Optimal enzyme profiles in unbranched metabolic pathways

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Abstract

How to optimize the allocation of enzymes in metabolic pathways has been a topic of study for many decades. Although the general problem is complex and non-linear, we have previously shown that it can be solved by convex optimization. In this paper, we focus on unbranched metabolic pathways with simplified enzymatic rate laws and derive analytic solutions to the optimization problem. We revisit existing solutions based on the limit of mass-action rate laws, and present new solutions for other rate laws. Furthermore, we revisit the known relationship between flux control coefficients and enzyme abundances in states of maximal enzyme efficiency. We generalize this relationship to models with density constrains on enzymes and metabolites, and present a new local relationship between optimal enzyme amounts and reaction elasticities. Finally, we apply our theory to derive kinetics-based formulae for protein allocation during bacterial growth.

Keywords
Metabolic pathway, enzymatic rate law, enzyme demand, optimization, protein allocation, bacterial growth law

Abbreviations
ECM – Enzyme Cost Minimization
PSA – Pathway Specific Activity
MDF – Max-min Driving Force method

1 Introduction

The idea that living beings show optimal shapes or behaviour has a very long history. A process like evolution, which combines random mutations with a selection for favorable properties, could potentially lead to optimization, but the question of *if* and/or *when* should we expect living beings to function optimally has been widely debated and is far from solved. In pratice, it is often useful to invoke optimality principles to seek insights and design principles that might be relevant also in naturally evolved systems [1]. Specifically, cellular metabolism has been often studied using this approach [2, 3], probably thanks to the powerful mathematical models that we have to describe it. But although natural selection has been the main inspiration for this study, the evolutionary aspects of pathway optimization are not discussed here, and are rather left for the reader to reflect upon.

Within cells, protein expression is arguably the most important and central resource, both in terms of contributing to fitness, but also since it requires large amounts of energy, metabolic precursors, and ribosomes and the proteins themselves occupy a significant portion of cellular space. Therefore, a cell should generally save protein wherever it can. This notion, specifically for enzymes, has been mathematically applied in genome-scale metabolic models [4, 5], models of core metabolism [6], and direct comparison between pathways [7, 8]. Interestingly, even a very simple linear chain model with two reactions, representing metabolism and protein synthesis, and a bound on the total protein budget has been successful in explaining bacterial growth laws and overflow metabolism [9, 10]. However, these bacterial growth law models did not consider enzyme kinetics.

Here, we focus on a special case of this cost/benefit analysis: the efficient use of metabolic enzymes in unbranched pathways operating at steady-state, giving priority to scenarios that can be solved analytically. We explore several possible kinetic rate laws and introduce the idea of bounding the total metabolite concentration (which is required in some cases). To define states of maximal enzyme efficiency, we consider two equivalent optimality problems: maximizing a production flux at a given enzyme budget or minimizing protein usage at a given required production flux. In both cases we, maximize production flux per enzyme usage within the given constraints. If the product of the pathway is directly tied to biomass, the overall enzyme efficiency, called "biomass/enzyme efficiency", can serve as a proxy for cell growth [6]. Furthermore, this optimality problem is also relevant in other contexts, such as metabolic engineering of synthetic pathways using a set of existing and/or new-to-nature enzymes with known kinetic parameters [11, 12].

Another perspective often used to analyze metabolic systems is through their control, e.g. the effect of changes in a level of an enzyme on the pathway flux [13]. In optimal metabolic states, each enzyme has an opportunity cost, and this cost must be balanced by a marginal benefit, given by the *flux control coefficient* – as defined in Metabolic Control Analysis (MCA) [14, 15, 13]). Hence, for systems in optimal states, there are simple relationships between enzyme abundance and flux control [16, 17, 18]. We will recapitulate these results in the following sections and demonstrate them using some of our analytic solutions.

Finally, we show how the analytic solutions derived here might be useful for modeling high-level phenomenon such as the Monod curve (i.e. the relationship between the concentration of a limiting nutrient and the growth rate of bacteria [19]). We use this model to demonstrate how each kinetic parameters should affect the growth rate under different conditions.

In summary, this paper revisits the question of optimal enzyme allocation and adds to previous results. We focus on unbranched metabolic pathways, extend the optimality problem, discuss new optimality conditions, and present analytic solutions that directly show how different factors determine optimal enzyme levels and fluxes. We discuss general principles, in particular how optimal enzyme levels reflect flux control and local reaction elasticities, and use our theory to derive formulae for kinetics-based bacterial growth.

2 Results

2.1 Optimality of linear unbranched pathways

One of the first attempts at finding an analytic solution to the enzyme allocation problem was published by Waley [20], who studied short pathways of 2-3 reactions while assuming the concentrations of metabolites (which were denoted *linking intermediates*) are much below saturation, and therefore affected the flux linearly. Given the total amount of catalytically active protein (bounded by $\varepsilon_{\mathrm{tot}}$), the relative enzyme concentrations should be such that they maximize rate (see Figure 1). Based on these assumptions, one can derive simple formulae for the optimal enzyme levels and maximal pathway flux. Later studies repeated this result and generalized it to linear chains of

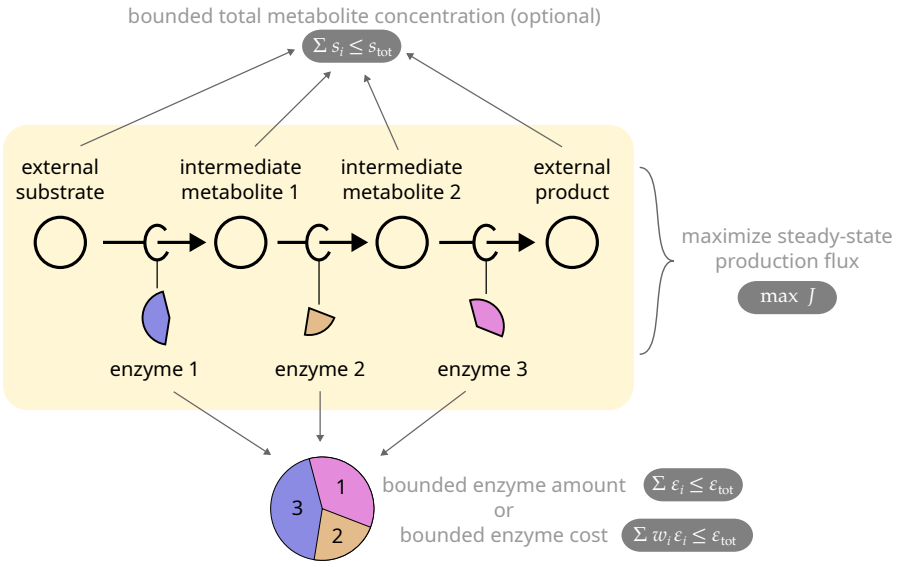


Figure 1: Optimal enzyme levels in unbranched metabolic pathways. In the basic optimality problem in this paper, we consider a chain of reactions and ask how a given protein budget should be spent on metabolic enzymes for a maximal steady-state flux. If the turnover rates of all the enzymes were known, the steady-state flux would determine the enzyme levels, and there would be nothing to optimize. Here we assume that the enzyme efficiencies can be adjusted by choosing the metabolite concentrations (not shown), and we search for the optimal metabolite and enzyme profile. The aim is to maximize the flux at a given total enzyme amount (bottom) and possibly under a constraint on metabolite concentrations (top). The flux ratios between reactions are predefined, for example assuming equal fluxes in all the reactions (right).

arbitrary size [21, 17, 18]. Here, we will present this general solution and further expand it to other types of rate laws beyond the one considered by Waley [20] (which, from now on, we will refer to as *mass-action*).

