_e}{s_e} = 0$$

and we conclude that

$$\frac{p_e}{s_e} = e^{\frac{-\Delta\mu^{0'}_r}{RT}},$$

with $\Delta\mu^{0'}_r = \mu^{0'}_P - \mu^{0'}_S$. The ratio of the equilibrium product concentrations over the equilibrium substrate concentrations is called the equilibrium constant K_{eq} of the reaction. We can conclude that,

$$\frac{p_e}{s_e} = e^{\frac{-\Delta\mu^{0'}_r}{RT}} = \frac{V^+_{max} K_{M,P}}{V^-_{max} K_{M,S}} = \frac{k^+_1 k^+_2 k^+_3}{k^-_1 k^-_2 k^-_3} = K_{eq}. \tag{11}$$

The relations in equation 11 are called the Haldane relations which are obeyed at thermodynamic equilibrium and constrain enzyme kinetic parameter values.

The following relation holds for all states of the enzyme – dynamic, steady-state and thermodynamic equilibrium –,

$$\Delta\mu_r = \underbrace{\mu_P^{0\prime} - \mu_S^{0\prime}}_{-RT\ln K_{eq}} + RT\ln\frac{p}{s} = RT\ln\frac{p}{sK_{eq}}, \;\Rightarrow\; \frac{p}{sK_{eq}} = e^{\frac{\Delta\mu_r}{RT}}.$$

which indicates that a reaction with a free energy potential of a reaction $\Delta\mu_r \neq 0$ is removed from thermodynamic equilibrium such that $\frac{p}{s} \neq K_{eq}$ and that therefore $v \neq 0$ (which we will conclude next).

Now we return to the rate equation of the enzyme we are considering (eq. 10) and identify its forward rate v^+ and backward rate v^-,

$$v = v^+ - v^-, \;\; v^+ = \frac{V_{max}^+ \frac{s}{K_{M,S}}}{1 + \frac{s}{K_{M,S}} + \frac{p}{K_{M,P}}}, \;\; v^- = \frac{V_{max}^- \frac{p}{K_{M,P}}}{1 + \frac{s}{K_{M,S}} + \frac{p}{K_{M,P}}}$$

Given these we can conclude that

$$v = v^+\left(1 - \frac{v^-}{v^+}\right), \;\; \frac{v^-}{v^+} = \frac{V_{max}^- \frac{p}{K_{M,P}}}{V_{max}^+ \frac{s}{K_{M,S}}} = \frac{p}{s\frac{V_{max}^+ K_{M,P}}{V_{max}^- K_{M,S}}} = \frac{p}{sK_{eq}} = e^{\frac{\Delta\mu_r}{RT}}. \tag{12}$$

Thus, we can write the reversible MM rate equation, using relations 12, also as

$$v = \frac{V_{max}^+ \frac{s}{K_{M,S}} \overbrace{\left(1 - \frac{p}{sK_{eq}}\right)}^{1-e^{\frac{\Delta\mu_r}{RT}}}}{1 + \frac{s}{K_{M,S}} + \frac{p}{K_{M,P}}} \tag{13}$$

Why is equation 13 worth mentioning and is equation 10 not enough for modelling purposes? The reason is the Haldane relation (eq. 11),

$$K_{eq} = e^{\frac{-(\mu_P^{0\prime} - \mu_S^{0\prime})}{RT}} = \frac{V_{max}^+ K_{M,P}}{V_{max}^- K_{M,S}}.$$

Since the standard formation energies of P and S are independent of the enzyme, they are tabulated. Therefore, the equilibrium constant of the reaction is independent of the (amino acid structure of the) enzyme. Thus, all enzymes with the same reactants have the same equilibrium constant – so all hexokinases, aldolases, etc., regardless of the species and their (allosteric) regulation. So, when you know the reactants of a reaction, you can calculate, or look up, its equilibrium constant. Now look at the previous equations. When you know the equilibrium constant you know only three of the kinetic parameters

of the enzyme need to be known and not four, i.e. $V^-_{max} = \frac{V^+_{max} K_{M,P}}{K_{eq} K_{M,S}}$. Hence, we often write the reversible MM rate equation (eq. 10) as equation 13.

What is free energy and why is it lost in a chemical reaction?‡

Consider once more equation 12,

$$v = v^+ \left(1 - \frac{v^-}{v^+}\right), \quad \frac{v^-}{v^+} = e^{\frac{\Delta\mu_r}{RT}}, \quad \Delta\mu_r = RT \ln \frac{v^-}{v^+},$$

from which we conclude that

$$v = v^+ \left(1 - e^{\frac{\Delta\mu_r}{RT}}\right),$$

which is a general equation: valid for all reversible chemical reactions described by mass-action and enzyme kinetics. This equation indicates that

1. $v > 0$ when $\Delta\mu_r < 0$ since then $e^{\frac{\Delta\mu_r}{RT}} < 1$
2. $v = 0$ when $\Delta\mu_r = 0$ since then $e^{\frac{\Delta\mu_r}{RT}} = 1$
3. $v < 0$ when $\Delta\mu_r > 0$ since then $e^{\frac{\Delta\mu_r}{RT}} > 1$

Thus, the rate of a reaction has an opposite sign when compared to the sign of its free energy potential,

$$sign(v) = -sign(\Delta\mu_r).$$

From the last equation we conclude that the following law is obeyed by every (bio)chemical reaction, the product of its rate and minus its free energy potential, i.e. $-\Delta\mu_r$, is larger than or equal to 0,

$$-v\Delta\mu_r = (v^+ - v^-)RT \ln \frac{v^+}{v^-} \geq 0$$

From relations 12 we also infer that

$$-\Delta\mu_r = RT \ln \frac{v^+}{v^-}.$$

This relation indicates that a reaction with a more negative free energy potential (thus $\Delta\mu_r < 0 \rightarrow \Delta\mu_r << 0$) favours the forward reaction over the backward reaction, i.e. $v^+ > v^-$. When there exists no net potential, so $\Delta\mu_r = 0$, then the forward and backward reaction have an equal rate.

Thus, what is 'free energy'? It biases reactions (or generally processes) to particular directions. This is what '(chemical) work' means.

Consider the reaction $S \rightleftharpoons P$ and that $\Delta\mu_r < 0$. Now we know that $v > 0$ and $v^+ > v^-$; thus, P is made and S is lost. How does

In fact, $-v\frac{\Delta\mu_r}{T}$ equals entropy production and from basic thermodynamics you may remember that entropy S always increases or stays constant. Hence $dS/dt = -v\frac{\Delta\mu_r}{T} \geq 0$ and $\frac{-\Delta\mu_r}{T}$ is then called the thermodynamic driving force. These relations show that $sign(ds/dt) = -sign(\Delta\mu)$. Since, $dS/dt \geq 0$ for all reactions inside reaction networks, it is also true for the entire network. We will return to this later, it leads to the conclusion that metabolic network consume free energy and produce entropy to maintain themselves at a steady state that it life supporting.

this change in concentration of S and P at time t influence $\Delta\mu_r$ at the next moment in time? Do we get more free energy from that or do we loose it? Thus, does $\Delta\mu_r$ rise (and we loose energy) and become less negative. Or do we gain energy? When we are loosing Gibbs free energy, then the system consumes it – this consumption or free energy loss is called dissipation. To address this we need to know whether $\frac{d\mu_r}{dt}$ is positive. Thus, consider,

$$\begin{aligned}
\frac{d\mu_r}{dt} &= \frac{\partial\mu_r}{\partial s}\frac{ds}{dt} + \frac{\partial\mu_r}{\partial p}\frac{dp}{dt} \\
&= \left(-\frac{\partial\mu_r}{\partial s} + \frac{\partial\mu_r}{\partial p}\right) v \qquad (note: \mu_r(t) = RT\ln\frac{p(t)}{s(t)K_{eq}}) \\
&= \left(-\frac{\partial \ln\frac{p}{sK_{eq}}}{\partial s} + \frac{\partial \ln\frac{p}{sK_{eq}}}{\partial p}\right) v \\
&= \left(\frac{1}{s} + \frac{1}{p}\right) v > 0. \qquad (14)
\end{aligned}$$

This shows that the free energy is lost while the reaction proceeds in the positive direction as the free energy becomes less negative with time. Thus, when $\Delta\mu_r < 0$ such that $v > 0$ and the reaction makes P from S, $d\Delta\mu_r/dt > 0$ and therefore $\Delta\mu_r$ becomes less negative, we are loosing Gibbs free energy. As a consequence, v decreases, because v^+/v^- reduces, and P is made less quickly from S by the enzyme. This continues until $\Delta\mu_r = 0$, then $v = v^+ - v^- = 0$ and then $p_e/s_e = K_{eq}$. Thus chemical reactions dissipate free energy unless it is lost (and all has been turned into entropy) and thermodynamic equilibrium is reached .

Note that this not made that entropy production now always implies heat production, as some chemical reactions are exergonic (heat producing) and others are endergonic (heat consuming). So you can make a refrigerator with chemical reactions!

The speed limit of enzyme is set by reactant diffusion and provides an upper bound for $k^+_{cat}/K_{M,S}$[‡]

Consider again equation 13, but now without product,

$$v = V^+_{max}\frac{s}{s + K_{M,S}}.$$

When the concentration of the substrate is very low, say because the enzyme is consuming it so rapidly, then the rate equals $\frac{V^+_{max}}{K_{M,S}}s$ and is therefore proportional to

$$v \propto \frac{V^+_{max}}{K_{M,S}} \propto \frac{k^+_{cat}}{K_{M,S}}.$$

The parameter $\frac{k^+_{cat}}{K_{M,S}}$ is a very important property of enzymes and generally considered to reduce during evolution.

See, for instance: Bar-Even et al. The Moderately Efficient Enzyme: Evolutionary and Physicochemical Trends Shaping Enzyme Parameters, Biochemistry, 50, 21, 4402-4410, 2011.

An enzyme that has a very high value of $\frac{k_{cat}^+}{K_{M,S}}$ is considered very efficient as it has a high rate when the substrate concentration is low. When does this occur? From above, we know that

$$k_{cat}^+ = \frac{k_2^+ k_3^+}{k_2^- + k_2^+ + k_3^+}, \quad K_{M,S} = \frac{k_1^- k_2^- + k_1^- k_3^+ + k_2^+ k_3^+}{k_1^+ (k_2^- + k_2^+ + k_3^+)},$$

which leads to (since all k's are positive)

$$\frac{K_{M,S}}{k_{cat}^+} = \frac{1}{k_1^+} + \frac{k_1^-}{k_1^+ k_2^+} + \frac{k_1^- k_2^-}{k_1^+ k_2^+ k_3^+} \geq \frac{1}{k_1^+},$$

and therefore,

$$\frac{k_{cat}^+}{K_{M,S}} \leq k_1^+.$$

Thus, the speed limit of a reversible MM enzyme per unit enzyme and substrate is set by the rate of the reaction $E + S \xrightarrow{k_1^+ \cdot e \cdot s} ES$ divided by the enzyme concentration and substrate concentration so by k_1^+.

You may find this confusing. Isn't the speed limit of an enzyme determined by its V_{max}^+? That is indeed the speed limit when the concentration of S is very high and this concentration is then considered as fixed. In a metabolic network, the concentration of S is a variable and a very speedy enzyme will reduce its concentration because it consumes it so fast. When an enzyme evolves and operates ever faster, its rate becomes more and more comparable to that of the rate of its diffusional encounters with substrate. Eventually it becomes limited by those diffusional encounters and the enzyme is effectively always waiting for a new encounter and, hence, its rate has become diffusion limited. Then, the time for the reaction $S \rightarrow P$ is much shorter than the waiting time for the next diffusional encounter. Then, you have the fastest enzyme possible and evolution cannot speed it up any more. An example of such an enzyme is carbonic anhydrase.

The rate constant k_1^+ has to do with the diffusional encounter of the enzyme with its substrate and the probability that such an encounter leads to binding. Thus, one can think of the reaction $E + S \xrightarrow{1} ES$ as involving a transition state complex $ES^\ddagger$, i.e. $E + S \overset{1a}{\rightleftharpoons} ES^\ddagger \xrightarrow{1b} ES$ (according to Eyring's transition state theory). When $k_{1b} >> k_{1a}^-$ nearly all encounters are productive and when $k_{1b} >> k_{1a}^+$ the enzyme-substrate complex formation is diffusion limited.

What is the maximal value of k_1^+? Earlier we already used the relationship that the time between diffusional encounter of two molecules A and B equals,

$$\tau = \frac{V}{4\pi(D_A + D_B)(r_A + r_B)}.$$

This equation we can turn into k_1^+ via an unit conversion. The unit of k_1^+ is $M^{-1}s^{-1}$ while the unit of τ is s (for two single molecules). So,

$$k_1^+ = \frac{VN_A}{\tau} = 4\pi(D_A + D_B)(r_A + r_B)N_A,$$

which has unit: $dm^3/(mol \cdot s) = M^{-1}s^{-1}$ with D in dm^2/s and r in dm. When we consider glucose with a diffusion coefficient of $600\mu m^2/s$ in water and an enzyme with a radius of 5 nm then we obtain

$$\begin{aligned} k_1^+ &= \frac{VN_A}{\tau} \approx 4\pi D_{glc} r_{enzyme} N_A \\ &= 4\pi 600 \frac{(10^{-5}dm)^2}{s} \cdot 5 \cdot 10^{-8} dm \cdot 6 \cdot 10^{23} \frac{1}{mol} \\ &\approx 10^{10} M^{-1}s^{-1}. \end{aligned} \tag{15}$$

An analysis of enzyme data from databased by Bar Even et al. (dx.doi.org/10.1021/bi2002289) indicates that the average enzyme has $k_{cat} \approx 10\ s^{-1}$ and $k_{cat}/K_M \approx 10^5\ M^{-1}s^{-1}$, so way below the speed limit. As one of the fastest enzyme of the planet, they report superoxide dismutase anhydrase, which has a $k_{cat}/K_M \approx 10^9\ M^{-1}s^{-1}$ and a $k_{cat} \approx 2 \cdot 10^5\ s^{-1}$.

The affinity-vs-rate trade-off for enzymes with an equilibrium constant that is close 1 (e.g. facilitated diffusion transporters)‡

Above we deduced that

$$K_{eq} = \frac{V^+_{max} K_{M,P}}{V^-_{max} K_{M,S}}$$

Now consider a transporter for glucose that operated according to a facilitated diffusion mechanism. Thus we are considering

$$glc_{out} \rightleftharpoons glc_{in}.$$

This reaction has an equilibrium constant that equals 1 (the physico-chemical conditions in and outside of the cell are sufficiently similar). Then,

$$\frac{V^+_{max}}{K_{M,S}} = \frac{V^-_{max}}{K_{M,P}}$$

and if one realises that the affinity of the enzyme for S equals $A_S = K^{-1}_{M,S}$ and $A_P = K^{-1}_{M,P}$ for P then

$$V^+_{max} A_S = V^-_{max} A_P.$$

This relation indicates that an enzyme with a high affinity and high maximal import rate for the substrate also has a high value of $V^-_{max} A_P$ and therefore can not profit from those features very much, as the export works equally good. It also indicates that a higher affinity for the substrate is always accompanied by a lower maximal import rate, V^+_{max}, when $V^-_{max} A_P$ is considered constant. Enzymes with an equilibrium constant that far exceeds 1, e.g. 100, do not suffer from this because they obey

$$V^+_{max} A_S = 100 V^-_{max} A_P.$$

A 4-state catalytic cycle of an enzyme as a generic model for motor proteins, transporters and metabolic enzymes‡

A 4-state catalytic cycle leads to an rate equation that has a broad applicability, e.g. a motor protein, nutrient transporter, or metabolic

enzyme (a 2-substate, 1-product enzyme or a 1-substrate, 2-product enzyme) (Fig. 27). It is therefore worthwhile to take a moment and derive its rate equation, using the steady-state method.