Consider the following unbranched pathway [22] (Figure 1):

$$\mathrm{S}_0 \overset{\mathrm{v}_1}{\rightleftharpoons} \mathrm{S}_1 \overset{\mathrm{v}_2}{\rightleftharpoons} \dots \overset{\mathrm{v}_n}{\rightleftharpoons} \mathrm{S}_n \tag{1}$$

In a kinetic model, each variable s_i represents the concentration of a metabolite i and each variable ε_i represents the level (molar concentration or mass concentration) of the enzyme catalyzing reaction i. Imagine that the total enzyme level in the pathway is bounded by ε_{tot}, i.e.

$$\sum_i \varepsilon_i \leq \varepsilon_{\text{tot}} \,. \tag{2}$$

What would be the optimal strategy for distributing this resource between the reactions in the pathway in order to maximize the steady state flux? To answer this question, we require more information as the rate of each reaction depends on the levels of enzyme, substrate, product, as well as kinetic parameters. For some rate laws, we can solve the optimization problem and obtain an analytic solution which describes exactly how much of each enzyme should be allocated. Below, we will also consider a variants of this problem with an extra bound on the sum of metabolite levels or with fixed initial and final metabolites (s_0 and s_n).

Since single analytic solutions are rare but instructive, we explore them in this article. In this paper we consider four different rate laws (summarized in Figure 2): the general Haldane rate law (saturable and reversible) which has now analytic solution, and three solvable approximations derived from it. As explained above, "enzyme levels" ε_i can either refer to molar concentrations or mass concentrations (depending on the modeler's preference). In the case of molar concentrations, the k^{cat} values are catalytic constant (e.g. in units of $1/\text{s}$). In the case of mass concentrations, k^{cat} are specific activities (e.g. in units of $\mu\text{mol} \times \text{min}^{-1} \times \text{mg}^{-1}$).

- **Reversible saturable rate law ("Haldane")** As a general type of rate law for a reaction $\mathrm{S} \leftrightarrow \mathrm{P}$, we consider here the reversible saturable rate law

$$v = \varepsilon \, \frac{k_+^{\text{cat}} \; s/K_{\mathrm{S}} - k_-^{\text{cat}} \; p/K_{\mathrm{P}}}{1 + s/K_{\mathrm{S}} + p/K_{\mathrm{P}}} \tag{3}$$

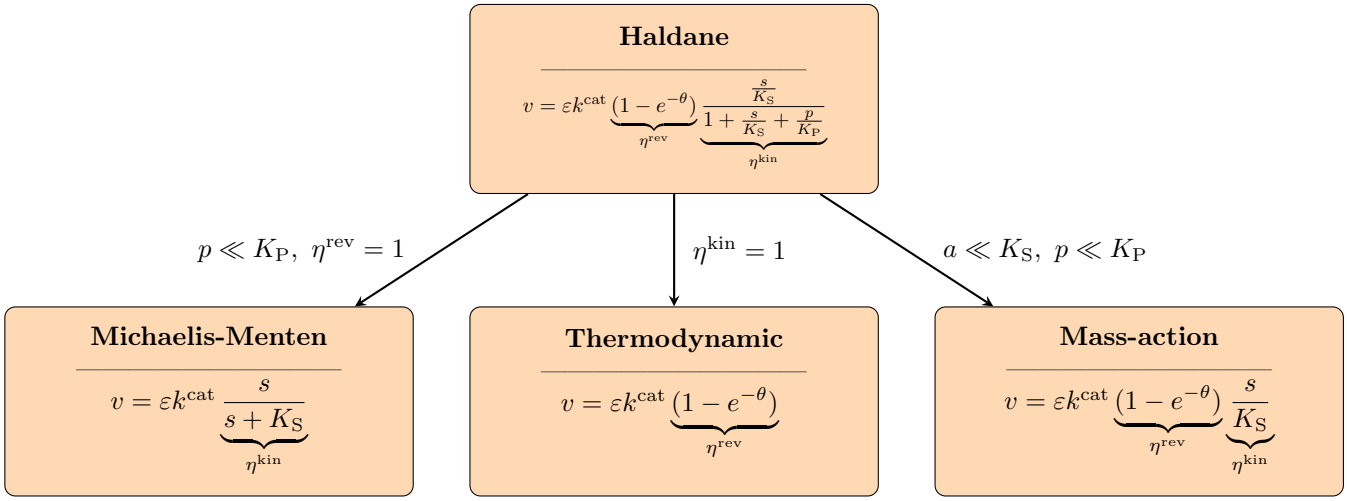


Figure 2: The Haldane rate law and some simplified rate laws. Simplified rate laws are obtained as limiting cases by setting efficiency terms η^{rev} or η^{kin} to 1 or another constant value (see Figure 3) or by assuming small reactant concentrations (in the case of the mass-action rate law). For the enzyme optimality problem in unbranched pathways, we do not know of any analytic solutions for the Haldane rate law. We report here solutions for the other, simplified rate laws (where the solution for the thermodynamic rate law contains an unknown auxiliary parameter).

with Michaelis-Mention constants K_S and K_P, which can be factorized into

$$v = \varepsilon\ k_+^{\mathrm{cat}}\ \underbrace{(1 - e^{-\theta})}_{\eta^{\mathrm{rev}}(\theta(s,p))}\ \underbrace{\frac{s/K_S}{1 + s/K_S + p/K_P}}_{\eta^{\mathrm{kin}}(s,p)}, \tag{4}$$

with the driving force $\theta(s,p) = \ln(K^{\mathrm{eq}}\ s/p)$, the thermodynamic efficiency η^{rev} and the kinetic efficiency η^{kin} (see Figure 3). The two efficiency terms can assume values between 0 and 1. These two formulations of the Haldane rate law are equivalent, based on the constraint that $K^{\mathrm{eq}} = k_+^{\mathrm{cat}}/k_-^{\mathrm{cat}} \cdot K_P/K_S$, which is commonly known as the *Haldane relationship* (see [23] for more details).

This rate law is the most realistic one that we discuss in this work, as it requires only a few assumptions. However, it is also the most mathematically complex and therefore most questions we raise don't have analytic solutions. So, in addition, we will consider three simplified rate laws as limiting cases.

- **Mass-action rate law** One of the most common approximations for enzymatic rate laws (the one also made by Waley [20]) is based on the limit of low metabolite concentrations ($s \ll K_S$ and $p \ll K_P$). In this case, the concentration-dependent terms in the denominator (i.e., $s/K_S + p/K_P$) are negligible so we get:

$$v = \varepsilon\ (k_+^{\mathrm{cat}}\ s/K_S - k_-^{\mathrm{cat}}\ p/K_P) \tag{5}$$

There are many equivalent ways to write down this rate law. For instance, we can apply the same approximation to the factorized form in Eq. (4) to get $v = \varepsilon\ k_+^{\mathrm{cat}}\ (1 - e^{-\theta})\ s/K_S$, and we can further replace θ with its explicit definition based on the reactant concentrations and write $v = \varepsilon\ k_+^{\mathrm{cat}}/K_S\ (s - p/K^{\mathrm{eq}})$.

Another common form for this rate law is based on the "first-order rate constants" $k_+ \equiv k_+^{\mathrm{cat}}/K_S$ and $k_- \equiv k_-^{\mathrm{cat}}/K_P$. Using them in Eq. (5) looks like this: $v = \varepsilon\ (k_+ s - k_- p)$. As it resembles mass-action (although here the enzyme level appears as a prefactor) we will refer to this as the "mass-action" rate law. Throughout this paper we will switch between these four different notations based on convenience.