We start again from the rate of change equations for the concentration of the enzyme species,

$$\begin{aligned}
\frac{d}{dt}e_0 &= v_4 - v_1 \\
\frac{d}{dt}e_1 &= v_1 - v_2 \\
\frac{d}{dt}e_2 &= v_2 - v_3 \\
\frac{d}{dt}e_3 &= v_3 - v_4
\end{aligned}$$

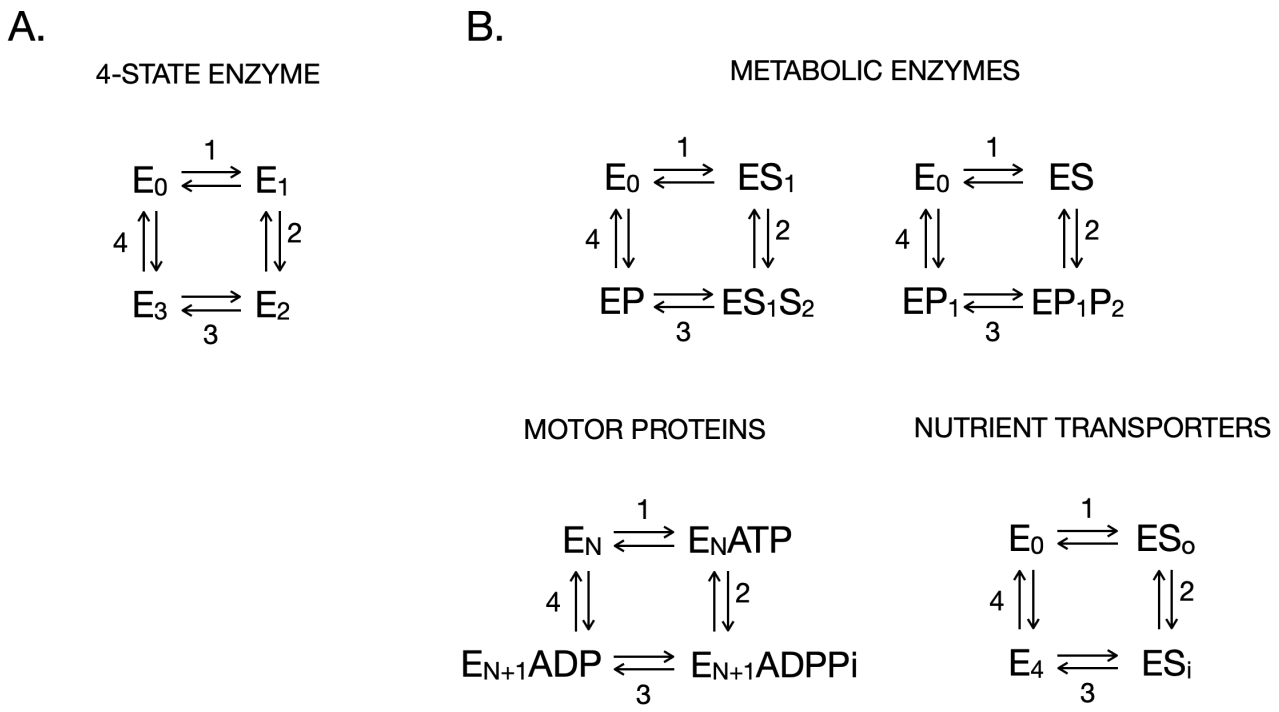


Figure 27: **A 4-state enzyme mechanism and examples showing its versatility.** A. A 4-state enzyme mechanism is shown. B. Four examples of 4-state enzyme mechanisms are shown: two metabolic enzymes catalysing either $S_1 + S_2 \rightleftharpoons P$ or $S \rightleftharpoons P_1 + P_2$, a motor protein that takes a step along polymer from position N to $N+1$ by hydrolysis of ATP ($E_N + ATP \rightleftharpoons E_{N+1} + ADP + Pi$) and a nutrient transporter transporting a nutrient S ($S_o \rightleftharpoons S_i$) that can flip inside the membrane, either facing the binding site of S on the extracellular site (E_0 state) or the intracellular site (E_4 state).

Since the different examples systems have different locations in the mechanism where reactants bind the unit of the elementary rate constants are not always the same. For instance, k_4^- is a second order rate with unit $conc^{-1}time^{-1}$ in all the schemes except for the nutrient transporter than it is a first order rate constant with unit $time^{-1}$. To

accommodate this variation we write the rate equations as,

$$\begin{aligned} v_1 &= \kappa_1^+ e_0 - \kappa_1^- e_1 \\ v_2 &= \kappa_2^+ e_1 - \kappa_2^- e_2 \\ v_3 &= \kappa_3^+ e_2 - \kappa_3^- e_3 \\ v_4 &= \kappa_4^+ e_3 - \kappa_4^- e_0. \end{aligned}$$

The κ's have interpretations in terms of elementary rate constants and concentrations that are dependent on the associated enzyme mechanism (e.g. those shown in Fig. 27).

Again we have the restriction that the total enzyme concentration is fixed,

$$e_T = e_0 + e_1 + e_2 + e_3$$

and that due to that a linear combination exists between the rate of change equations, i.e.

$$\frac{d}{dt}e_0 + \frac{d}{dt}e_1 + \frac{d}{dt}e_2 + \frac{d}{dt}e_3 = 0,$$

which implies that the following four equations,

Can you think of another set of equations we could have used instead?

$$\begin{aligned} v_4 - v_1 &= 0 \\ v_1 - v_2 &= 0 \\ v_2 - v_3 &= 0 \\ e_0 + e_1 + e_2 + e_3 &= e_T, \end{aligned}$$

should be used to solve for the (quasi-) steady state concentrations of the enzyme species (i.e. $e_{0,s}$, $e_{1,s}$, $e_{2,s}$, and $e_{3,s}$) , either by hand, linear algebra or software. This gives some quite complex equations, which we do not show here.

The (quasi-steady state) rate of the enzyme is given by one of the rates of the enzyme-state transitions in the mechanism (since they are equal at steady state) with the quasi-steady state concentrations of the enzyme species substituted. This leads to the rate equation,

$$v = e_T \frac{\left(\kappa_1^+\kappa_2^+\kappa_3^+\kappa_4^+ - \kappa_1^-\kappa_2^-\kappa_3^-\kappa_4^-\right)}{\kappa_2^-\kappa_3^- + \kappa_2^+(\kappa_3^- + \kappa_3^+))(\kappa_1^+ + \kappa_4^-) + (\kappa_2^+\kappa_3^+ + \kappa_1^+(\kappa_2^- + \kappa_2^+ + \kappa_3^+))\kappa_4^+ + \kappa_1^-((\kappa_3^- + \kappa_3^+)\kappa_4^- + \kappa_3^+\kappa_4^+ + \kappa_2^-(\kappa_3^- + \kappa_4^- + \kappa_4^+)}.$$

This is a rather lengthy rate equation as you can see. Much lengthier than the rate equation we saw in the previous chapter when using the rapid-equilibrium assumption. It is however a better description of the rate of an 4-state enzyme than its rapid-equilibrium approximation.

Again we proceed now, which is what is commonly done, and use the Cleland formalism to identify enzyme-kinetic parameters such as K_M's, K_I's and V_{max}'s.

Exercise

1. Identify the interpretation of the κ's in the last equations in terms of elementary rate constant and reactant concentrations for all the

examples shown in figure 27.

2. Consider the numerator of the rate equation for the motor protein. How much more often does the enzyme step forward when the ratio $ATP/(ADP \times Pi)$ is doubled?

Why cells use enzymes

There are at least four reasons why cells exploit enzymes, possibly more. They are considered one by one below.

To speed up reactions and make spontaneous, hazardous chemical reactions negligible. Multi-substrate chemical reactions always involve two steps: productive collisions of the substrates leading to binding followed by the conversion. This is, however, not how we often write reactions; hence,

$$A + B \rightarrow AB$$

is not written as,

$$A + B \rightleftharpoons AB^{\ddagger} \rightarrow AB,$$

with $AB^{\ddagger}$ as the so called transition-state complex. The latter two-step depiction of the reaction represents, however, the current understanding of how reactions work and why some are more active than others.

The rate of the two-step reaction is dependent on the rate of productive collisions – leading to the transition-state complex – and the subsequent conversion. One can think of the transition-state complex as a 'barrier' that needs to be overcome in order for the reaction to occur. According to Eyring's rate theory (`https://en.wikipedia.org/wiki/Transition_state_theory`), enzymes speed up the reactions by reducing this barrier. The magnitude of the barrier is associated with a free energy that A and B require to stably bind. The energy for spontaneous reactions is of the order of $k_b T$ and if the needed free energy is higher than this the stable formation of AB may be very unlikely. What enzymes do is that they offer physicochemical conditions in their catalytic site such that the free energy barrier is lower, which makes the stable formation of AB more likely and, therefore, speeds up the rate. For instance, the enzyme adenylate kinase increases the reaction by several orders of magnitude (Kerns, S., et al. Nat Struct Mol Biol 22, 124–131, 2015). There is a catch, however, enzymes cannot change the equilibrium constant

of reactions such that when they speed up a reaction they do this by increasing the forward and backward reaction by the same factor, in agreement with the Haldane relationships we saw before.

By speeding up reactions with enzymes, cells also 'lift' all life-supporting reactions onto a time scale that is much faster than that of spontaneous (uncatalysed) chemical reactions, which ensures that spontaneous, life-unsupporting chemical reactions have negligible rates.

TO REGULATE RATES OF REACTIONS BY ALTERING ENZYME CONCENTRATIONS AND ALLOSTERIC REGULATION. Usage of enzymes as catalysts gives cells the opportunity to modulate rates according to conditions, via changes in concentrations of enzymes and allosteric effectors (via feedback or feedforward loops). Changes in enzyme concentrations occur via gene expression, which acts on a slower time scale than changes in effector concentrations. This means that cells exploit a mode of fast and slow regulation of enzyme activities.

TO REVERT THE DIRECTION OF A REACTION INTO ITS ENERGY-DEMANDING DIRECTION BY COUPLING IT TO ANOTHER ENERGY-LIBERATING REACTION. The net rate v of a reversible reaction with forward rate v^+ and backward rate v^- equals

$$v = v^+ - v^-.$$

This rate is related to the change in its free energy (or Gibbs energy) $\Delta\mu$ as

$$v = v^+ \left(1 - \frac{v^-}{v^+}\right), \ \frac{v^-}{v^+} = e^{\frac{\Delta\mu}{RT}}.$$

For a reaction with a single substrate and product, e.g. $A \xrightarrow{1} B$ the change in the free energy is given by

$$\Delta\mu_1 = \mu_B - \mu_A,$$

with the formation free energy of compound X at concentration x defined as $\mu_X = \mu^{0'} + RT \ln x$.

From these relations, we conclude

$$\begin{aligned} v < 0 &\Rightarrow \Delta\mu > 0 \\ v = 0 &\Rightarrow \Delta\mu = 0 \\ v > 0 &\Rightarrow \Delta\mu < 0. \end{aligned}$$

Thus the sign of reaction is always opposite of that of the sign of its free energy change. Thus a reaction proceeds in the direction of a loss of free energy, i.e. 'downhill of its free energy gradient' (Fig. 28).

Note that these $\mu - v$ relations imply that each reaction obeys

$$-v \ln \frac{v^-}{v^+} = -v\Delta\mu \geq 0,$$

This can be shown to be proportional to the entropy production, dS/dt, which is either zero or positive (for a cell and its environment together), due to the second law of thermodynamics. For a network of reactions, the entropy production equals $\frac{dS}{dt} = -\sum_i \frac{v_i \Delta\mu_i}{T} \geq 0$ where the sum is over all its reactions (intracellular and in-/exporting reactions). Also, for each reactions we have the relation: $sign(v_i) = -sign(\Delta\mu_i)$.

Thus when $\Delta\mu_1 = \mu_B - \mu_A < 0$, A is turned into B, the rate is positive, and free energy is produced by the reaction. (Hence, B contains less free energy than A or, in other words, B costs less Gibbs energy to form reference compounds than A). Enzymes can use such produced free energy to run another reaction – perform chemical work –, for instance, $C \xrightarrow{2} D$, in the direction that goes against its free energy gradient, i.e. in its $\Delta\mu_2 = \mu_D - \mu_C > 0$ direction. This feasible if and only if,

$$\Delta\mu_3 = \underbrace{\Delta\mu_1}_{\ll 0} + \underbrace{\Delta\mu_2}_{>0} < 0.$$

The net reaction catalysed by the enzyme is then

$$A + C \xrightarrow{3} B + D,$$

and the free energy liberated in reaction $A \xrightarrow{1} B$ is used to drive $C \xrightarrow{2} D$ uphill its free energy gradient, since $\Delta\mu_2 > 0$. This is a great feat of enzyme catalysis!

For example, ATP synthase achieves this reaction coupling when it uses the free energy liberated by proton flow along its downward concentration gradient to make ATP from ADP and Pi, which is an energy demanding reaction. Thus,

$$ADP + Pi \xrightarrow{1} ATP,\ \Delta\mu_1 > 0$$

while

$$H_o^+ \xrightarrow{2} H_i^+,\ \Delta\mu_2 << 0$$

such that

$$H_o^+ + ADP + Pi \xrightarrow{ATP\ synthase} H_i^+ + ATP,\ \Delta\mu_{ATP\ synthase} = \Delta\mu_1 + \Delta\mu_2 < 0,$$

provided that the enzyme can achieve sufficient free energy transduction from reaction 2 to 1 to make $\Delta\mu_3 < 0$. Note that ATP synthase functioning requires also the pumping of protons against their gradient to reestablish the proton motive force: $H_i^+ \xrightarrow{2} H_o^+$. The required

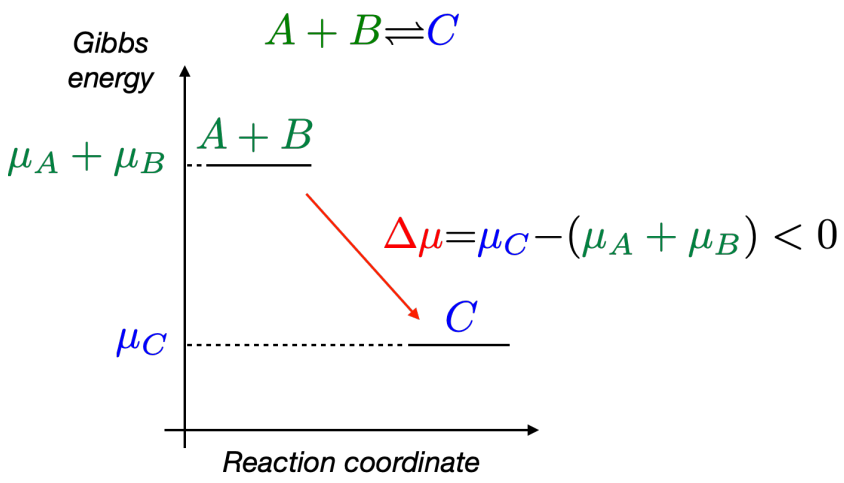


Figure 28: **All reactions proceed in a direction that liberates Gibbs free energy and is therefore in a direction of reduced free energy.** Thus, free energy is lost – 'dissipated' – in agreement with the second law of thermodynamics. Here we considered the reaction $A + B \rightleftharpoons C$ as an example, but the principle applies to all reactions.

free energy is obtained, for instance, from the respiration of glucose into carbon dioxide and water.

It is important to realise that these arguments about the direction of reactions did not involve any kinetics, only free energies. The free energy of a reaction with rate $v = v^+ - v^-$ equals the ratio of its backward over its forward rate, i.e. $v^-/v^+ = e^{\frac{\Delta\mu}{RT}}$, which no longer contains $time^{-1}$ in its unit in contrast to the rates. This conclusion is universally valid, i.e. for enzyme-catalysed and uncatalysed reactions (enzyme kinetics and mass-action kinetics).

To allow for evolutionary tuning of reaction kinetics

Evolution tinkers with the properties of enzymes via mutations in their genes. As a result, the rate of a reaction at specific reactant, effector and enzyme concentrations is altered – presumably in a direction of enhanced evolutionary fitness. A key aspect of life is that enzyme properties are evolvable by changes in their amino-acid sequence.

Exercise

1. To become a bit more familiar with the enzymes of glycolysis consider once more the website `https://pdb101.rcsb.org/motm/50`, showing all the glycolytic enzymes.

   (a) Make a table with the identity of the glycolytic enzymes as its rows and their following information in its columns: the enzyme name, the number of subunits, the reaction it catalyses, the equilibrium constant of the reaction, the k_{cat} of the reaction and the k_{cat}/K_M (use a K_M of one of the substrates).

The last three pieces of information you can obtain from the two supplementary Excel files of a paper by Flamholz et al. (Glycolytic strategy as a tradeoff between energy yield and protein cost, PNAS, 110(24), 10039-10044, 2013). You can calculate the equilibrium constant K_{eq} using the following relationship $K_{eq} = e^{\frac{-\Delta G_r^{0'}}{RT}}$ (the value of $-\Delta G_r^{0'}$ is in the second supplementary Excel file).

(b) Verify for two reactions in glycolysis that Lavoisier's principle of 1987 and that mass, and therefore also chemical elements, is conserved in a chemical reaction.

Hence, with chemistry we cannot turn lead into gold – despite all the efforts of alchemists. This can only be done by nuclear fusion, which is what happens in stars. In stars, all chemical elements are made from lighter elements (e.g. protons) made during the Big Bang. The larger the star, the heavier the chemical elements are that can be formed in it by nuclear fusion. This is how the chemical elements of the 'periodic table of chemical elements' are formed in the universe in a process called nucleosynthesis (`https://shorturl.at/ACR01`).

(c) The rate v of a reaction is zero at thermodynamic equilibrium and proportional to its displacement from thermodynamic equilibrium,

$$v \propto 1 - \frac{\prod_i p_i}{\prod_j s_j K_{eq}}$$

with p_i and s_j as the concentrations of the products and substrates of the reaction so we were considering the reaction (with all stoichiometric coefficients equal to 1)

$$\sum_j S_j \rightleftharpoons \sum_i P_i.$$

From the above, we conclude that a rate is positive when

$$\frac{\prod_i p_i}{\prod_j s_j} < K_{eq}.$$

This means that inequality bounds exists for the concentrations of the reactants of all the reactions in glycolysis that need to be met in order to ensure that they all occur in the glycolytic direction from glucose to pyruvate. Assume that $ATP/ADP = 10$, $NADH/NAD = 0.1$, and $Pi = 1\ mM$ (which we took from Noor et al., `https://doi.org/10.1371/journal.pcbi.1003483`). Determine all those inequalities for the concentrations.

At equilibrium $\frac{\prod_i p_{i,e}}{\prod_j s_{j,e}} = K_{eq}$ and the concentration of the reaction's reactants have attained their equilibrium values denoted by the subscript 'e'.

This type of reasoning was used in this great paper `https://doi.org/10.1016/j.ymben.2011.02.005` by Canelas et al. on thermodynamic aspects of glycolysis.

Suggestions for further reading

1. Hill, T. (2012). Free energy transduction in biology: the steady-state kinetic and thermodynamic formalism. Elsevier.

2. Hill, Terrell L. Cooperativity theory in biochemistry: steady-state and equilibrium systems. Springer Science & Business Media, 2013.

3. Cornish-Bowden, Athel. Fundamentals of enzyme kinetics. John Wiley & Sons, 2013.

4. Segel, I. H. (1975). Enzyme kinetics: behavior and analysis of steady-state and rapid equilibrium enzyme systems. Willey-Interscience, New York.

5. Phillips, R., Kondev, J., Theriot, J., & Garcia, H. (2012). Physical biology of the cell. Garland Science.

Part II

Models of Metabolism

Basics of kinetic models of metabolism

Steady states and dynamics of metabolic networks

A microbe requires about 250-300 metabolic reactions to grow in a particular nutrient broth (`https://doi.org/10.1073/pnas.93.19.10268`). It then makes all the precursors for macromolecules from nutrients, make those macromolecules from them, using the energy extracted from the nutrients which act as the energy source in its catabolism.

In an entire metabolic network (Fig. 29) all enzymes are coupled via mass flow – the sequential conversion of reactants – and allosteric regulation, of which some effects are rather distant: for instance, in *E. coli*, phosphofructokinase is regulated by citric acid cycle intermediates and glucose import by the phosphotransferase system is regulated by 2-oxoglutarate (the C-skeleton provider for amino acid biosynthesis).

Note that the minimal number of needed reactions for growth can also be calculated from a flux balance analysis. How would you do that and how do you then guarantee that the set of computed reactions is the minimal set?

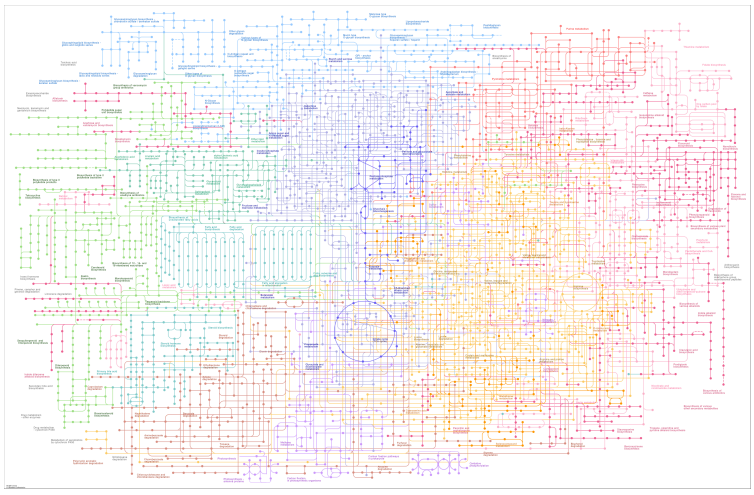


Figure 29: **An overview of cellular metabolism from the KEGG database.** Microbial genomes encode huge metabolic networks. All reactions are coupled via mass flow and in addition by allosteric interactions, which are not shown here, for coordination of fluxes. That coordination remains poorly understood.

When microbes grow in a constant environment (constant (or excess) nutrient concentrations, constant temperature, pH, etc.) then the specific growth rate of a population of cells eventually settles to a fixed value. The metabolic network (of the average cell) is then op-

erating at a steady state. At such a steady state, the concentration of all the metabolic intermediates occurring in the network remain constant, because their net synthesis rate is balanced by their net degradation rate. Such states can be calculated with mathematical models of metabolic networks, from the kinetics and allosteric regulation of all the enzymes and a specification of the environment of the cell. These models can also predict the dynamics of a metabolic network when changes are made to the environment of the cell. These models are called kinetic models and they are the topic of this chapter.

Before we set out to explore complex, realistic models we will study some toy models illustrating some specific aspects of metabolic networks. Those models are deliberately made simple so that they do not contain any features that can distract from their main message.

Not all mathematical models of metabolism settle to a steady state, some oscillate

Glycolysis in yeast can display oscillations during constant environmental conditions (Fig. 30). This indicates that metabolism does not always settle to a steady state – characterised by constant metabolite concentrations and reaction activities (fluxes) – when the external conditions are constant. This has been known already for a long time. The first models that explained glycolytic oscillations proposed the curious regulation of phoshofructokinase as a reason for the onset of oscillations. A good overview about such models a paper by Goldbeter & Dupont (Goldbeter & Dupont, Allosteric regulation, cooperativity and biochemical oscillations, Biophysical Chemistry, 37, 341, 1990). Their model is highly simplified, gives rise to oscillations and it proposes that oscillations are caused by the MWC kinetics of phosphofructokinase. Have a look at their paper if you are interested. We will focus on steady states in what follows.

The steady state of a metabolic network is characterised by steady-state concentrations and fluxes

When a metabolic network has attained a steady state then all the metabolite concentrations that are considered as variables in the experiment or model have attained steady-state and constant concentrations. It is customary to refer to reaction rates at steady state as fluxes. Thus at steady-state, the net synthesis fluxes of all metabolites equal their net degradation fluxes – they balance. Thus, the steady-state concentrations have attained such values that all these fluxes balance, for each metabolite.

This does not mean that the metabolism in individual cells cannot be in an oscillating state as those would only be visible when all cells would oscillate in synchrony – which, by the way, they can do under particular, but rare, conditions.

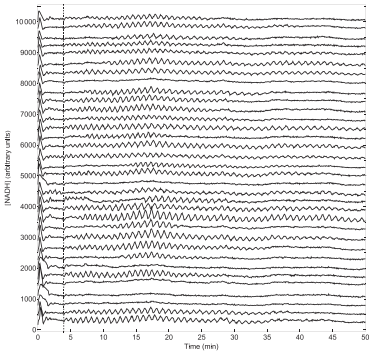


Figure 30: **Dynamics of single yeast cells showing glycolytic oscillations.** From Gustavsson A.K., et al. Sustained glycolytic oscillations in individual isolated yeast cells, FEBS Journal, 279, 2837-2847, 2012. This is about 60 years after the start of this scientific field when the first glycolytic oscillations were observed by Britton Chance. If you want to play with the mathematical model that was used to analyse this data go to `https://jjj.bio.vu.nl/models/gustavsson1/simulate/`.

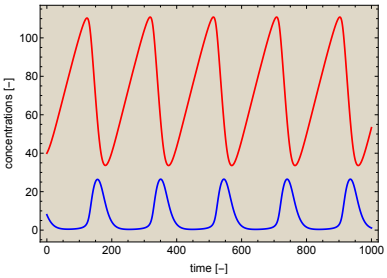


Figure 31: **Golbeter's model of glycolytic oscillations from Goldbeter & Dupont, Biophysical Chemistry, 1990.**

Mathematically, this means that for all variable metabolites we have the following rate of change equation of its concentration,

$$\text{Dynamic conditions: } \frac{dx_i}{dt} = \sum_j n_{ij} v_j(\mathbf{x}, e_j, \text{kinetic parameters})$$
$$\text{Steady-state conditions: } \sum_j n_{ij} J_j(\mathbf{x}_s, e_j, \text{kinetic parameters}) = 0 \;\; \text{(steady state)}$$

where v_j is the rate of reaction j, and J_j its steady state flux, and metabolite x_i is either used ($n_{ij} < 0$, made ($n_{ij} > 0$) or is not involved ($n_{ij} = 0$) in this reaction. The rate v_j are, in principle, described by equations that follow the reversible MWC rate equation and are therefore functions of concentrations of reactants and effectors (in the concentration vector $\mathbf{x}$, the concentration of the catalysing enzyme and kinetic parameters of this enzyme). When we consider the enzyme concentration as fixed – so we are considering a fast, metabolic time scale – then they are parameters, like the kinetic parameters, i.e. we know their values. Then the steady-state condition is best written as,

$$\text{for all variable metabolites } i: \; \sum_j n_{ij} J_j(\mathbf{x}_s) = 0,$$

indicating that if we know the metabolite concentrations at steady state that we know all the flux values, the J_j's. Since we have as many variable metabolite concentrations as $dx_i dt = 0$ equations, we have as many equations as unknowns and, hence, we can solve the steady-state concentrations (typically using numerical methods).

Consider once more the steady-state equation, $\sum_j n_{ij} J_j(\mathbf{x}_s) = 0$, without thinking about the concentrations,

$$\sum_j n_{ij} J_j = 0.$$

we have as many of these equations as variable metabolites. Can you think of an example where we have more fluxes than the number of such equations? How many fluxes do you need to know, as prior knowledge, if you want to determine all fluxes when you have 1, 2, 3, or 4 more fluxes than equations?

Perhaps this is best illustrated with an example. Go to the JWS Online website (`https://jjj.bio.vu.nl/models/teusink/simulate/`) choose steady state and perform a steady state simulation. The steady-state concentration of the model are calculated and the fluxes. Confirm that 2-phosphoglycerate is synthesised at the same that it is degraded and calculate those rates using the kinetics of the associated enzymes, given the associated steady-state concentration of the reactants.

Consider the linear pathway,

$$\underline{S} \overset{1}{\rightleftharpoons} X \overset{2}{\rightleftharpoons} Y \overset{3}{\rightleftharpoons} Z \overset{4}{\rightleftharpoons} \underline{P},$$

with the concentrations of S and P fixed. We have the following rate of change equations,

$$\frac{dx}{dt} = v_1(x) - v_2(x,y), \; \frac{dy}{dt} = v_2(x,y) - v_3(y,z), \; \frac{dz}{dt} = v_3(y,z) - v_4(z)$$

and at steady state,

$$0 = v_1(x_s) - v_2(x_s, y_s), \; 0 = v_2(x_s, y_s) - v_3(y_s, z_s), \; 0 = v_3(y_s, z_s) - v_4(z_s),$$

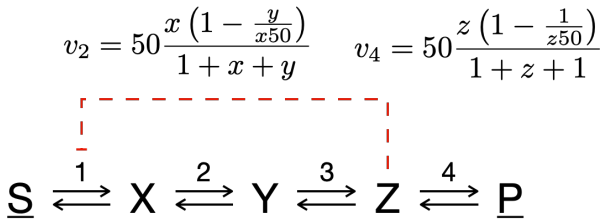
From this we also conclude that at steady state: $v_1 = v_2 = v_3 = v_4$.

indicating that we have as many equations, i.e. 3, as unknown concentrations x_s, y_s and z_s. So we can determine the concentrations if we would know how each rate is described in terms of a rate equation from enzyme kinetics. Then giving those concentrations we can calculate the steady state reaction rates. Consider now that we do not know any of the rate equations, then we cannot determine the concentrations. But we still now that

$$0 = v_1 - v_2,\ 0 = v_2 - v_3,\ 0 = v_3 - v_4.$$

Now we have four unknown fluxes and three equations, so we need to know 1 flux value to determine all steady-state flux values.

E.g. $v_1 = 3s/(1+x+s)$, $v_2 = 4x/(1+x+y)$, $v_3 = 15y/(1+y/2+z)$, $v_4 = 2z/(1+4z+2p)$ and $s = 10$ and $p = 5$.



$$v_1 = 10\frac{\left(1+1+\frac{x}{10}\right)^3\left(1-\frac{x}{100}\right)}{\left(1+1+\frac{x}{10}\right)^4+0.5\left(1+z\right)^4} \qquad v_3 = 50\frac{y\left(1-\frac{z}{y50}\right)}{1+y+z}$$

Figure 32: **A 4-enzyme pathway with a negative feedback of the last metabolite on the first enzyme.** The rate equations for all the enzymes are shown and are according to the general MWC equation. Enzyme 1 has 4 subunits and the remaining enzymes all have 1 subunit.

Negative feedback regulation leads to homeostasis of the feedback metabolite

Consider figure 32, it shows a linear pathway with four enzymes, a negative feedback loop and the rate equations of the enzymes. We consider the steady state of this model. All the enzyme rates are then equal. To understand the function of the feedback loop we plot the steady state flux of the subsystem composed of the first three enzymes as function of the concentration of the feedback metabolite Z. This steady state obeys $v_1(x_s, z) = v_2(x_s, y_s) = v_3(y_s, z)$ where z is a parameter. We also plot the rate of reaction 4 as function of the concentration of Z. This is shown in figure 33A where the number of subunits of the feedback-inhibited enzyme (reaction 1) was varied. The two lines intersect at the steady state of the entire system at the steady-state concentration of Z and then $v_1(x_s, z_s) = v_2(x_s, y_s) = v_3(y_s, z_s) = v_4(z_s)$, which is shown in figure 33B and C. A change in the maximal rate of the fourth enzyme changes the steady state and enhances the steady state flux. When the feedback is strongest the change in the steady-state concentration of Z is smallest, indicating

negative feedback leads to homeostasis of the feedback metabolite and that this is achieved with multi-subunit enzyme (more subunits is then better).