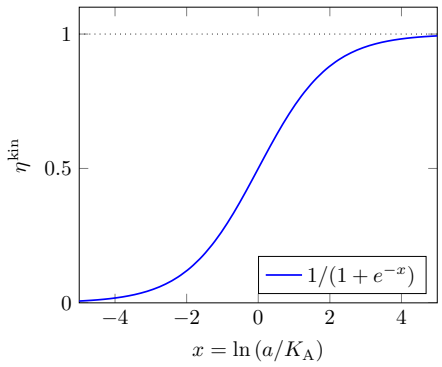
- **Thermodynamic rate law** If η^{kin} is approximated by 1 (e.g. in the limit $s \gg K_S$ and $p \ll K_P$), we obtain:

$$v = \varepsilon\ k_+^{\mathrm{cat}}\ \left(1 - \frac{p}{s}\ \frac{1}{K^{\mathrm{eq}}}\right) = \varepsilon\ k_+^{\mathrm{cat}}(1 - e^{-\theta}). \tag{6}$$

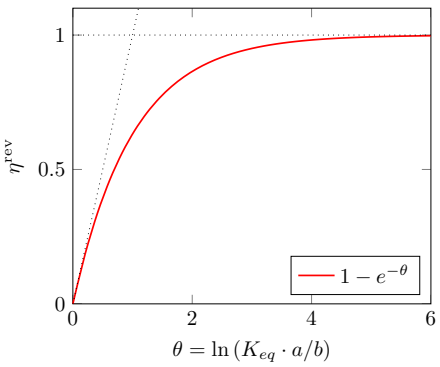
We will also consider a special case of this rate law where $p/s \approx K^{\mathrm{eq}}$, i.e. the reaction is close-to-equilibrium and therefore $\theta \to 0$. In this case the rate law becomes $v = \varepsilon\ k_+^{\mathrm{cat}}\ \theta$.

$$v = \varepsilon \; \frac{k_+^{\mathrm{cat}} \; a/K_\mathrm{A} - k_-^{\mathrm{cat}} \; b/K_\mathrm{B}}{1 + a/K_\mathrm{A} + b/K_\mathrm{B}} = \varepsilon \; k^{\mathrm{cat}} \; \underbrace{\left(1 - \frac{b}{aK^{\mathrm{eq}}}\right)}_{\eta^{\mathrm{rev}}} \; \underbrace{\frac{a/K_\mathrm{A}}{1 + a/K_\mathrm{A} + b/K_\mathrm{B}}}_{\eta^{\mathrm{kin}}}$$

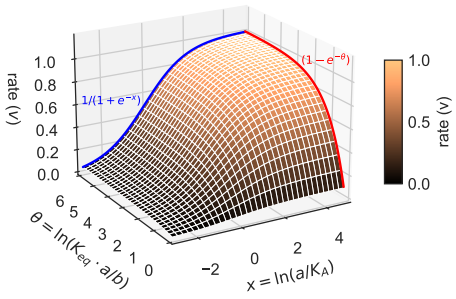
(a) Haldane Rate Law



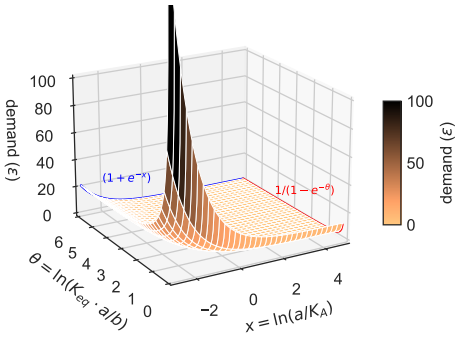
(b) Kinetic efficiency



(c) Thermodynamic Efficiency



(d) Rate laws



(e) Enzyme Demand

Figure 3: The Haldane rate law and approximations assuming constant efficiency terms. (a) The Haldane rate law. In the factorized form (right), thermodynamic and saturation effects are described by separate efficiency factors. (b) The thermodynamic efficiency as a function of θ (driving force). (c) The kinetic efficiency as a function of the product of the substrate (in log-scale), assuming $b \ll K_\mathrm{B}$. (d) A surface plot showing the rate (v) as a function of θ and the product of the two reactants (in log-scale). The parameters are $K^{\mathrm{eq}} = K_\mathrm{A} = k^{\mathrm{cat}} = \varepsilon = 1$ and $K_\mathrm{B} = 10$. (e) A surface plot showing the enzyme demand for a given rate ($v = 1$), all kinetic parameters are the same as in (d).

- **Irreversible saturable rate law ("Michaelis-Menten kinetics")** If we assume that $p \ll s \; K^{\mathrm{eq}}$ (which means that $\theta \to \infty$ and therefore η^{rev} can be approximated by 1) and also $p \ll K_\mathrm{P}$ (so we can drop p/K_P from the denominator in η^{kin}), we obtain the Michaelis-Menten rate law

$$v = \varepsilon \; k_+^{\mathrm{cat}} \; \frac{s}{s + K_\mathrm{S}}. \tag{7}$$

  Originally, Michaelis and Menten [24] developed this rate law by assuming that the rate of enzyme-substrate binding is very fast compared to catalysis, and that the catalytic step is irreversible. The assumptions made here lead to the same result but are less stringent.

In the approximations, we may assume that the thermodynamic or saturation efficiencies (one of them or both) are approximated by constant numbers smaller than 1; this has the same effect as setting the efficiency to 1, but instead of the k^{cat} value we obtain a smaller apparent value in the approximated rate law.

2.2 Metabolic states - what formulae are we interested in?

How can we characterize metabolic states? The aim here is to do this by using analytic formulae. For an unbranched pathway with a given type of rate laws (e.g. mass-action or Michaelis-Menten rate laws), we are interested in formulae for the following quantities:

1. **Metabolic steady state** Given the enzyme levels, external metabolite concentrations, and kinetic constants, can we directly compute the stationary fluxes and internal metabolite concentrations. For general metabolic networks, no explicit formulae are known, but for unbranched pathways with some simple rate laws, explicit formulae exist.

2. **Stability of steady state** If the Jacobian in a steady state has positive eigenvalues, the state is asymptotically unstable and is not able to persist under (inevitable) noise in the cell. Stability is also a prerequisite for metabolic control coefficients being defined. A sufficient (but not necessary) condition for stable steady states in unbranched metabolic pathways is given in Appendix D.7.

3. **Metabolic control** The response coefficients are defined as derivatives between steady-state concentrations or fluxes and model parameters (e.g. the enzyme levels). If two model parameters act (specifically) on the same reaction, their response coefficients towards all fluxes and steady-state concentrations will be the same (up to a proportional scaling). The control coefficients take this into account: they describe the same type of derivatives, but for a set of hypothetical parameters that individually act on reaction rates. In practice, assuming that reaction rates are proportional to enzyme levels, that each reaction is catalyzed by a single enzyme, and that each enzyme catalyzes a single reaction, we can define them as $C^X_{il}/(v_l/e_l)$.

   The control coefficients can be computed from the stoichiometric matrix and the elasticity matrix, and they satisfy summation and connectivity theorems. Therefore, the metabolic control coefficients can be computed in two ways: if an analytic formula for the metabolic steady state is known (as is the case in some unbranched pathway models studied below=, we may differentiate symbolically by the enzyme levels; otherwise, in theory, we may compute (symbolically) the elasticity coefficient matrices by differentiating the rate laws, and then compute the control coefficient matrices from them; however, since this involves a matrix inversion, the analytic formulae for this may be extremely complicated. Another way to compute control coefficients, which only works in optimal states, is described below.

4. **Optimal metabolic states** Below, in our basic metabolic optimality problem, we define optimal states as states in which a given enzyme budget (fixed sum of enzyme levels) is allocated to maximize a production flux. Kinetic constants and external metabolites are given, and we compute the optimal metabolite profile, the optimal enzyme profile, and the optimal flux. If the flux distribution is known (e.g. a steady flux in an unbranched pathway) and can only increase or decrease proportionally, this problem is equivalent to the ECM problem of minimizing the enzyme demand at a given (unit) flux. This (convex) problem can be solved numerically, but analytic solutions were known only for very few cases. Below we present some new analytic solutions. Formulae for optimal enzyme levels and the optimal achievable flux are shown below, in table 1. We also consider a related problem, maximizing the flux under a constraint on the total enzyme plus metabolite investment.

5. **Metabolic control in optimal states** The control coefficients tell us how a metabolic system responds physically to perturbations (resulting in a perturbed state that is again stationary, but probably non-optimal). For optimal states (with a constraint on the sum of enzyme levels), the enzyme-control rule states that enzyme levels and flux control coefficients must be proportional. Since we also know (from the summation theorem) that the control coefficients must sum to 1, we can conclude: whenever there is an analytic formula for optimal enzyme levels, we obtain a formula for the control coefficients.

2.3 Optimal metabolic states: analytic solutions

What are the general principles behind optimal enzyme allocation? One important principle, valid in optimal metabolic states, has been shown for pathways with mass-action rate laws [21] and later been confirmed for general rate laws [17]: in optimal states – where the metabolic flux has been maximized at a fixed enzyme budget enzyme

Name (assumptions)	**Enzymatic rate law:** $v_i/\varepsilon_i =$	**Comments / definitions**	**Optimal enzyme allocation:** ${\varepsilon_i}^* \propto$	**Minimum pathway cost per flux:** $\varepsilon_{\mathrm{tot}}/J^* =$
Trivial (irreversible, saturated)	k_i^{cat}	$\alpha_i \equiv 1/k_i^{\mathrm{cat}}$	α_i	$\|\|\boldsymbol{\alpha}\|\|_1$
Michaelis-Menten (irreversible, saturable)	$k_i^{\mathrm{cat}} \frac{s_{i-1}}{s_{i-1}+K_{\mathrm{M},i}}$	$\beta_i \equiv K_{\mathrm{M},i}/k_i^{\mathrm{cat}}$ $\sum_i s_i \leq s_{\mathrm{tot}}$	$\alpha_i + \sqrt{\beta_i \, \|\|\boldsymbol{\beta}\|\|_{\frac{1}{2}}}/s_{\mathrm{tot}}$	$\|\|\boldsymbol{\alpha}\|\|_1 + \|\|\boldsymbol{\beta}\|\|_{\frac{1}{2}} \big/ s_{\mathrm{tot}}$
Thermodynamic (reversible, unsaturable)	$k_i^{\mathrm{cat}} \cdot \left(1 - \frac{s_i}{s_{i-1}K_i^{\mathrm{eq}}}\right)$	$s_0 = \mathrm{const}$, $s_n = \mathrm{const}$ Find Ψ s.t. $\frac{1}{2}\ln(s_0 K_{\mathrm{tot}}^{\mathrm{eq}}/s_n) = \sum_i \ln\left(\sqrt{\Psi\alpha_i} + \sqrt{1+\Psi\alpha_i}\right)$	$\alpha_i \left(\frac{1}{2} + \frac{1}{2}\sqrt{1+(\Psi\alpha_i)^{-1}}\right)$	$\sum_i \alpha_i \left(\frac{1}{2} + \frac{1}{2}\sqrt{1+(\Psi\alpha_i)^{-1}}\right)$
Thermodynamic $K_{\mathrm{tot}}^{\mathrm{eq}}\, s_0/s_n \to 1$	$k_i^{\mathrm{cat}} \cdot \ln\left(\frac{s_{i-1}K_i^{\mathrm{eq}}}{s_i}\right)$	$s_0 = \mathrm{const}$, $s_n = \mathrm{const}$	$\sqrt{\alpha_i}$	$\|\|\boldsymbol{\alpha}\|\|_{\frac{1}{2}} \big/ \ln(K_{\mathrm{tot}}^{\mathrm{eq}}\, s_0/s_n)$
Mass-action [20] (reversible, unsaturable)	$k_i^{\mathrm{cat}} \cdot \left(1 - \frac{s_i}{s_{i-1}K_i^{\mathrm{eq}}}\right) \frac{s_{i-1}}{K_{\mathrm{M},i}}$	$s_0 = \mathrm{const}$, $s_n = \mathrm{const}$ $\gamma_i \equiv \beta_i \cdot \prod_{j=i}^{n} K_j^{\mathrm{eq}}$	$\sqrt{\gamma_i}$	$\|\|\boldsymbol{\gamma}\|\|_{\frac{1}{2}} \big/ (s_0 - s_n/K_{\mathrm{tot}}^{\mathrm{eq}})$
Haldane (reversible, saturable)	$k_i^{\mathrm{cat}} \cdot \left(1 - \frac{s_i}{s_{i-1}K_i^{\mathrm{eq}}}\right) \frac{s_{i-1}/K_{\mathrm{S},i}}{1+s_{i-1}/K_{\mathrm{S},i}+s_i/K_{\mathrm{P},i}}$	No known solution, use convex optimization solver		

Table 1: A summary of all rate laws considered in this paper, along with their solution for optimal enzyme allocation and minimum pathway cost per flux. The rate laws are roughly ordered by increasing level of complexity. The "trivial" rate law does not have its own section in the text, but is rather mentioned as the limit of the Michaelis-Menten rate law when $s_{i-1} \gg K_{\mathrm{M},i}$, as well as the thermodynamic rate law when $K_{\mathrm{tot}}^{\mathrm{eq}}\, s_0/s_n \to \infty$. Note that although the "mass-action" rate law appears in this table in a modified form (for the purpose of using the same set of kinetic parameters as the other rate laws), it is equivalent to the form used in previous studies [20, 16, 17]. In order to calculate the absolute optimal enzyme levels from the relative ones (given in the column titled *Optimal enzyme allocation*), one can use $\varepsilon_i^* = \varepsilon_{\mathrm{tot}} \frac{x_i}{\sum_j x_j}$ where x_i are the relative values.

levels and flux control coefficients must be proportional[1]:

$$ {\varepsilon_i}^* \propto {C_i^J}^*. \tag{8} $$

Here the star * denotes variables in the optimal state. Together with summation theorem for flux control coefficients [14, 15], $\sum_i C_i^J = 1$, we obtain the conversion formulae

$$ {C_i^J}^* = \frac{{\varepsilon_i}^*}{\sum_j {\varepsilon_j}^*}, \qquad {\varepsilon_i}^* = \varepsilon_{\mathrm{tot}}\, {C_i^J}^*. \tag{9} $$

The enzyme-control rule (8) provides a condition for metabolic states, independent of the type of rate laws considered. Importantly, it holds only in states of maximal flux, given a fixed total enzyme amount and no other constraints. More realistic models employ also bounds on metabolite levels [22, 5, 25] for different reasons. First, metabolite levels are bounded in real cells: while metabolite molecules may be small, there concentrations are high, and they contribute much more than macromolecules to osmotic pressure. Second, as we will see below, some models without metabolite bounds lead to paradoxical results. We will present a generalized version of the enzyme-control rule that takes metabolite bounds into account.

But what are the general shapes of optimal enzyme profiles, i.e. how do enzyme levels vary across the network? And on what factors (kinetic, thermodynamic, or cost factors) does this depend? To answer this, the enzyme-control rule alone would not be enough (because kinetic details do not appear in the rule). Also numerical studies would not be enough (because they apply to single cases and yield no general laws). Hence, to study this, it would be good to consider analytic solutions. Unfortunately, analytic solutions are not known for general metabolic model, but solutions exist for unbranched pathways with simple rate laws (mass-action rate law and saturable kinetics.

We now present analytic formulae for optimal metabolic states with different types of rate laws. We discuss the rate laws in increasing order of difficulty. An overview of all analytic solutions is given in Table 1.

[1] To explain this rule, we note that in an optimal state all enzymes have the marginal cost (contribution to the enzyme budget, per mol of enzyme), which must be balanced by the same marginal benefit (contribution to the production flux, per mol of enzyme). This means that all enzymes must have the same (unscaled) flux response coefficients dv^{st}/de_l, and so the (unscaled) flux control coefficients $dv^{\mathrm{st}}/de_l/(\frac{v}{e_l})$ must be proportional to the enzyme concentrations e_l.