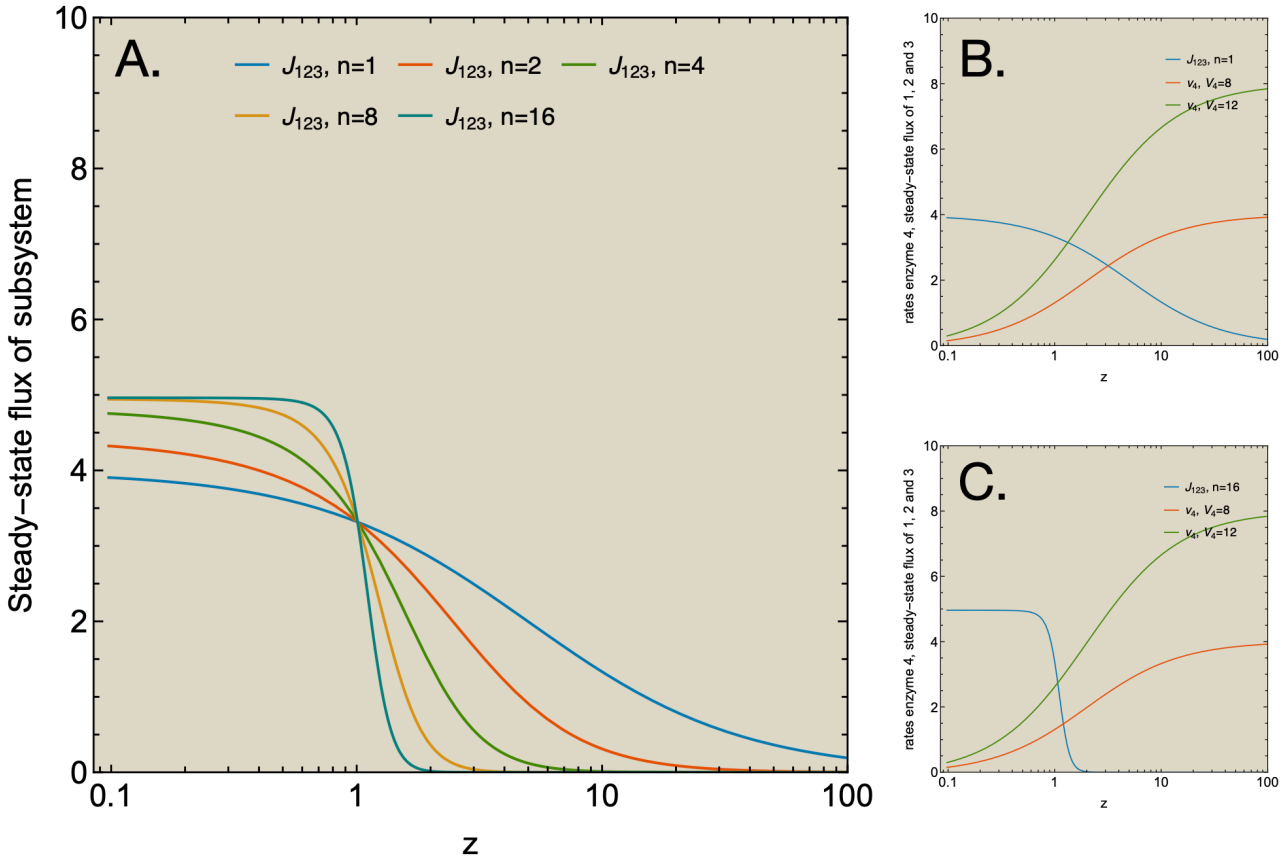


Figure 33: **Negative feedback in a linear pathway leads to homeostasis of the concentration of the feedback metabolite.** A. The steady-state flux of the subsystem, containing enzyme 1, 2 and 3, is plotted as function of the feedback metabolite z for different values of the number of subunits of the feedback inhibited enzyme. B and C. The steady-state flux of the subsystem, containing enzyme 1, 2 and 3, is plotted as function of the feedback metabolite z for a feedback-inhibited enzyme with 1 subunit (B.) and with 16 subunits (C.). The rate of enzyme 4 is also plotted as function as function of z for two values of its maximal rate V_4. These lines intersect at the steady state of the entire metabolic pathway. An increase of the number of subunits of the feedback-inhibited first enzyme makes its dependency on z steeper. Note that the change in the steady-state concentration of z, upon a rise in the V_{max} of the enzyme 4, is less when the feedback is stronger (when the subsystem's flux is more sensitive to z), showing that negative feedback leads to homeostasis of the feedback metabolite.

Reasoning about the effects of enzyme concentration changes on the steady state of a pathway

Changes in concentrations of enzymes change their maximal rates, i.e. $V_{max} = k_{cat}e$, and this induces a changes in the steady state of a metabolic network. This is, for instance, shown in figure 34 where we consider the metabolic pathway shown in figure 32 at a steady state with the maximal rate of enzyme 4 equal to 1. We then change this maximal rate 20 fold at time point 50 and determine the response of the system to this parameter change. The system responds by going to a new steady state with changes steady-state concentrations and pathway flux (Fig. 34).

The concentration of z increases while the others drop and the steady-state rate increases. Why is that?

Generally, it is the case that enzymes are inhibited by their products – such that the rate decreases when their concentrations rise – and activated by their substrates – the rate increases when their concentrations rise. Also, the rate of an enzyme increases when its concentration rises. In the model of the linear metabolic pathway (figure 32) all enzyme obey these rules. This is a general phenomenon, as product activation and substrate inhibition are rare. This enzyme-kinetic logic we can exploit to reason about the effects of changed enzyme concentrations on the change in steady-state concentrations and fluxes in a linear metabolic pathway, which we call its response.

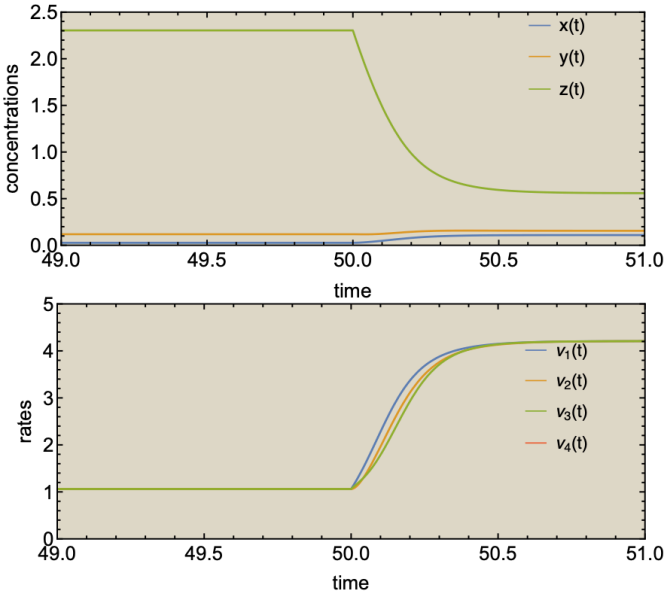


Figure 34: **A change in the concentration of an enzyme causes a metabolic pathway to choose from one steady state to another.** We considered the metabolic pathway shown in figure 32 that is at a steady state with maximal rate of enzyme 4 equal to 1. At time point 50, we change the maximal rate of enzyme 4 from 1 to 20. The response of the system is a drop in the steady state concentration of z and a rise in the steady state concentrations of x and y. Initially, the rates become unequal and then they equalise again after some time when the new steady state is reached. Why do some steady-state concentrations rise and some drop? Does the rate of a pathway always increase when we increase the activity of an enzyme, e.g. by increasing its maximal rate or, equivalently, its enzyme expression (by adjusting its gene control).

For instance, consider the linear pathway of figure 32 without the negative feedback loop. Say, we increase the concentration of enzyme 3. I do not think it requires any explanation that we can safely assume that steady-state flux will have gone up in the new steady state, since having more enzyme will speed up reactions. But what has happened to the steady-state concentrations of the reactants? Which ones increased and which decreased?

Since, we know that the flux has gone up and how changed metabolite concentrations influence enzyme rates, we can deduce which concentrations rose and which ones dropped. The easiest way to do this is to start either at the first or last enzyme in the linear pathway, since there depend only on a single reactant concentration. So, since the steady-state flux goes up:

- the rate of enzyme 1 increased in the new steady state. This can only have happened when its product concentration dropped to relief product inhibition if its enzyme concentration stays constant.

Thus, the concentration of X dropped.

- the concentration of Z must have increased since the only way that the rate of enzyme 4 could have increased in the new steady state is when the concentration of its substrate rose, since the concentration of enzyme 4 stayed constant.
- the rate of enzyme 2 also increased in the new steady state, but its substrate X dropped in concentration (which is reducing the rate of enzyme 2), so its product Y must have dropped too! This happens in order to relief product inhibition to such a degree that this can compensate for the rate decrease due to a reduced substrate concentration.
- So, X and Y drop in concentration and Z increases in concentration. Thus, the substrate of enzyme 3 (Y) decreased in concentration, while its product concentration Z rose. How can the rate then have increased in the new steady state? Well, this enzyme's concentration was increased!

If you understand this logic then you should be able to make the following exercises.

Here follows an example calculation for the linear pathway of figure 32 without the negative feedback loop when the starting values of the maximal rates of enzymes are 10 and then we change one of them to 20 and investigate the response of the steady state of the metabolic pathway, which you can determine by comparing the new steady state with the reference steady state when all maximal rates (the V_{max}'s) are 10.

	x_s	y_s	z_s	flux, J
reference condition	2.41752	2.04238	1.57709	4.35296
enzyme 1 increased	10.2935	5.88801	2.95401	5.92249
enzyme 2 increased	1.1406	2.52192	1.79434	4.67628
enzyme 3 increased	1.57917	0.846116	1.71386	4.56092
enzyme 4 increased	1.92433	1.31846	0.601982	4.47337

You can also generate such a table yourself of course using a simulation software package such as Copasi, Pycses, Mathematica or Matlab.

The flux always increases as you can see. When enzyme 1 its V_{max} increases all concentrations increase. When enzyme 2's V_{max} increases X drops and Y and Z increases. When enzyme 3's V_{max} increases X and Y drops and Z increases. When enzyme 4's V_{max} increases X, Y and Z drop. All of this follows from the logic that an enzyme is inhibited by its product and activated by its substrates and its own concentration.

Exercises

1. CHANGE OF A STEADY-STATE CONCENTRATION OF A REACTANT UPON A CHANGE IN ENZYME CONCENTRATION. To grasp the main concept consider the following, simplest metabolic network

$$\underline{S} \xrightarrow{1} X \xrightarrow{1} \underline{P}$$

and consider the following rate equations of the two enzymes

$$v_1 = V_1 \frac{\frac{s}{K_{1,S}}}{1 + \frac{s}{K_{1,S}} + \frac{x}{K_{1,X}}}$$
$$v_2 = V_2 \frac{\frac{x}{K_{x,X}}}{1 + \frac{x}{K_{2,X}} + \frac{p}{K_{2,P}}} \qquad (16)$$

with $V_1 = 2$, $s = 1$, $K_{1,S} = 0.2$, $K_{1,X} = 0.1$, $V_2 = 3$, $K_{2,X} = 0.3, K_{2,P} = 1$, $P = 0.1$.

a. Plot v_1 and v_2 as function of x in 1 figure. Write down the steady-state concentration of X and the steady state flux J.

b. Consider more enzyme 1, so change V_1 from 2 to 4. What happens to steady state concentration of X and the steady state flux?

c. Consider more enzyme 1, so change V_2 from 3 to 6. What happens to steady state concentration of X and the steady state flux?

d. Consider more enzyme 1 and 2, so change V_1 from 2 to 4 and V_2 from 3 to 8. What happens to steady state concentration of X and the steady state flux?

e. Consider more enzyme 1 and 2, so change V_1 from 2 to 4 and V_2 from 3 to 6. What happens to steady state concentration of X and the steady state flux?

2. STEADY-STATE RESPONSE OF A LINEAR PATHWAY WITHOUT FEEDBACK AND A PRODUCT-INSENSITIVE FIRST ENZYME. This is the result when all the enzyme V_{max} value are changed from 10 to 20, one-by-one (not simultaneously):

	x_s	y_s	z_s	flux, J
reference condition	4.24646	3.1216	2.04	5
enzyme 1 increased	1.74639×10^{52}	2.47872×10^{36}	-1.07044×10^{18}	10
enzyme 2 increased	1.45711	3.1216	2.04	5
enzyme 3 increased	2.11044	1.06773	2.04	5
enzyme 4 increased	2.78991	1.72107	0.693333	5

What's the explanation of the metabolic explosion and why does it only occur when the concentration of enzyme 1 is increased?

For a realistic example consult the phenotype of a tps1 mutant of *S. cerevisiae* in `https://doi-org.vu-nl.idm.oclc.org/10.1016/S0968-0004(98)01205-5` which displays a metabolic explosion.

3. STEADY-STATE RESPONSE OF A LINEAR PATHWAY WITH A NEGATIVE FEEDBACK ONTO THE FIRST ENZYME, BUT PRODUCT INSENSITIVE ENZYME. Now consider the network shown in figure 32, but without the product inhibition of the first enzyme by its product. So, we have the following steady-state fluxes relations indicating their dependencies on reactant concentrations:

$$\begin{aligned} \frac{dx}{dt} &= v_1(z_s) - v_2(x_s, y_s) = 0 \\ \frac{dy}{dt} &= v_2(x_s, y_s) - v_3(y_s, z_s) = 0 \\ \frac{dz}{dt} &= v_3(y_s, z_s) - v_4(z_s) = 0. \end{aligned} \tag{17}$$

   (a) How are these relations changed when the first enzyme would be inhibited by its product?

   (b) Consider now again the situation without product inhibition of the first enzyme. At steady state all fluxes are the same, such that $v_1(z_s) = v_4(z_s)$. Why is it that enzyme 2 and 4 do not influence the flux when their concentration is changed?

   (c) Remove the feedback, which enzyme does now only set the flux?

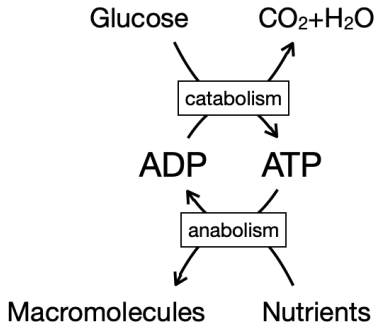


Figure 35: **Catabolism makes the ATP which drives analbolism.** What is suggested in this figure is that the total concentration of adenosine is fixed and is either in the ADP or ATP state. We often consider metabolism in this manner.

Moiety conservation is a common feature of metabolic models

A key aspect of metabolic pathways is that their is no net synthesis or degradation occurs of some chemical components (called moieties) of metabolites such that their total concentration remain constant when distributed over different metabolites. Although the cell has to make those compounds, their total concentrations may remain constant across conditions or when you make models you assume those total concentrations to be constant. For instance, the adenosine moiety of AMP, ADP and ATP is not changed at a minutes time scale when glycolysis responds to a change in the glucose concentration. Thus, none of these three concentrations can exceed their total sum and when one rises other have to decrease. Other examples are NAD, NADP and coenzyme A, these are can also each be considered conserved in total concentration under particular physiological conditions or scenarios. I write scenarios because whether conservation occurs depends on how you consider those concentrations. When you consider glycolysis with the concentrations of AMP, ADP and ATP as variables then the total adenosine pool is conserved but when you consider them fixed then this is not the case. Then you implicitly

assume a mechanism that keeps those concentrations fixed, independent of the state of the glycolysis, which may be considered weird since the function of glycolysis is to make ATP from ADP when it ATP has been used in anabolic reactions of the cell (Fig. 35).

When you consider the concentrations of AMP, ADP, and ATP variable, their concentrations change due to the occurrence of reactions. Because the total concentration of adenosine is considered fixed, your 'model' of metabolism should obey that the sum of rate of change equations of AMP, ADP, and ATP remains constant

$$\frac{dAMP}{dt} + \frac{dADP}{dt} + \frac{dATP}{dt} = 0,$$

since this is a direct consequence of their sum being constant, i.e.

$$AMP(t) + ADP(t) + ATP(t) = A_T.$$

This also implies that when you know the differential equations for AMP, ADP and ATP, you can deduce that their sum is constant because these equation sum to zero.

Consider, for instance, the model of Teusink et al. of yeast glycolysis as shown in figure 37, the sum of the differential equations of the concentrations of AMP, ADP and ATP indeed sum to 0, indicating that in this model the total concentration of adenosine is conserved.