2.3.1 Michaelis-Menten rate law

The first rate law we consider is the Michaelis-Menten rate law (i.e. irreversible reactions with simple saturation kinetics):

$$v_i = \varepsilon_i \cdot k_i^{\text{cat}} \ \frac{s_{i-1}}{s_{i-1} + K_{\text{M},i}} \ . \tag{10}$$

In an unbranched pathway, at steady-state all rates must be equal. To describe them, we introduce a new variable J called the pathway flux, and assume that $\forall i \ v_i = J$. Now we can use Eq. (10) to find a relationship between the substrate and the enzyme level:

$$\varepsilon_i = \frac{J}{k_i^{\text{cat}}} \cdot \left(1 + \frac{K_{\text{M},i}}{s_{i-1}}\right) = J \left(\alpha_i + \frac{\beta_i}{s_{i-1}}\right) \tag{11}$$

where we define $\alpha_i \equiv 1/k_i^{\text{cat}}$ and $\beta_i \equiv K_{\text{M},i}/k_i^{\text{cat}}$ for convenience.

Combining this equation with the upper bound on total enzyme from Eq. (2) we get that:

$$\varepsilon_{\text{tot}} \geq \sum_i \varepsilon_i = \sum_i J \ \left(\alpha_i + \frac{\beta_i}{s_{i-1}}\right) = J \left(\sum_i \alpha_i + \sum_i \frac{\beta_i}{s_{i-1}}\right) \ . \tag{12}$$

and by rearranging we get:

$$J \leq \frac{\varepsilon_{\text{tot}}}{\sum_i \alpha_i + \sum_i \frac{\beta_i}{s_{i-1}}} \tag{13}$$

Maximizing J would mean that we reach the upper bound and therefore we can treat this as an equality. Since ε_{tot} is constant and the only free variables are the metabolite concentration, the maximal flux is reached when the denominator on the right-hand side is minimized. The problem is that it is a monotonically decreasing function in s_i (for each i) and since metabolite concentrations are unbounded, the optimum is at $s_i = \infty$. In reality, of course, the range of physiological osmotic pressure does impose some constraint on the concentrations of small molecules. As a proxy for this effect, we can add another constraint to bound the sum of all metabolite concentrations, $\sum_i s_i \leq s_{\text{tot}}$. Thus, one can show (see Appendix D.1) that the optimal allocation of enzymes will obey:

$${\varepsilon_i}^* \propto \alpha_i + \sqrt{\beta_i} \cdot \frac{\sqrt{||\boldsymbol{\beta}||_{\frac{1}{2}}}}{s_{\text{tot}}} \tag{14}$$

where $\boldsymbol{\beta}$ is the vector of all β_i and $||\cdot||_{\frac{1}{2}}$ is the $\text{l}_{1/2}$ norm. In this case, the maximal flux would be:

$$J^* = \varepsilon_{\text{tot}} \cdot \left(||\boldsymbol{\alpha}||_1 + ||\boldsymbol{\beta}||_{\frac{1}{2}}/s_{\text{tot}}\right)^{-1} \ . \tag{15}$$

Note that the solution looks essentially the same even if we constrain the first metabolite (s_0) to have a fixed concentration (see Appendix D.1.4). We will revisit this case in section 2.5 in the context of a course-grained model of a growing cell.

If the metabolite density constraint is not very tight (i.e. s_{tot} is large enough), we can ignore the second term in Eq. (14) which would be equivalent to assuming all enzymes are substrate-saturated. In this case, the optimal allocation of enzymes will be proportional to α_i (or inversely proportional to k_i^{cat}) and therefore:

$$\lim_{s_{\text{tot}} \to \infty} J^* = \varepsilon_{\text{tot}} \cdot \left(||\boldsymbol{\alpha}||_1\right)^{-1} \ . \tag{16}$$

Interestingly, $||\boldsymbol{\alpha}||_1$, which is equal to the sum of k^{cat} reciprocals, is in fact the inverse of the *Pathway Specific Activity* as originally defined by Bar-Even et al. [11]. Indeed, the idealized scenario considered in that study (where all enzymes are irreversible and saturated) provides a sort of upper bound on the maximal flux achievable.

One reason that the irreversible Michaelis-Menten model is unrealistic is that it ignores reactions that are close to equilibrium and therefore suffer from a counter-productive reverse flux. This is yet another reason why it might be impossible for some metabolites to reach very high concentrations: they might be products of unfavorable reactions. In most metabolic networks, about half of the reactions are reversible and therefore it would be more realistic to use a reversible rate law like such as Haldane's. However, using the Haldane equation would typically create a system of equations with an infinite number of solutions. So, first, we will now consider reversible rate laws that reverse fluxes into account.

2.3.2 Thermodynamic rate law

Irreversible rate laws like the Michaelis-Menten kinetics depend only on the substrate concentration; in the formula there is no product-dependent reverse term that decreases the total rate or could make it become negative. However, according to thermodynamics, such laws can only be approximations: thermodynamically feasible rate laws must contain a reverse term and must depend on the thermodynamic imbalance of substrate and product concentrations expressed by the thermodynamic force. Some rate laws can even be written as functions of the thermodynamic driving force alone. Here we describe a "thermodynamic" rate law, where v is proportional to the thermodynamic efficiency $1 - \mathrm{e}^{-\theta}$, while the kinetic efficiency η^{kin} is assumed to be constant:

$$v_i = \varepsilon_i \; k_i^{\mathrm{cat}} \left(1 - e^{-\theta_i}\right) = \varepsilon_i \; k_i^{\mathrm{cat}} \left(1 - \frac{s_i}{s_{i-1}} \; \frac{1}{K_i^{\mathrm{eq}}}\right) . \tag{17}$$

This type of kinetics approximates cases where all reactions are saturated ($s_i \gg K_{\mathrm{M},i}$). The parameters here are the turnover numbers (k_i^{cat}) and the equilibrium constants (K_i^{eq}). However, as we will soon learn, the individual K_i^{eq} do not change the result, and only the overall equilibrium constant ($K_{\mathrm{tot}}^{\mathrm{eq}} = \prod_i K_i^{\mathrm{eq}}$) matters.

As before, we can define the steady-state pathway flux J and apply the upper bound on the total enzyme to get:

$$\begin{aligned} \varepsilon_{\mathrm{tot}} \geq \sum_i \varepsilon_i = \sum_i \frac{J}{k_i^{\mathrm{cat}} \left(1 - e^{-\theta_i}\right)} = J \cdot \left(\sum_i \frac{1}{k_i^{\mathrm{cat}}} \cdot \frac{1}{1 - e^{-\theta_i}}\right) \\ J \leq \varepsilon_{\mathrm{tot}} \cdot \left(\sum_i \frac{1}{k_i^{\mathrm{cat}}} \cdot \frac{1}{1 - e^{-\theta_i}}\right)^{-1} . \end{aligned} \tag{18}$$

Unfortunately, there is no closed-form analytic solution for the maximal rate of this thermodynamic rate law. But, we do have something very close which requires rather simple computations. First, we need to invert the functional relationship between the overall driving force (θ_{tot}) and an auxiliary variable Ψ which is defined by the inverse function of:

$$\ln\left(\frac{s_0}{s_n} K_{\mathrm{tot}}^{\mathrm{eq}}\right) = \theta_{\mathrm{tot}} = 2 \sum_i \ln\left(\sqrt{\Psi / k_i^{\mathrm{cat}}} + \sqrt{1 + \Psi / k_i^{\mathrm{cat}}}\right) . \tag{19}$$

The expression on the right is analytic and strictly increasing in the range $\Psi \in [0, \infty)$, so there is a unique solution which can be found by simple numerical methods. Then, we can use that value to directly calculate the optimal driving forces, enzyme levels and pathway flux:

$$\begin{aligned} {\theta_i}^* &= 2 \ln\left(\sqrt{\Psi / k_i^{\mathrm{cat}}} + \sqrt{1 + \Psi / k_i^{\mathrm{cat}}}\right) \\ {\varepsilon_i}^* &= J^* \cdot \left(\frac{1}{2} + \frac{1}{2}\sqrt{1 + k_i^{\mathrm{cat}} / \Psi}\right) \Big/ k_i^{\mathrm{cat}} \\ J^* &= \varepsilon_{\mathrm{tot}} \cdot \left(\sum_i \left(\frac{1}{2} + \frac{1}{2}\sqrt{1 + k_i^{\mathrm{cat}} / \Psi}\right) \Big/ k_i^{\mathrm{cat}}\right)^{-1} . \end{aligned} \tag{20}$$

The full derivation of this solution can be found in Appendix D.2.2. An example of what the relationship between the driving force and the optimal flux looks like for a pathway with two enzymes is illustrated in Figure 4(a) and 4(b).