Identification of conserved moieties is often done by performing some linear algebra procedures on the stoichiometric matrix, i.e. gaussian elimination (row reduction) on the rows of the stoichiometric matrix, and reducing it to its reduced row echelon form. In this manner, you can also determine the rank of the stoichiometric matrix. Perhaps you have ever done this in a linear algebra class.

Exercise

1. Confirm that adenosine is conserved in the scheme of figure 37. What happens when you run this model with starting concentrations of AMP, ADP and ATP equal to 0? Check the differential equation of AMP. What do you conclude when AMP is at steady state? What happens to the steady state when you make AK irreversible in the model?

2. METABOLISM IS THE CUTTING AND PASTING OF PIECES OF CHEMICAL COMPOUNDS. Make a drawing of glycolysis from glucose to ethanol (considering them both fixed). Consider adenylate kinase ($2ADP \rightarrow AMP + ATP$) and an ATP consuming reaction catalysing $ATP \rightarrow ADP + P$. Write all the reactants in the following manner write glucose as C_6e_2 (so a six carbon-atom containing molecule with 2 electrons), ATP as AP3, ADP as AP2, AMP as AP1, Pi as P, NADH as Ne, and NAD as N. Now follow the logic of all the chemical conversions of glycolysis, e.g. dihydroxyacetonephosphate is $C3Pe$, glyceraldehyde-3-phosphatse is $C'3Pe$. What are the moieties that are being conserved? Do glycolysis, and finding the conserved moieties, make more sense now, because

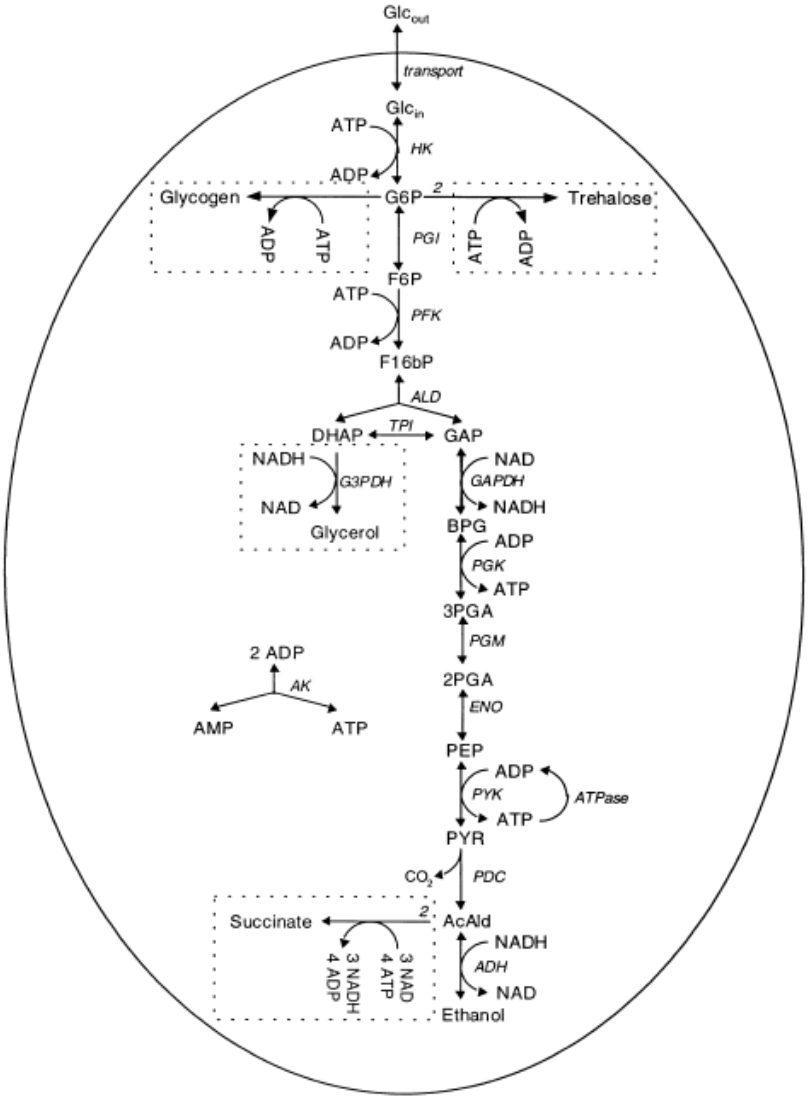


Figure 36: **Glycolysis in yeast, according to the model of Teusink et al. `https://doi.org/10.1046/j.1432-1327.2000.01527.x`.**

you have considered which chemical conversions, which cut and past chemical building blocks, they are subjected to?

Regulation of phosphofructokinase by ATP

What we have been sweeping under the rug until now is the complexity of rate regulation of cooperative proteins due to effector-effector and effector-reactant interactions. When we take this into consideration the regulatory potential of cooperative proteins becomes clear, suggesting that they can be really complex integrative devices.

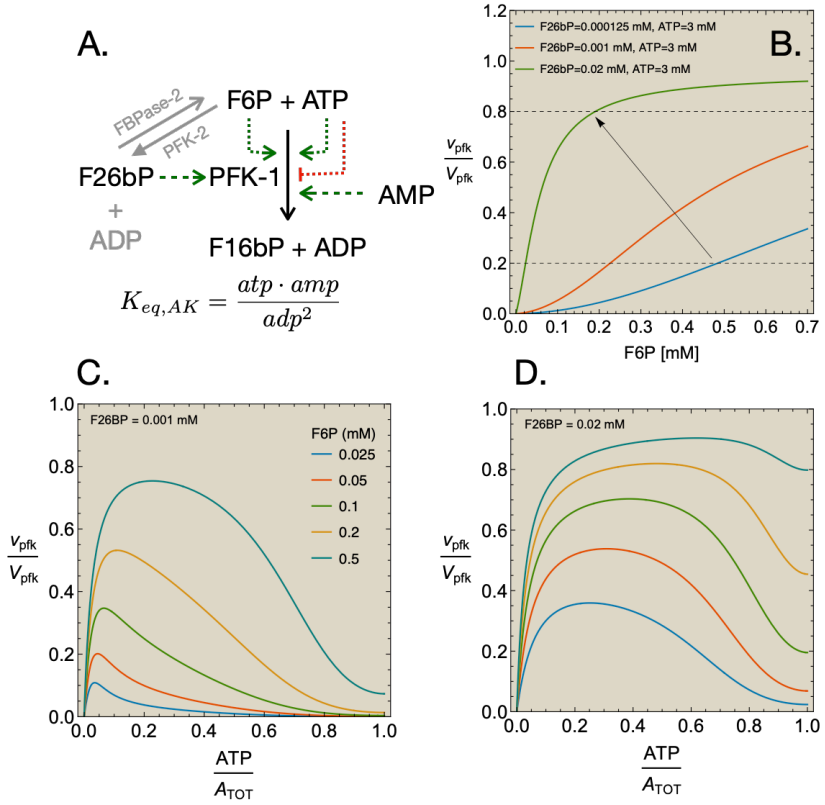


Figure 37: **The regulatory potential of cooperative proteins is illustrated by complexity of phosphofructokinase.** These curves were made using the rate equation of phosphofructokinasen (from Teusink et al. `https://doi.org/10.1046/j.1432-1327.2000.01527.x` in the presence of adenylate kinase operating at thermodynamic equilibrium. Here we assume that the total adenosine concentration is fixed. The ADP and AMP concentration then follow the thermodynamic equilibrium of adenylate kinase, given a concentration of ATP. When ATP is high, AMP is low. **A.-C.** The regulation of PFK by several of its reactants and effectors. Thus, at low ATP glycolysis is activated while at high ATP it is inhibited. This suggests that the cell aims to control ATP homeostatically, within a small range of concentrations.

A flavour of this complexity is shown in figure 37, where the regulation of phosphofructokinase (PFK) is shown by both effectors and substrates. It suggests that the cell aims to control ATP homeostatically, within a small range of concentrations. It achieves this by its regulation of PFK by ATP and AMP, the conservation total adenosine,

$$A_T = atp + adp + amp$$

and the equilibrium relation of adenylate kinase

$$K_{eq,AK} = \frac{atp \cdot amp}{adp^2},$$

which gives rise to a relation between the concentration of ATP and ADP,

$$K_{eq,AK} = \frac{atp \cdot amp}{(A_T - amp - atp)^2} \Rightarrow amp = f(atp),$$

making AMP a function of AMP. Since AMP is an effector of the rate of PFK, ATP acts as a substrate and an implicit effector of PFK via its influence of AMP. Taken together, this leads to the suggestion that via this PFK control the cell aims to keep its ATP concentration within bounds.

The cell is unfortunately rather populated by multimeric enzyme which may all be regulated by these effector-effector and effector-reactant interactions. Examples are glutamine synthetase (`https://doi.org/10.1002/9780470123089.ch2`) and aspartate transcarbamoylase (`https://doi.org/10.1111/febs.12483`) for which such interactions have been shown .

Regulation of branch point fluxes‡

Branch points occur often in metabolism and often one of the two enzymes directly after the branch point is subject to regulation, e.g. feedback or covalent modification, that changes, for instance, the maximal activity of this enzyme. An example of that is isocitrate dehydrogenase (IDH) in *E. coli*, which is regulated by phosphorylation, while the other branch point enzyme, isocitrate lyase (ICL), is unregulated. ICL is part of the glyoxylate pathway, needed for growth on for instance acetate, while IDH is part of the TCA cycle needed for growth on glucose. The regulation of IDH is there to regulate the switch between these two pathways. In this case, the branch point metabolite is the common substrate for IDH and ICL, isocitrate, synthesised by aconitase.

IDH has a higher affinity for isocitrate than ICL, i.e. 8 versus 600 μM (LaPorte et al., J Biol Chem. 1984 Nov 25;259(22):14068-75). When isocitrate is therefore around 1 mM in concentration both pathways are active, if none of the two enzymes is regulated. Also we can conclude that when ICL is active e.g. when isocitrate is $\approx$ 600 μM than IDH is also active (and at maximal activity). Thus, having ICL active and IDH inactive requires that the activity of IDH is regulated by, for instance, covalent modification (e.g. phosphorylation). This is because IDH has the higher affinity such that it is always active when ICL is active. That a high affinity enzyme at a branch point is regu-

lated is an interesting principle in metabolism at branch points where each branch can be active without activity of the other branch.

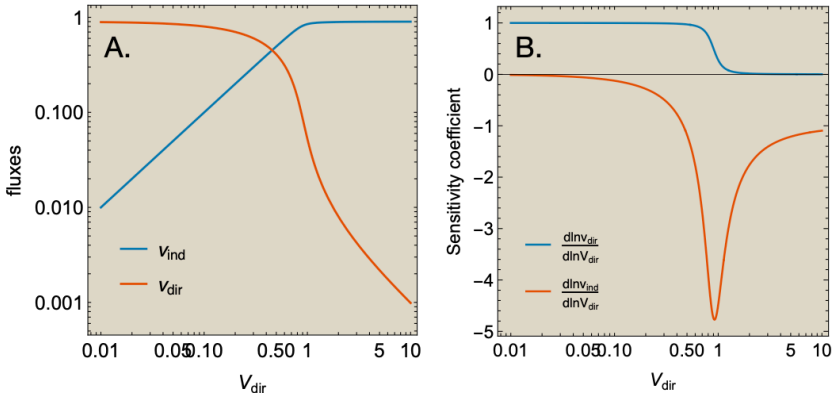


Figure 38: **Ultrasensitivity of branch fluxes to a change in the maximal affinity of the high-affinity enzyme.** The fluxes of two reactions, i.e. v_{dir} and v_{ind}, immediately following a branch point in metabolism are shown as function of the maximal activity V_{dir} of the enzyme with the highest affinity for the branch point metabolite. A. The flux values as function of V_{dir}. B. The sensitivity coefficients of the fluxes shown in A to V_{dir}. This work was inspired by LaPorte et al. (The branch point effect, JBC, 259, 22, 14066-14075, 1984). Parameters: $v_T = 0.9$, $V_{ind} = 1$, $K_{dir} = 1$, and $K_{ind} = 1$.

To understand how this works we can consider the V_{max} of the regulated, high-affinity enzyme as a parameter that is controlled by a regulatory system. LaPorte et al. (The branch point effect, JBC, 259, 22, 14066-14075, 1984) studied this case, inspired by the regulation of IDH and ICL, and made a small model of it. They considered a branch point metabolite S and the following three reactions, one making it and two consuming it. One of the consuming reactions is directly regulated by a change in its V_{max} and referred as "dir" and the rate of other reaction is indirectly regulated via a change in the concentration of the branch point metabolite S, i.e.

$$\begin{aligned} &\xrightarrow{T} && S \\ S &\xrightarrow{dir} \\ S &\xrightarrow{ind} \end{aligned}$$

They considered the following rate of change equation of s and kinetics of the reactions,

$$\frac{ds}{dt} = v_T - V_{dir}\frac{s}{K_{dir}+s} - V_{ind}\frac{s}{K_{ind}+s}.$$

The steady state concentration s_s is set by this relation

$$v_T - V_{dir}\frac{s_s}{K_{dir}+s_s} - V_{ind}\frac{s_s}{K_{ind}+s_s} = 0.$$

When we know this concentration than we can determine the steady-state values of the rates v_{ind} and v_{dir}.

In figure 38A we plot these rates as function of the maximal rate of the directly regulated reaction. What we observe is that the flux of the indirectly regulated enzyme, the one with the lowest affinity

for S, is very sensitive – called ultrasensitive – to the change in the maximal rate of the directly regulation enzyme. The sensitivity of these fluxes to the maximal rate of the directly regulated enzyme is shown in the figure 38B.

ULTRASENSITIVITY OF KINASE AND PHOSPHATASE COUPLES CONTROLLING ACTIVITY OF METABOLIC ENZYMES. Consider an enzyme with a single substrate and a rate equation described by irreversible Michaelis-Menten kinetics. Such an enzyme operates in its zero-order regime when its substrate concentration greatly exceeds the K_M constant. The rate of the enzyme is then nearly independent of the substrate concentration and therefore depends on this concentration to zero-th order, i.e. $v \approx V_{max} \propto s^0$. When a kinase and phosphatase of the same target protein operate in this regime, the steady-state phosphorylation fraction of the enzyme reacts ultrasensitivily to changes in concentrations of effectors of the kinase and/or the phosphatase. This can be illustrated and explained with the following model,

$$\text{kinase: } E \xrightarrow{v_k} EP, \quad v_k = V_k \frac{e_T - ep}{K_k + e_T - ep},$$

$$\text{phosphatase: } EP \xrightarrow{v_p} E, \quad v_p = V_p \frac{tp}{K_k + ep},$$

$$\text{conservation of target protein concentration } \quad e_T = e + ep,$$

We are considering steady states, so

$$v_k = v_p \quad \Rightarrow \quad V_k \frac{e_T - ep_s}{K_k + e_T - ep_s} = V_p \frac{ep_s}{K_k + ep_s}$$

and we like to solve this equation for the steady-state concentration of ep denoted by ep_s. We can do this by hand, but this leads to a quadratic equation, so this is best done with a numerical investigation shown in Figure 39.

Next, consider now that the V_{max} of a metabolic enzyme is proportional to the concentration of its active state and let's assume that this the phosphorylated state then,

$$V_{max} = k_{cat} ep = k_{cat} \frac{ep}{e_T} e_T,$$

which indicates that the V_{max} of an enzyme is proportional to the total expressed concentration of the enzyme e_T – set by gene expression – and by the phosphorylated fraction ep_s/e_T – set by the activity of its associated kinase and phosphatase.

This analysis indicates that the activity of an enzyme can be regulated by a kinase and phosphatase pair, where the kinase is, for instance, regulated by a (distant) metabolite that acts as a kinase effector, and if this pair operates in its zero-order regime than this activity regulation can be very steep.

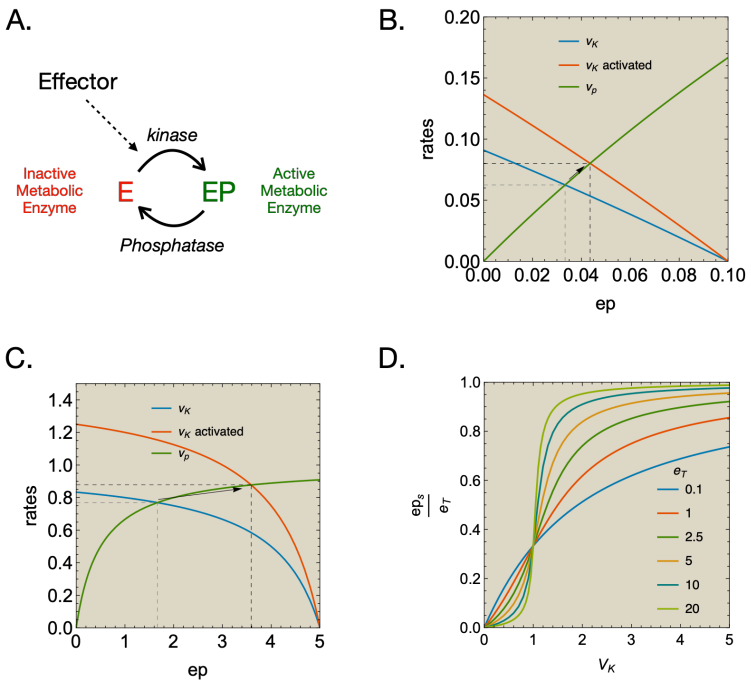


Figure 39: **Illustration of ultrasensitive phosphorylation of a metabolic enzyme.** A. When the kinase and the phosphatase do not operate in their first-order regime, an activation of the kinase by 50% leads to moderate change in the steady-state phosphorylation level of the metabolic enzyme. B. When the kinase and the phosphatase do operate in their first-order regime, an activation of the kinase by 50% leads to huge change in the steady-state phosphorylation level of the metabolic enzyme. The kinase and phosphatase were put in their zero-order regime by enhancing the concentration of the metabolic enzyme. C. Illustration of the increased sensitivity of the phosphorylated enzyme fraction as function of the maximal rate of the kinase when the total concentration of the metabolic enzyme is increased. This analysis is in line with the regulation of isocitrate dehydrogenase of *E. coli* as reported by La Porte and Kosland (La Porte and Kosland, Phosphorylation of isocitrate dehydrogenase as a demonstration of enhanced sensitivity in covalent modification, Nature, 305, 22, 1983.)

Quite some example exist in metabolism of this mode of regulation. For instance, pyruvate dehydrogenase is regulated by phosphorylation in this manner in eukaryotic cells and isocitrate dehydrogenase (IDH) is regulated by phosphorylation in *E. coli*. The kinase and phosphatase of IDH are regulated by 3-phosphoglycerate, a glycolytic and gluconeogenic intermediate – it activates the phosphatase and inhibits the kinase (isocitrate does this also). As the phosphorylated form of IDH is inactive, increased concentrations of 3-phosphoglycerate enhance the activity of IDH. This enhances the flux through the TCA cycle at the expense of the flux through the glyoxylate shunt. Thus, during growth on acetate, when gluconeogenesis is active, IDH is inactivated by high 3-phosphoglycerate concentrations, while it is active in the presence of glucose. During growth on acetate and glucose the situation can be more complex, the glyoxylate pathway and the TCA cycle can be used simultaneously (Walsh & Koshland, JBC, 260, 14, 8430-8437, 1985).

In fact, a recent study estimates that about 20% of all proteins in *E. coli* can be phoshorylated and regulated in this manner. Protein phosphorylation of metabolic enzymes may turn out to be a lot more important than we often think.

Exercise

1. Use your favourite plotting tool (can also be Excel) to reproduce figure 39B to D. The best approach is to analytically solve $V_k \frac{e_T - ep_s}{K_k + e_T - ep_s} = V_p \frac{ep_s}{K_k + ep_s}$ for ep_s so that you can plot ep_s/e_T as function of V_k and change the enzymes away and towards their zero-order regime.

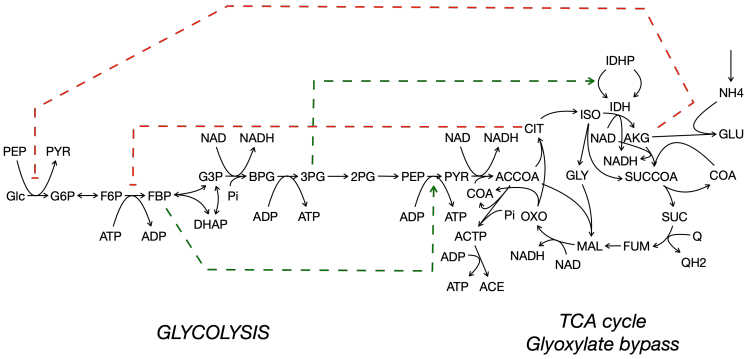


Figure 40: **Cross regulation between glycolysis and the citric acid cycle (see also Figure 5).** Illustration of the regulation of IDH by 3-phosphoglycerate and of glucose uptake (PTS system) by $\alpha-$ketoglutarate `https://doi.org/10.1038/nchembio.685`.

The thermodynamic driving force of a metabolic network‡

In figure 41, the fermentation of glucose into ethanol and carbon dioxide is shown. Its overall reaction is

$$C_6H_{12}O_6 \xrightarrow{J_{glc}} 2CO_2 + 2C_2H_6O.$$

From the standard Gibbs free energies of formation of these 3 chemical compounds, we can calculate the equilibrium constant that is associated with this conversion. Whether the reaction runs in the direction of ethanol depends on the sign of the Gibbs free energy change of the reaction (it needs to be negative) and the concentrations of the reactants.

Earlier, we already deduced that the rate of a reaction has an opposite sign than its Gibbs free energy, i.e.

$$sign(v_i) = -sign(\Delta\mu_i) \;\Rightarrow\; -v_i\Delta\mu_i \geq 0.$$

A positive flux is therefore associated with a negative Gibbs free energy change (and the flux is zero when the free energy change is zero). In Figure 41, we define all the reactions positive when they run in the direction of their arrow. They therefore all have a negative free energy change.

The overall reaction is the net outcome of glycolysis plus pyruvate decarboxylase, ATPase, adenylate kinase and alcohol dehydrogenase. The overall reaction is satisfied when the metabolic system is running under steady state conditions such that none of the intermediates accumulate. This requires fixed concentrations of the reactants of the overall reaction (hence, they are underlined in figure 41). All the other reactants of the metabolic network are considered with variable concentrations, which are determined by the kinetics of the enzymes and the steady-state requirement.

Each of the associated reactions has a free energy change which makes it run in the direction of ethanol formation. These driving

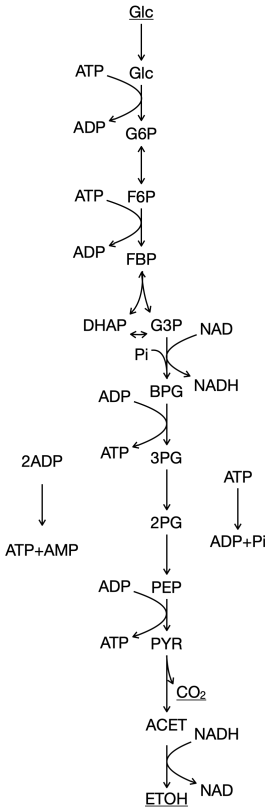


Figure 41: **Cross regulation between glycolysis and the citric acid cycle (see also Figure 5).** Illustration of the regulation of IDH by 3-phosphoglycerate and of glucose uptake (PTS system) by $\alpha-$ketoglutarate `https://doi.org/10.1038/nchembio.685`.

forces are however not independent, since enzyme depend on the concentrations of reactants which are also reactants of at least one other enzyme. Thus, free energy changes are interdependent and eventually also related to the concentrations of the boundary reactants: glucose, carbon dioxide and ethanol. How does this work?

Consider the following sum (we consider steady-state rates, so fluxes, now),

$$\sum_{i=1}^{\text{all reactions}} -J_i \Delta\mu_i \geq 0$$

Assume also that all the fluxes are a multiple of the glycolytic flux (the metabolic system has a single independent flux), which we define as the rate of glucose uptake, and denote by J_{glc},

$$J_i = r_i J_{glc}.$$

When we substitute the previous relation into the former then

$$-J_{glc} \sum_{i=1} r_i \Delta\mu_i \geq 0$$

What turns out is that

$$\sum_{i=1} r_i \Delta\mu_i = 2\mu_{ETOT} + 2\mu_{CO2} - \mu_{GLC} \leq 0,$$

which relates the driving forces of all the reactions to the driving force of glycolysis as a whole.

Exercise

1. In this exercise, you will confirm the main results of the previous section. We are considering glucose fermentation as shown in Figure 41 at steady state.

(a) Show that

$$\begin{pmatrix} J_{glt} \\ J_{hk} \\ J_{pgi} \\ J_{pfk} \\ J_{ald} \\ J_{tpi} \\ J_{gapdh} \\ J_{pgk} \\ J_{pgm} \\ J_{pyk} \\ J_{pdc} \\ J_{adh} \\ J_{ak} \\ J_{atpase} \end{pmatrix} = \underbrace{\begin{pmatrix} 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 2 \\ 2 \\ 2 \\ 2 \\ 2 \\ 2 \\ 0 \\ 2 \end{pmatrix}}_{\mathbf{r}} \times J_{glc}$$

(b) Show that

$$\sum_{i=1} r_i \Delta\mu_i = 2\mu_{ETOT} + 2\mu_{CO2} - \mu_{GLC}$$

(c) Show that $2\mu_{ETOT} + 2\mu_{CO2} - \mu_{GLC} \approx 2\mu^{0'}_{ETOT} + 2\mu^{0'}_{CO2} - \mu^{0'}_{GLC}$ and that therefore the contrations of glucose, carbon dioxide and ethanol do not matter much for the driving force.

(d) What is Gibbs free energy potential adenylate kinase at steady state?

Biochemical characteristics of a pathway regulated by negative feedback

Negative feedback inhibition is ubiquitous in metabolic networks (Fig. 42). In this section, we consider what the biochemical requirements are for a negative feedback to function properly. It turns out that for answering this question it suffices to consider the metabolic segment as function of the concentration of the negative-feedback inhibitor when we consider it fixed (Fig. 43). The results will be obtain do not depend on the length of this metabolic segment.

In a previous exercise, it already became clear that the steady-state flux is set by the first enzyme when this enzyme is insensitive to the concentration of its product. Then we only need to know the kinetic parameters of this enzyme and the concentration of its substrates in order to determine that value of the pathway flux at steady state. (That flux is then independent of the kinetics of all the other

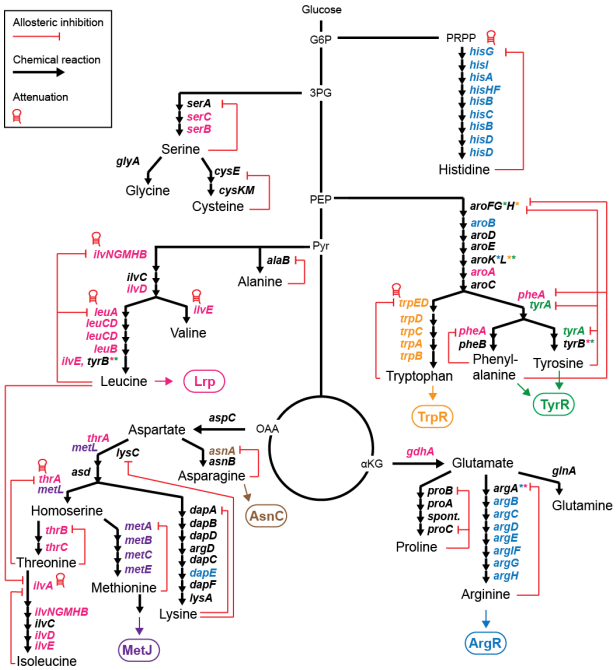


Figure 42: **The occurrence of negative feedback in amino acid metabolism of *E. coli*.** From the introduction of the PhD thesis of Timor Sander (Understanding and engineering metabolic feedback regulation of amino acid metabolism in *Escherichia coli*, Philipps-Universität Marburg, 2019.)

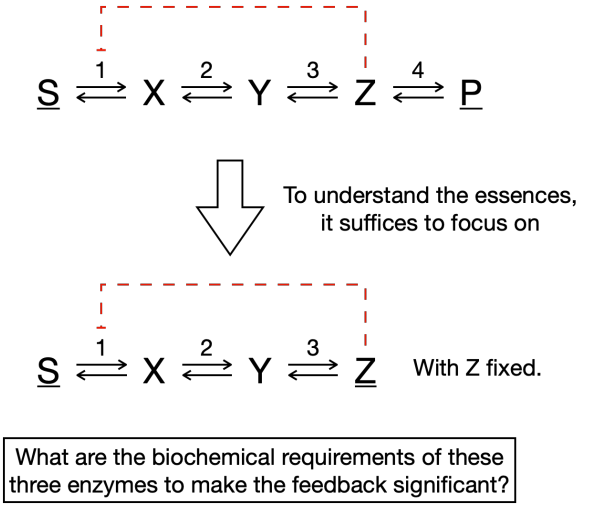


Figure 43: **Biochemical requirements of a functional negative feedback in a linear metabolic pathways.**

enzymes. Their kinetics do however determine whether a steady state indeed exists in case when a negative feedback is absent.) Not many examples are however known of enzymes that are completely insensitive to changes in their product concentrations, but enzymes may have kinetic characteristics that make them nearly insensitive and that is often enough for a potent negative feedback. One of these is the enzyme has a very high equilibrium constant (such that it is irreversible) as then,

$$v = k_{cat} \cdot e \cdot f(\mathbf{x};\mathbf{p}) \prod_i \frac{s_i}{K_{s_i}} \left(1 - \frac{\prod_j p_j}{\prod_i s_i K_{eq}}\right) \Rightarrow v \approx k_{cat} \cdot e \cdot f(\mathbf{x};\mathbf{p}) \prod_i \frac{s_i}{K_{s_i}}$$

Note that the substrate saturation function of the enzyme $f(\mathbf{x};\mathbf{p}) = \frac{\mathcal{C}_\mathcal{R}{}^{n-1}}{\mathcal{C}_\mathcal{R}{}^n + L\frac{\mathcal{A}_\mathcal{T}{}^n}{\mathcal{A}_\mathcal{R}{}^n}\mathcal{C}_\mathcal{T}{}^n}$ for the general rate equation, derived within the MWC framework.

and the enzyme is only sensitive to the product concentration via the function $f(\mathbf{x};\mathbf{p})$. How the enzyme is sensitive to its products, e.g. in a noncompetitive, uncompetitive or competitive manner with respect to other reactants, depends on the precise mechanism of the enzyme (its binding polynomials), which is subject to evolutionary tuning. We can therefore ask the question which biochemical mechanisms of product interactions with enzyme 1 are most desired for a properly functioning feedback of Z onto enzyme 1? We have already concluded that the enzyme needs to be effectively irreversible.