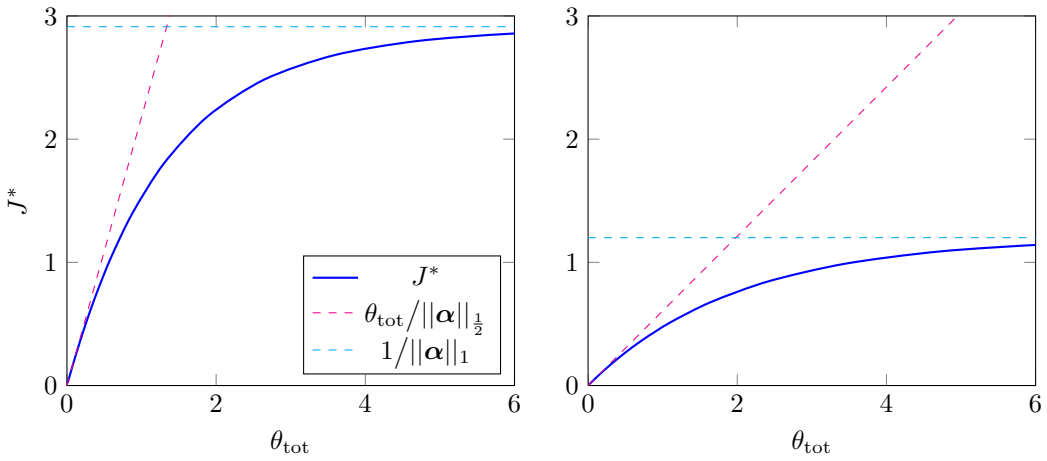
These formulae cannot be directly evaluated because of the unknown parameter Ψ. To obtain solutions that do not depend on this parameter, we now consider two limiting cases in which Ψ is either infinitely high (very high driving force) or infinitely low (very low driving force).

When the driving forces are very high (i.e. $\theta_{\mathrm{tot}} \to \infty$), the solution for Ψ in Eq. (19) will approach infinity and the optimal flux becomes

$$\lim_{\theta_{\mathrm{tot}} \to \infty} J^* = \lim_{\Psi \to \infty} \frac{\varepsilon_{\mathrm{tot}}}{\sum_i 1/k_i^{\mathrm{cat}} \cdot \left(\frac{1}{2} + \frac{1}{2}\sqrt{1 + k_i^{\mathrm{cat}} / \Psi}\right)} = \frac{\varepsilon_{\mathrm{tot}}}{\sum_i 1/k_i^{\mathrm{cat}}} = \varepsilon_{\mathrm{tot}} \cdot \left(||\boldsymbol{\alpha}||_1\right)^{-1} \tag{21}$$

where we use the same definition as before for the set of parameters $\alpha_i = 1/k_i^{\mathrm{cat}}$. This solution indeed makes sense as it is equivalent to the fully saturated limit in the Michaelis-Menten case (see Section 2.3.1, Eq. (16)).

However, when there is a limited amount of driving force in the pathway (Ψ has a finite positive value), enzymes with a higher k_i^{cat} value will pay a higher penalty due to their driving force being closer to 0. On the other



(a) $k_1^{\text{cat}} = 3s^{-1}$ $k_2^{\text{cat}} = 100s^{-1}$ (b) $k_1^{\text{cat}} = 3s^{-1}$ $k_2^{\text{cat}} = 2s^{-1}$

Figure 4: The relationship between the maximal flux per enzyme and overall driving force for the thermodynamic rate law. Even though we do not have a closed-form solution for J^* as a function of θ_{tot}, we can still plot $J^*(\Psi)$ against $\theta_{\text{tot}}(\Psi)$ for varying values of Ψ using Eq. (19) and Eq. (20). Here, we show this relationship for a pathway with 2 steps. The parameters are: $\varepsilon_{\text{tot}} = 1$, $K_i^{\text{eq}} = 1$, $k_1^{\text{cat}} = 3\ s^{-1}$, and the k^{cat} of the second enzyme is either (a) $k_2^{\text{cat}} = 100\ s^{-1}$ or (b) $k_2^{\text{cat}} = 2\ s^{-1}$. The magenta dashed line represents the approximations for very small driving forces based on Eq. (23), and similarly in cyan for very large driving forces – Eq. (21).

hand, slow enzymes will have more driving force, which will help them by having a smaller fraction of reverse flux. Notably, the distribution is *not* dependent on the reaction equilibrium constants (only on the overall $K_{\text{tot}}^{\text{eq}}$). Perhaps it is not that surprising if we consider the fact that the concentrations intermediate substrates and products are unconstrained and therefore we have enough degrees of freedom to adjust to any value of K_i^{eq} as long as the total driving force stays the same.

One can also consider the other extreme where all reactions are close to equilibrium, which means that θ_{tot} is close to 0 (and therefore also each $\theta_i \to 0$). In this case, we can approximate Eq. (18) by:

$$J \leq \varepsilon_{\text{tot}} \cdot \left(\sum_i \frac{1}{k_i^{\text{cat}}} \cdot \frac{1}{1 - e^{-\theta_i}} \right)^{-1} \approx \varepsilon_{\text{tot}} \cdot \left(\sum_i \frac{1}{k_i^{\text{cat}}\ \theta_i} \right)^{-1} \tag{22}$$

Maximizing J under the constraint $\sum_i \theta_i = \theta_{\text{tot}}$ yields the solution (see the full derivation in appendix D.2.3):

$$\begin{aligned} {\theta_i}^* &= \theta_{\text{tot}} \frac{\sqrt{1/k_i^{\text{cat}}}}{\sum_i \sqrt{1/k_i^{\text{cat}}}} \\ J^* &= \frac{\theta_{\text{tot}}\ \varepsilon_{\text{tot}}}{\left(\sum_i \sqrt{1/k^{\text{cat}}}\right)^2} = \varepsilon_{\text{tot}} \cdot \frac{\theta_{\text{tot}}}{||\boldsymbol{\alpha}||_{\frac{1}{2}}} \,. \end{aligned} \tag{23}$$

2.3.3 Mass-action rate law

Next, we consider the same unbranched pathway but assuming mass-action rate laws:

$$v_i = \varepsilon_i\ k_i^{\text{cat}} / K_{\text{M},i}\ (s_{i-1} - s_i / K_i^{\text{eq}}) = \varepsilon_i\ \beta_i^{-1}\ (s_{i-1} - s_i / K_i^{\text{eq}}) \,. \tag{24}$$

Where, as before, we define $\beta_i \equiv K_{\text{M},i}/k_i^{\text{cat}}$. Note that instead of Eq. (5) we use a form of the mass-action rate law that does not require the turnover rate and K_{M} of the product (and instead uses K_i^{eq}), to avoid confusing indexation of forward and backward parameters.