An analysis of parameters is shown in figure 44. The yellow and green line in figure 44A have approximately the same steepness and flux range, making them almost equally good showing that competition inhibition of Z with X brings no benefit. Binding of Z to a binding site on the T state in the absence of competition with X gives the steepest inhibition over the largest flux range.

$$v_1 = \frac{10(1 + s + x + c_1 z)^3 \left(1 - \frac{x}{sK_{eq,1}}\right)}{(1 + s + x + c_1 z)^4 + L(1 + c_2 z)^4 (1 + s + x + c_3 z)^4}$$

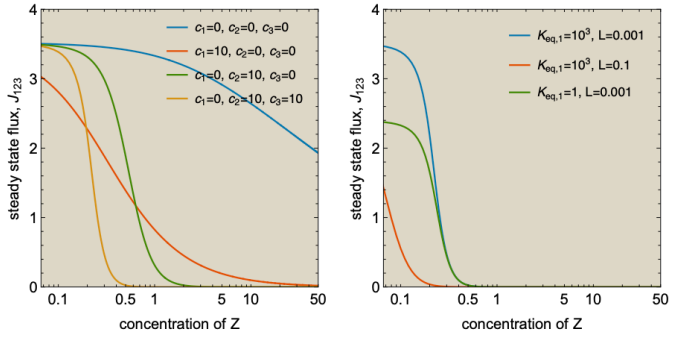


Figure 44: **Illustration of the effects of equilibrium constants and inhibition parameters on feedback of enzyme 1 by Z.** The rate equation for the feedback inhibited first enzyme is shown. The remaining enzymes have a kinetics $v_2 = 10x/(1 + x + y)$ and $v_3 = 10y/(1 + y + z)$. A. $K_{eq,1} = 10^3$, $L = 0.001$ and the inhibition parameters were changed. B. $c_1 = 0$, $c_2 = 10$, $c_3 = 10$ and the equilibrium constants were varied.

Self-regulating metabolic pathways

So far, we have mostly considered metabolic networks with fixed enzyme concentrations and, therefore, also fixed V_{max} values. This is, however, only meaningful at steady state or on time scales during which no appreciable changes in enzyme concentrations have occurred. A more realistic case is shown in figure 45 where gene expression of metabolic enzymes is regulated by the reactants of those same enzymes. This resembles, for instance, the control of enzyme concentrations in amino acid metabolism, which occurs via an transcription attenuation or a transcription involving the amino acid itself.

Remember: $V_{max} = k_cat \cdot e$ with e as the enzyme concentration.

See Wikipedia `https://en.wikipedia.org/wiki/Trp_operon` and, for an influential paper on this, see `https://doi.org/10.1016/S0021-9258(19)35394-3`.

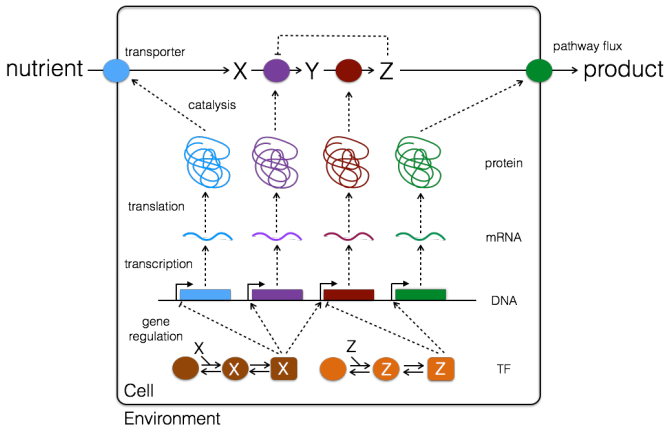


Figure 45: **The central dogma of molecular biology and the hierarchy of control mechanisms in a cell.** How does the cell regulates its own metabolic activity? One way is shown in this figure and occurs via the binding of metabolic intermediates X and Z to transcription factors that regulate the expression of the genes coding for the enzymes that metabolise X, Y and Z.

What an analysis of the regulation of amino acid metabolism shows is that regulation occurs on two time scales. Metabolic regulation is fast and occurs on a time scale of tens of seconds to minutes, via negative feedback of amino acids on the start of their biosynthesis pathways (figure 42). Gene-expression regulation is slower and occurs on time scale of tens of minutes to hours, via the influence of the amino acid on the biosynthesis of its synthesising enzymes, via transcription attenuation or transcription factors (`https://en.wikipedia.org/wiki/Trp_operon`).

The paper by Chassagnole et al. (`https://doi.org/10.1042/0264-6021:3560415`) describes a kinetic model of the metabolic regulation of threonine biosynthesis, using rate equations that are in line with experimental data. One interesting mode of regulation occurs in this model is the occurrence of multiple negative feedback on the same enzyme, which is common in amino-acid metabolism. For instance, in their model aspartate kinase is inhibited both by threonine and lysine, but they, interestingly, inhibit two different isoenzymes of aspartate kinase. The introduction of this paper is quite an interesting read on this. In any case, this paper is

a good example of a model made with realistic, not-too-complex enzyme kinetics. An accompanying paper by the same authors (https://doi.org/10.1042/0264-6021:3560433) studies the influence of enzymes concentrations on the flux through the threonine pathway and is a good read if you want to become familiar with how models can be used to study metabolism.

You can play with this model on the JWS Online modelling website, e.g. https://jjj.biochem.sun.ac.za/models/chassagnole1/

Rationalising the control of amino acid biosynthesis pathways

What has remained unclear so far is why amino acids generally inhibit their own biosynthesis in two different ways: i. on a metabolic time scale, via a negative feedback on the first enzyme of their biosynthesis pathway, and ii. on a gene-expression time, via a negative feedback onto the gene expression of the enzymes in their biosynthesis pathway.

We will first consider the inhibition of gene expression. Why does this occur? One of the current ideas is based on the fact that cells have a finite amount of biosynthetic resources for gene expression and protein synthesis, such as nucleic acids, amino acids, RNA polymerase and ribosomes. A consequence of this is that the enhanced synthesis of one protein lowers that of others. Thus proteins should be used optimally and their concentration should be the minimally required concentration for a task to ensure that all tasks can be carried out as good as possible, given the constraint of finite biosynthetic resources.

When we translate this economic use of protein to the fluxes of amino-acid biosynthesis then this flux should be attained with the least amount of enzyme expressed in the pathway. Say those enzyme concentrations are too high, given the current consumption rate of this amino acid by translation, then the steady-state concentration of the amino acid increases. This would then signal an excess of biosynthetic protein of this amino acid and the corresponding genes should be inhibited by this amino acid. Is this indeed what happens?

To address this, we consider a model of the biosynthesis of an amino acid with a negative feedback of the amino acid on the first enzyme (i.e. like figure 43). We consider the following optimisation problem. We demand a flux of 1 through the pathway. We vary the concentration of S and minimise the concentrations of enzyme 1 to 4 needed to give rise to a flux 1 at a minimal total enzyme cost. Figure 46 gives the outcome of the optimisations at different concentrations of S. The figures indicate that the supply curve (the steady state flux of enzyme 1 to 3; blue) varies with the concentration of S only in the left and right direction, while the demand rate (red) moves down. When the concentration of S rises the steady-state concentration

of X_3 rises a little. Both systems are used close to their maximal capacity, indicating that the enzyme concentrations are tuned to optimal usage.

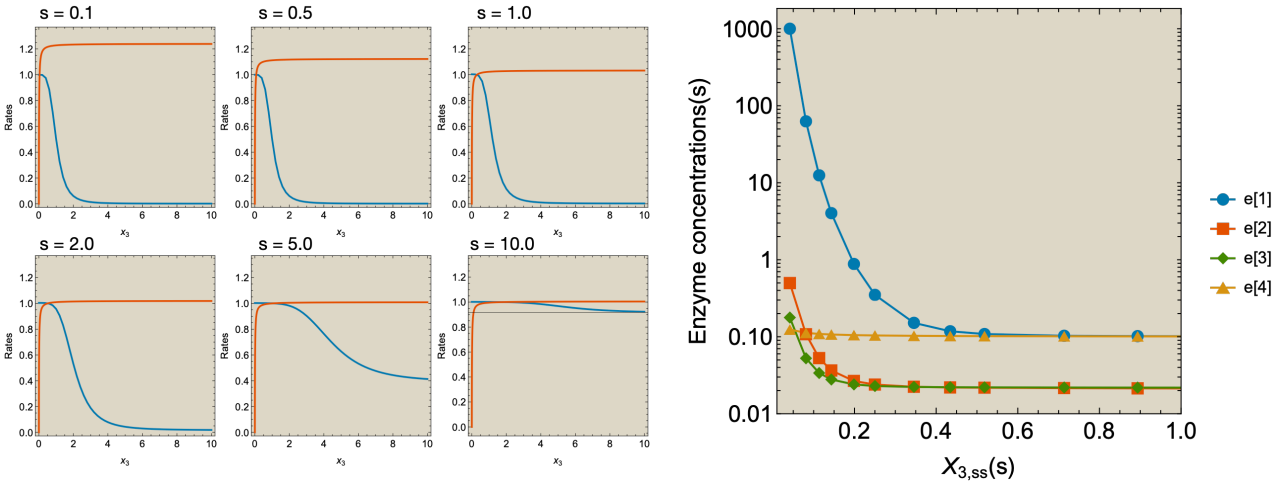


Figure 46: **Optimisation of the biosynthetic flux of an amino acid by minimisation of protein investment.** The plots on the left show the steady-state flux of enzyme $1-3$ (blue) and the rate of enzyme 4 (red), at the optimal enzyme concentrations, their intersection is the steady state (always at flux equal to 1). The plot on the right shows the optimal enzyme concentration as function of the feedback metabolite, X_3, which is our proxy of an amino acid. In this plot, the shown optimal values correspond to different values of the concentration of the pathway substrate S.

The plot on the right shows the optimal concentrations of the enzymes as function of the steady-state concentration of X_3. It is striking that enzyme 4 hardly changes in its optimal concentration, while the concentration of enzyme 1 to 3 go down. Indeed, the activity of the genes of those three enzymes are controlled by the concentration of X_3 (the amino acid) in reality and enzyme 4 not. Note that we did now not consider gene expression control, instead we used optimisation to calculate the enzyme concentrations (by minimising them to reach a flux of 1). So, effectively we predicted the behaviour of an optimal gene expression circuit that has x_3 as an input and the concentrations of enzymes 1, 2 and 3 as output. The plot on the right shows that the optimal behaviour is that the concentrations of those three enzyme decrease with the concentration of X_3: hence, X_3 should inhibit their synthesis by inhibiting the corresponding gene activities! Which is indeed what many cells are doing.

Now that we know the optimal relation between the steady-state concentration of enzyme $1-3$ needed for optimal behaviour (the right plot), we can infer which type of gene activity control is needed. It turns out that the three curves for the optimal steady-state concentrations of enzyme $1-3$ as function of x_3 can be fitted by the following functions,

Note that we omit mRNA here. This does not change the method.

$$e_1 = \frac{a_1 + \frac{b_1}{1+2\frac{\left(\frac{x_3}{K}\right)^4}{\left(1+\frac{x_3}{K}\right)^4}+c_1\left(\frac{\left(\frac{x_3}{K}\right)^4}{\left(1+\frac{x_3}{K}\right)^4}\right)^2}}{d_1},\quad e_2 = \frac{a_2 + \frac{b_1}{1+c_2\frac{\left(\frac{x_3}{K}\right)^4}{\left(1+\frac{x_3}{K}\right)^4}}}{d_2},\quad e_3 = \frac{a_3 + \frac{b_1}{1+c_3\frac{\left(\frac{x_3}{K}\right)^4}{\left(1+\frac{x_3}{K}\right)^4}}}{d_3}$$

Indicating that their corresponding differential equations should look like,

$$\begin{aligned}
\frac{de_1}{dt} &= a_1 + \frac{b_1}{1 + 2\frac{\left(\frac{x_3}{K}\right)^4}{\left(1+\frac{x_3}{K}\right)^4} + c_1\left(\frac{\left(\frac{x_3}{K}\right)^4}{\left(1+\frac{x_3}{K}\right)^4}\right)^2} - d_1 e_1 \\
\frac{de_1}{dt} &= a_2 + \frac{b_1}{1 + c_2\frac{\left(\frac{x_3}{K}\right)^4}{\left(1+\frac{x_3}{K}\right)^4}} - d_2 e_2 \\
\frac{de_1}{dt} &= a_3 + \frac{b_1}{1 + c_3\frac{\left(\frac{x_3}{K}\right)^4}{\left(1+\frac{x_3}{K}\right)^4}} - d_3 e_3
\end{aligned}$$

Thus, a basal synthesis rate of the three enzymes is needed: a_1, a_2, a_3 in addition to a regulated rate. Those regulated rates indicates that we need a transcription factor composed of four subunits, each with a binding site for X_3 and a binding polynomial $1 + \frac{x_3}{K}$. On the gene promoters of enzyme 1 two of these transcription factors have to bind and on the promoters for e_2 and e_3 only one. All enzymes are in addition degraded at rates $d_1 e_1$, $d_2 e_2$, $d_3 e_3$.

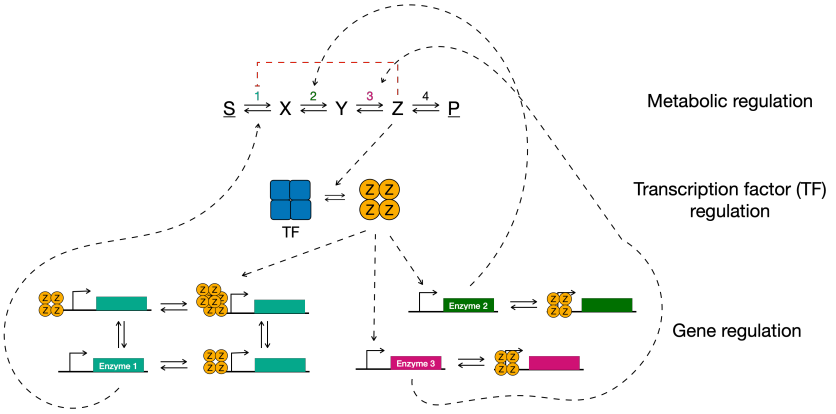


Figure 47: **The structure of the optimal gene network that ensures minimal enzyme usage for a steady-state flux equal to 1 as function of the concentration S.** The minimisation of the enzyme concentrations as function of S for a steady-state flux of 1 lead to a relationship between the optimal steady-state concentration of Z and the optimal concentrations e_1, e_2 and e_3 (enzyme 4 had constant optimal concentrations as function of S). This information constraints the steady-state behaviour of the optimal gene network.We subsequently deduced that the gene network structure that gives rise the optimal relationship between Z – the input of the gene network – and the enzyme concentrations – the output of the gene network. The resulting design resembles that of amino acid metabolic pathways as found in bacteria.

Note that we inferred this optimal gene-regulatory circuitry (Fig. 47) from the rate equations of the metabolic enzymes– those therefore suffice to predict optimal gene regulation. Note also that adding the previous three differential equations to the those of the metabolic reactants gives a dynamic model, with metabolites and enzyme concentrations as variables, that always reaches an optimal steady state as function of the concentration of S.

I am of the opinion is that this a very useful method to figure out which gene-expression control is needed for a certain task and to predict the likely function of known regulatory circuitry. For this section, we conclude that the negative feedback of an amino acids

We prioneered this method for the first time for the regulation of the Gal operon in *S. cerevisiae* (`https://doi.org/10.1038/srep01417`) by ensuring that the gene expression is optimal given the relation of the growth rate as function of the galactose concentration.

on the gene expression of its synthesising enzymes exists to ensure economic usage of proteins and prevent protein overexpression (that would reduce the performance of other cellular tasks). We already concluded that the negative feedback of an amino acid on the first enzyme of its biosynthesis pathway leads to a homeostatic concentration of the amino acid. I think that this occurs to ensure that the ribosome maintains a constant activity per unit ribosome, but I will not analyse this further here (see `doi:10.1111/febs.13258`).

Exercise

1. Consider the method outlined of the previous section.

   (a) A simplified binding model of a transcription factor composed out of four 4 subunits was proposed. Make it one step more realistic by deriving a MWC binding model, again with 4 subunits, but now with two subunits state, R and T. Assume that the R_4Z_4 binds to the gene promoter and that Z binds to the R and T state with different affinities.

   (b) mRNA was omitted in the previous section. Add this to the model and show that this not change the model in a significant way and that the procedure then still work without problems. mRNA was omitted to keep the model simple.

Suggestions for further reading

1. Fell, David. Understanding the control of metabolism. Vol. 2. London: Portland press, 1997

2. Heinrich, R., & Schuster, S. (2012). The regulation of cellular systems. Springer Science & Business Media.

3. Reich, J. G., & Sel'kov, E. E. (1981). Energy metabolism of the cell: a theoretical treatise. Academic press.

4. Strogatz, S. H. (2018). Nonlinear dynamics and chaos: With applications to physics, biology, chemistry, and engineering. CRC press.

5. Christopher P. Fall, Eric S. Marland, John M. Wagner, John J. Tyson, Computational Cell Biology, Springer New York, NY, 2002.

Realistic kinetic models of metabolism: introduced via a series of exercises

The paper by Mulquiney and Kuchel (Biochemical Journal, 342, 581-596, 1999) on glycolysis in human erythrocytes is a great introduction to a kinetic model of metabolism with an emphasis on its use for the prediction of steady state data. Open the paper let's skim through it.

In their scheme 1, the metabolic network is shown that is considered in this model. Now move to the Appendix on page 588 in the paper, there you see the different mechanisms of the enzymes as well as the corresponding rate equations. As you will see some of the rate equations have been derived with the steady-state method, while others (such as the kinetics of PFK, eqs. A4-6, and PK eq. A12) use the rapid-equilibrium method and the MWC formalism. Steady-state enzyme kinetics can become very complicated, consider for instance the rate equation of GAPDH (eq. A9), it is huge and reliant on a large number of kinetic parameters. I advise you to go from enzyme to enzyme in the Appendix to get an impression of the mechanisms and the associated rate equations. Those underlie all the model predictions.

A relevant exercise is to derive a rapid-equilibrium rate equation of this three substrate and three product reaction that has the smallest number of kinetic parameters. What is this number?

Some of these predictions are reported in Tables 1 and 2 of their paper. As you can see most metabolite concentrations are in the mM range, typically below it.

Open Teusink's paper on glycolysis in *S. cerevisiae* next to Mulquiney's paper and compare the concentration of glycolytic intermediates (Table 1 and 4 in Teusink versus Table 1 in Mulquiney), again mM concentrations (although all about a factor of 10 higher). By the way, sub mM concentrations are in general a good estimate of metabolite concentrations, except for metabolites with functions associated with their concentrations (such as osmolites).

Since concentrations are in the mM regime, affinity constants such as K_M's and K_D's are so too, see Teusink's Table 2 and the Appendix of Mulquiney, again sub mM reflecting the concentrations of reac-

tants.

Teusink's paper gives also an overview of the used maximal rates of the enzymes – their V_{max}'s. Pick a rate equation from Teusink's model and calculate the steady-state flux J. What is the ratio of the flux over the V_{max} for all the enzymes? Which enzymes are close to rate limiting given this proxy?

You can calculate the flux by hand or you can use the website: `https://jjj.biochem.sun.ac.za/models/teusink/`, using the Simulate and Steady State buttons.

An example of a realistic dynamic model of metabolism

Teusink's model later adapted to dynamic conditions under which glycolysis oscillates by Dupreez et al. (`https://dx.doi.org/10.1111/j.1742-4658.2012.08665.x`). One of their oscillations models can be run at `https://jjj.biochem.sun.ac.za/models/dupreez4/` by clicking Simulate, Time Evolution and then Go!. Now see all the glycolytic intermediates oscillating in a manner that reflects experimental data. Compare the steady state concentrations of the Teusink to these oscillations, are the concentrations still in the same regime?

Now you have had a look at three different models of glycolysis of two different species, in a steady-state and dynamic setting and all quantitatively based on measured enzyme kinetics, giving rise to predictions that are in agreement with data and leading to concentrations in a sub mM regime.

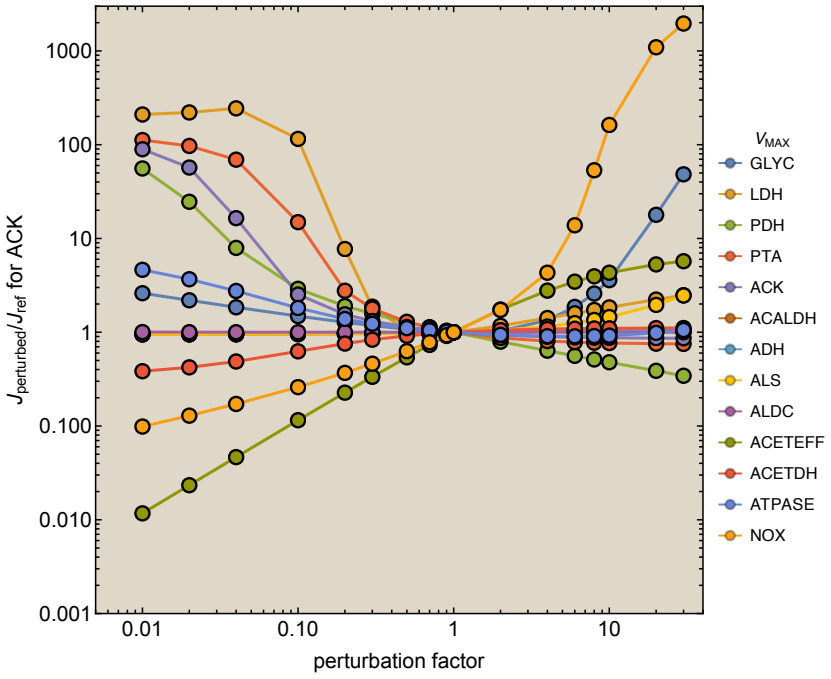


Figure 48: **Illustration of the effect of changing all the V_{max}'s of Hoefnagel's model (`https://dx.doi.org/10.1099/00221287-148-4-1003`) on the production flux of acetate.** PTA, NOX and LDH appear to be important for influencing the acetate production flux.

Model sensitivity to enzyme concentrations in biotechnological applications

LET'S NOW DO SOMETHING USEFUL WITH A STEADY-STATE MODEL. Open the paper by Hoefnagel et al. (`https://dx.doi.org/10.1099/00221287-148-4-1003`) and the model of that paper (`https://jjj.biochem.sun.ac.za/models/hoefnagel1/`). Perform a steady-state simulation of this model and compare the calculated flux values with figure 2A of the paper. Confirm that those numbers match. Make all the mutants considered in figure 2 of this paper and compare your calculations with those reported values.

Consider figure 48, it shows the response of the acetate flux (by acetate kinase (ACK)) to a change in the V_{max}'s of all the enzymes in the model. Interestingly, ACK itself is not so important (Why!?) for an increase its own flux. If you perform an analysis of the flux control coefficients on `https://jjj.biochem.sun.ac.za/models/hoefnagel1/` with Simulate, Steady State and then Flux Control Matrix. Then you also see that the flux control coefficient of ACK (v_{11}) on itself, so $C_{v_{11}}^{v_{11}}$ is very small. If you look into its row in the matrix then you find that $C_{v_4}^{v_{11}}$ has the largest value of 0.486, which happens to be the reaction $NADH + O_2 \rightleftharpoons NAD$ catalysed by NOX, which is in agreement with figure 48. Reproduce some of the lines of figure 48 with a parameter scan on `https://jjj.biochem.sun.ac.za/models/hoefnagel1/` .

By the way, a flux control coefficient $C_{v_i}^{J_k}$ is defined as the change in the steady-state flux through reaction k $d \ln J_k = \frac{dJ_k}{J_k}$ upon a change in the rate of enzyme i by $d \ln v_i = \frac{dv_i}{v_i}$. Thus, $C_{v_i}^{J_k} = \frac{d \ln J_k}{d \ln v_j}$ and colloquially its interpretation is that flux k changes by $C_{v_i}^{J_k}$ % when the rate of enzyme i is changed by 1%.

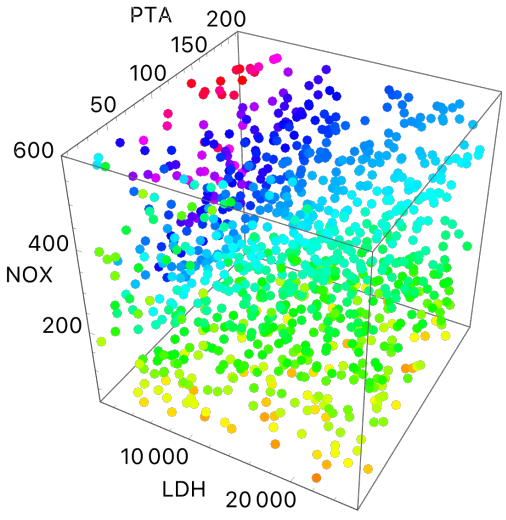


Figure 49: **Illustration of the effect of changing all the V_{max}'s of Hoefnagel's model (`https://dx.doi.org/10.1099/00221287-148-4-1003`) on the production flux of acetate.** PTA, NOX and LDH appear to be important for influencing the acetate production flux.

IN ADDITION TO NOX, LDH AND PTA ARE ALSO IMPORTANT ACCORDING TO THE RESULTS OF FIGURE 48. In figure 49 the result

is shown of the influence of their V_{max}'s on the production flux of acetate, by plotting 1000 random combinations of their values. Such a plot may indicate whether the combined change of these parameters has a different influence on the acetate flux than expected from the response of the acetate when they were each changed independently. Do the results from the combined change shown in figure 49 agree with your expectations from figure 48?

Epilogue

First of all I hope that this little book has been useful for you and inspired you to think about the mind-boggling fact that cell behaviour is the outcome of hundreds to thousands of interacting proteins, each tinkered by evolution to work more coherently in a fitness-enhancing manner.

I hope to have convinced you that quantitative biochemistry provides the fundament of microbial physiology and biotechnology and is truly an interesting field. Although, I did not discuss many of the applied aspects in much depth, I hope that these were sufficiently implied throughout the text. At the very least, I hope that this text enables you to appreciate quantitative biochemistry more in your own research projects.

Many topics were not covered in this text, because this would make it too lengthy or too advanced. One example that would made it too lengthy is the measurement of enzyme kinetics in cell free extract and the subsequent determination of the enzyme mechanism or enzyme-kinetic parameters. The books of enzyme kinetics by Athel Cornish Bowden are a great reference for this.

Cornish-Bowden, Athel. Fundamentals of enzyme kinetics. John Wiley & Sons, 2013.

What I will cover in the future, but have not yet added is the insights one can gain from metabolic control analysis about the design and functioning of metabolic networks. I think that considering metabolic control analysis however only makes sense after one has played with complex models to have a feeling for their complexity and the important role of enzyme concentrations in them – so, after this book. Since, I wrote this book for a 3-day course, I did not yet add this. I also wonder what the right way of teaching this is – separate or integrated with quantitative biochemistry? For now, I think David Fell's book about control of metabolism is the best resource.

What I also did not discuss in great depth is analysis of the stoichiometric matrix, leading to insights into moiety conservation, independent fluxes, and flux modes, using linear algebra. This is rather classical and can be found, for instance, in a paper by Jannie Hofmeyr and Athel Cornish Bowden (`https://doi.org/10.1006/jtbi.2002.2547`) and the great teaching notes of Jannie Hofmeyr. I

think that this topic is however best explained by first considering the composition of metabolism's reactants in terms of their chemical components (moieties) that are being cut and pasted in enzymatic conversions, as these flow and branch through metabolism, while, at the same time, keeping track of all this with linear algebra. In this way, you learn most about how metabolism works and what its stoichiometric constraints mean in terms of chemistry and linear algebra. I also have some teaching material about this, but that can certainly improve. Some basic ideas about this are however already in this book, although not in a rigorous manner. This should be added in the future.

A more modern stoichiometric view is the one obtained from the concepts of elementary flux modes, which follows after stoichiometric analysis, and its connections to the steady-state flux space and constraint flux-optimisations with linear programming, associated with flux balance analysis and optimal resource allocation. This concerns some of our current work. I find our current understanding too immature at the moment to end up in a book form like this. These ideas still need to develop a bit more such that the theory interdependencies and general insights are more clear, as well as the best way of explaining this without ending up in complex mathematics. For instance, it allows for the decomposition of the entire cell metabolism in terms of stoichiometric subnetworks, each making a single macromolecular constituent of a cell with its own nutrient and energy demand. It is unclear at the moment whether studying metabolism in this manner also leads to a better understanding of the regulation of whole-cell metabolism.

Bruggeman, F. J., Planqué, R., Molenaar, D., & Teusink, B. (2020). Searching for principles of microbial physiology. FEMS Microbiology Reviews, 44(6), 821-844.

What I hope to have conveyed is that we really do not yet have a very good understanding of the coordinated usage of different metabolic pathways by the cell, involving regulation by feedback and feedforward circuitry, cooperative proteins, interdependencies via mass flow, gene-expression control and signalling processes. I have the impression that this is partially due to lack of quantitative data on kinetics and regulation of enzymes. This is also not really considered anymore in teaching and research. For instance, one problem is that we require data that relies on methods that are no longer carried out in many labs anymore – at least not to the degree that they were about 30-40 years ago –: i.e., the laborious methods from enzymology.

If microbial cell biology, physiology and biotechnology really aim to become predictive by becoming more quantitative – as opposed to, for instance, being driven by machine learning – then those methods will eventually be needed again. Hopefully, by then, high-throughput methods (e.g. lab robotics) exist for measurement of enzyme kinetics

and regulatory interactions. This realisation starts with teaching.

A quantitative biochemical view on whole-cell metabolism and its regulation can have a major impact on predictive microbial physiology and biotechnology, when used in combination with metabolomics and proteomics. Some studies by Jörg Stelling's and Uwe Sauer's lab are great examples of this.

https://doi.org/10.1038/nbt.2489; https://doi.org/10.1126/scisignal.2005602; https://doi.org/10.1038/msb.2013.66

Another aspect of modern enzyme biochemistry and regulation of metabolism in systems biology is the hope that principles exist that are broadly applicable across (evolutionarily-distant) (micro)organisms – so that we do not have to measure each one of them in painstaking details. These appear to exist for metabolic strategies – explaining why cells shift from respiration to respiro-fermentation –, but it is unclear how predictive these principles are in applied settings. The hope is that principles can help us make quantitative predictions without having to know all the kinetic parameters of enzyme rate equations. Such principles would be really welcome, since methods based on simplified usage of enzyme kinetics (e.g. linlog approximation, biochemical system's theory) have not proven useful and fitting of models with simplified kinetics leads to models that can be poorly extrapolated to new conditions (e.g. a shift in nitrogen or carbon source). Thus, we may need to identify principles from metabolic models with biochemically realistic kinetics which poses an experimental and a theoretical challenge. Considering recent advances in mathematics of metabolic networks, I really believe that this is possible.

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I am of the opinion that the reversible MWC rate equation is the best compromise between ease-of-use, general applicability, solid fundaments, and number of parameters for use in detailed, predictive models. I hope that its explanation in terms of binding polynomials of the binding sites of enzymes made the MWC equation insightful and useable to you. These ideas are based on concepts pioneered by Terrell Hill, Ken Dill and Rob Phillips in texts that are more targeted to a biophysics audience. I hope that I made it understandable to biotechnologists, systems biologists and biochemists without a proficiency in equilibrium thermodynamics. I did therefore not discuss the relation of MWC models to partition functions of thermodynamics.

1. Dill, K., & Bromberg, S. (2010). Molecular driving forces: statistical thermodynamics in biology, chemistry, physics, and nanoscience. Garland Science.
2. Hill, Terrell L. Cooperativity theory in biochemistry: steady-state and equilibrium systems. Springer Science & Business Media, 2013.
3. Phillips, R., Kondev, J., Theriot, J., & Garcia, H. (2012). Physical biology of the cell. Garland Science.

Quite some models of complex metabolic networks have been made in the last decades, with the bigger ones containing tens of enzymes – so still small relative to the entire metabolic network of the cell. Some of those we briefly touched upon in this text when illustrating ways to work with them and understand the role of regulation of enzyme activity in them. I personally think that such models can be useful in applications, in particular when used in combina-

tion with optimisation methods. Although I only shortly mentioned this, I believe this offers a great toolkit to make predictions under parameter uncertainty for medical or biotechnological applications.

Finally, I hope that you enjoyed this text as much as I did when writing it. An important part of science is communicating our ideas and hypotheses in order to influence each others' ways of thinking, and contribute in this way to scientific solutions of important open problems (fundamental and applied). I hope that this book did just that.