Like with the previous rate laws, we can define the pathway flux J and apply the upper bound on the total enzyme

levels:

$$\begin{aligned} \varepsilon_{\text{tot}} \geq \sum_i \varepsilon_i = \sum_i \frac{J\ \beta_i}{s_{i-1} - s_i/K_i^{\text{eq}}} = J \sum_i \frac{\beta_i}{s_{i-1} - s_i/K_i^{\text{eq}}} \\ J \leq \varepsilon_{\text{tot}} \cdot \left(\sum_i \frac{\beta_i}{s_{i-1} - s_i/K_i^{\text{eq}}} \right)^{-1} . \end{aligned} \tag{25}$$

Again, we maximize the flux at a constrained total enzyme level. Optimization with Lagrange multipliers yields the following expressions for the optimal individual enzyme levels ($\varepsilon_j{}^*$) and the maximal flux (J^*) (see full derivation in Appendix D.3):

$$\begin{aligned} \varepsilon_j{}^* &= \varepsilon_{\text{tot}} \sqrt{\frac{\gamma_j}{||\boldsymbol{\gamma}||_{\frac{1}{2}}}} \\ J^* &= \varepsilon_{\text{tot}} \cdot \frac{s_0\ K_{\text{tot}}^{\text{eq}} - s_n}{||\boldsymbol{\gamma}||_{\frac{1}{2}}} \end{aligned} \tag{26}$$

where γ_j and $||\cdot||_{\frac{1}{2}}$ are defined as

$$\gamma_i \equiv \beta_i \prod_{j=i}^{n} K_j^{\text{eq}} \qquad \text{and} \qquad ||\boldsymbol{\gamma}||_{\frac{1}{2}} \equiv \left(\sum_{j=1}^{n} \sqrt{\gamma_j} \right)^2 . \tag{27}$$

Of course, the exact value of this maximum would depend on all the different system parameters. However, it is interesting to consider a naïve assumption where all the γ_j parameters are identical (i.e., where all $K_i^{\text{eq}} = 1$). In such a case, the flux in the pathway would decrease quadratically with the number of steps [26]. Of course, we know that in real metabolic pathways the equilibrium constants are typically not close to 1, and therefore this approximation might not have many applications in biology.

What would happen if a mutation improved the catalytic rate of only one of the enzymes ε_i by a factor of a (i.e. β_i decreases by a factor of a, but the equilibrium constant K_i^{eq} remains the same). In this case, γ_i would be divided by a but the optimal enzyme concentration for this reaction $\varepsilon_i{}^*$ would only decrease by a factor of $\sqrt{a}$. This saving would then be distributed proportionally among all the n enzymes and contribute to an increase in the pathway flux J. On the flip side, if for some reason the activity of one enzyme is decreased by a multiplicative factor b, it would need to "pay" (increase its concentration) only by a factor $\sqrt{b}$, and this increase would be "funded" by all of the enzymes together in order to keep the same ε_{tot}, thus lowering J.

2.3.4 Haldane rate law

So far we analyzed three cases where all enzymes could be described by a special kinetic rate law: mass-action, Michaelis-Menten, or thermodynamic. Although these rate laws can reliably describe some enzymes in specific conditions, it is very unlikely that such an approximation would apply to *all* the reactions in a single pathway (except for the trivial case of a 1-reaction pathway). A more realistic model would allow all reactions to be reversible and saturable. Here, we will analyze such a case based on the factorized rate law with one substrate and one product. Note, that although it is equivalent to Eq. (3), we prefer this formulation because it is easier to separate thermodynamics from saturation effects.

$$v_i = \varepsilon_i \cdot k_i^{\text{cat}} \cdot \left(1 - e^{-\theta_i}\right) \cdot \frac{s_{i-1}/K_{S,i}}{1 + s_{i-1}/K_{S,i} + s_i/K_{P,i}} . \tag{28}$$

As always, we can assume all fluxes are equal to J and use the total enzyme budget to get an upper bound:

$$\begin{aligned} \varepsilon_{\text{tot}} \geq \sum_i \varepsilon_i &= \sum_i \frac{J}{k_i^{\text{cat}} \cdot \left(1 - e^{-\theta_i}\right) \cdot \frac{s_{i-1}/K_{S,i}}{1 + s_{i-1}/K_{S,i} + s_i/K_{P,i}}} \\ &= J \cdot \left(\sum_i \frac{1}{k_i^{\text{cat}}} \left(1 - e^{-\theta_i}\right)^{-1} \left(1 + \frac{K_{S,i}}{s_{i-1}} + \frac{s_i K_{S,i}}{s_{i-1} K_{P,i}} \right) \right) \\ J &\leq \varepsilon_{\text{tot}} \cdot \left(\sum_i \frac{1}{k_i^{\text{cat}}} \left(1 - e^{-\theta_i}\right)^{-1} \left(1 + \frac{K_{S,i}}{s_{i-1}} + \frac{s_i K_{S,i}}{s_{i-1} K_{P,i}} \right) \right)^{-1} \end{aligned} \tag{29}$$

where we can now appreciate how this is a generalization of both Eq. (13) (Michaelis-Menten) and (71) (thermodynamic). We can see that the maximal pathway flux would be realized when the term in parentheses is minimized with respect to the s_i, i.e.:

$$\text{minimize}_{\mathbf{s}} \sum_i \frac{1}{k_i^{\text{cat}}} \left(1 - e^{-\theta_i(\mathbf{s})}\right)^{-1} \left(1 + \frac{K_{\text{S,i}}}{s_{i-1}} + \frac{s_i K_{\text{S,i}}}{s_{i-1} K_{\text{P,i}}}\right) . \tag{30}$$

Unfortunately, as we have already discussed, this problem is not solvable analytically in the general case.

2.4 Optimal metabolic states: insights from Metabolic Control Analysis

2.4.1 Enzyme-control rule and enzyme-elasticity rule

We start with some further observations about the enzyme-control rule. The enzyme-control rule Eq. (8) states that enzyme levels adn flux control coefficients must be proportional: $\varepsilon_i^* \propto C_i^{J^*}$. From the summation theorem, we know that the flux control coefficients must sum to 1; therefore, the optimal enzyme levels must be given by the flux control coefficients, multiplied by the (predefined) total enzyme level. For optimal states, we obtain the simple conversion $C_i^J = \frac{\varepsilon_i}{\varepsilon_{\text{tot}}}$ and $\varepsilon_i = \varepsilon_{\text{tot}}\ C_i^J$. When the aim is to compute control coefficients in optimal states (at a given enzyme budget ε_{tot}), the enzyme-control rule comes in handy. To what types of optimality problems does the rule apply? If the metabolic system is not an unbranched pathway, but a general network with one target flux, the rule will still hold (where the flux control coefficients refer to this target flux. This also hold for models with metabolite dilution. In the basic forma of the rule we put a constraint on the enzyme mass, and assume that enzyme levels are mass densities. If the enzyme levels are molar concentrations, and differently weighted in the constraint, the weights can be taken into account by modifying the rule

However, there are some limitations. Importantly, the rule holds only in states in which control coefficients are well-defined. This condition may seem unproblematic, but below we will see that it is actually violated in the chain with Michaelis-Menten rate laws because in the optimal state, any variation of a single enzyme level would not only make the state non-optimal, but would in fact break the steady state (the reason being that in this case, the optimal state, all internal metabolite elasticities are zero). We discuss this in Appendix A.1. One way of avoiding this problem is to introduce a bound on metabolite levels. For example, we may consider a problem with a generalized density constraint on enzyme levels and metabolite concentrations

$$\text{Maximize } \mathbf{z} \cdot \mathbf{v} \quad \text{s.t.} \quad \mathbf{a} \cdot \boldsymbol{\varepsilon} + \mathbf{b} \cdot \mathbf{s} \leq \rho$$

with a linear flux objective $\mathbf{a} \cdot \boldsymbol{\varepsilon}$ instead of a single target flux, and enzyme weights a_l and metabolite weights b_i, and an upper ρ on the molecule density. This problem leads to a generalized form of the enzyme-control rule. For an unbranched pathway with equal weights (e.g. molecular masses) a for enzymes and b for metabolites,

$$\varepsilon_l^* = \varepsilon_{\text{tot}}^*\ C_l^{J*} - J^*\ \frac{b}{a}\ \sum_i C_l^{s_i *}. \tag{31}$$

where $\varepsilon_{\text{tot}}^*$ is the sum of enzymes emerging in the optimal state (for derivation and details, see Appendix A.3).

The enzyme-control rule has a direct and useful consequence. Since enzyme levels (in optimal states) are proportional to flux control coefficients, they need to satisfy a connectivity theorem. Connectivity theorems relate the elasticities of of a given metabolite i to the control coefficients of reactions around this metabolite. In the case of flux control coefficients, the right-hand side of the theorem is zero, and we obtain an equation of exactly the same form for the optimal enzyme levels. We call this formula the enzyme-elasticity rule (see Appendix B). A similar rule for small adaptations of enzyme levels, instead of enzyme levels themselves, has been shown in [27]. For a linear chain, the enzyme-elasticity rule entails a simple result: in an optimal state, for each metabolite i and its producing reaction $j = i - 1$ and its consuming reaction i, the ratio of enzyme levels $\frac{\varepsilon_i}{\varepsilon_j}$ must be equal to the inverse ratio of the elasticities $E_{c_i}^{v_j} / E_{c_i}^{v_i}$, where the negative sign of $E_{c_i}^{v_j}$ is ignored. An example of this, with the mass-action rate law, is explicitly shown in Appendix B. In contrast to the enzyme-control rule (where there is only one for the entire pathway), there is an enzyme-elasticity rule for every internal metabolite, which determines the ratio of the enzyme levels around this metabolite. Together with the known sum of enzyme levels, these rules therefore determine the entire optimal enzyme profile (of course, given all the elasticities in the optimal state). In Appendix B, we show the explicit derivation for a concrete example, the mass-action rate law.

2.4.2 Analytic formulae for different rate laws

We learned that enzyme levels in optimal states (under a constraint on total enzyme, and for a maximization of steady-state flux) satisfy two general laws, the enzyme-control rule and the enzyme-elasticity rule. Hence, whenever we have a formula for the optimal enzyme levels and can trust the enzyme-control rule, we also know the flux control coefficients. The enzyme-elasticity rule (which comes from the connectivity theorem) relates optimal enzyme levels to elasticities. All this holds generally whenever the rules apply. However, for didactic reasons, we computed some of the control coefficients for different rate laws (also demonstrating different ways to compute them) and checked some of the rules. Here we summarize this only briefly, details can be found in the Appendix.

- **Michaelis-Menten rate law** In metabolic pathways with irreversible rate laws, the flux control coefficients are simple: 1 for the first reaction, 0 for all remaining reactions (assuming that we are in a steady state. But under an optimization, all metabolite levels go to infinity and all elasticities go zero. Mathematically, the optimal state doe not exist (because it would entail infinite concentrations), but even if we assume that enzymes can be completely saturated, any enzyme variation would break the steady state: the control coefficients are not defined, and so the enzyme-control rule does not apply (see Appendix A.1). To obtain meaningful results, we therefore consider a different optimality problem with enzyme and metabolite constraints. In this case, metabolite levels remain finite and the control coefficients remain defined.

- **Thermodynamic rate law** With the thermodynamic rate law, we can derive a simple formula for the flux control coefficients that contains only the enzyme levels, the k^{cat} values, and the flux (for which we don't have an explicit formula). Nevertheless we can verify that the flux control coefficients satisfy summation and connectivity theorems, and that the enzyme-control rule is satisfied in the optimal state. In the metabolic chain with thermodynamic rate laws, we do not have a closed formula for the flux as a function of enzyme levels, and so it seems impossible to find a closed formula for the flux control coefficients. However, a formula can be obtained with a trick. From the rate law, we obtain a relation between enzyme levels, external metabolite levels, kinetic constants, and flux:

$$\prod_{i=1}^{n}(1 - J/\varepsilon_i k_i^{\text{cat}}) = \frac{s_n}{s_0}\frac{1}{K_{\text{tot}}^{\text{eq}}} \tag{32}$$

  (see Eq. (68) in the Appendix for the derivation). While we cannot solve this for the flux J directly, we can obtain the response coefficients $R_{\varepsilon_l}^J = \partial J/\partial \varepsilon_l$ by implicit differentiation. This directly yields the flux control coefficients (derivation in Appendix D.2.5):

$$C_l^J = \frac{(\varepsilon_l\ k_l^{\text{cat}} - J)^{-1}}{\sum_{i=1}^{n}(\varepsilon_i\ k_i^{\text{cat}} - J)^{-1}} \tag{33}$$

  The control coefficients are proportional to $(\varepsilon_l\ k_l^{\text{cat}} - J)^{-1}$ and normalized to a sum of 1, as required by the summation theorem. For didactical reasons, we explicitly checked the connectivity theorem (Appendix D.2.4) and the enzyme-control rule (Appendix D.2.7).

- **Mass-action rate law** With the reversible mass-action rate law, we can explicitly compute the flux control coefficients and verify that they satisfy summation and connectivity theorems. To verify that the enzyme-control rule is satisfied in the optimal state, we use the explicit formula (25) for the steady state flux and take derivatives to obtain the flux control coefficients

$$C^J = \frac{\gamma_l/\varepsilon_l}{\sum_j \gamma_j/\varepsilon_j} \tag{34}$$

  with γ_l defined as above (derivation in Appendix D.3.3). The coefficients sum to 1 as required by the summation theorem. Again, we also verified the connectivity theorem (Appendix D.3.4) and the enzyme-control rule (Appendix D.3.5) for didactical reasons.

2.5 A cell model with enzyme kinetics and metabolite constraints

As an illustrative example, we apply our formulae to a simple model of a growing cell, as shown in Figure 5. The model comprises an unbranched chain of 3 reactions describing overall transport, overall metabolism, and protein synthesis, as well as two upper bounds: one on the sum of enzyme levels (ε_{tot}) and another for metabolite levels (s_{tot}). The fluxes (i.e. steady-state flux J) are proportional to the cell growth rate.

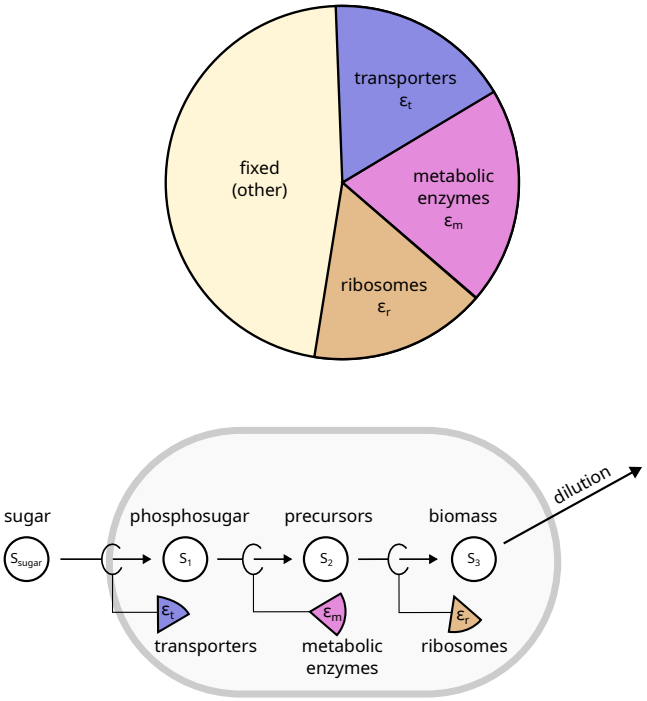


Figure 5: A growing cell described as a chain of reactions. Top: Following the "bacterial growth law" cell model in [9], we assume a fixed protein budget, consisting of a fixed fraction and fractions for transporters, metabolic enzymes, and ribosomal proteins. The proportions of the last three fractions can be optimized to maximize growth. Bottom: We describe a cell as a chain of reactions, each catalyzed by one of the protein fractions. For a constant biomass production, the three reactions must be in steady-state.

Obviously, none of the rate laws that we considered in this work can fully capture the dynamics of a growing cell. Having one single reaction representing metabolism is a gross oversimplification (and likewise for transport and translation). Nevertheless, we might still be able to draw insights from this model if we make the right assumptions. This approach has been successful in the past, for instance by assuming enzyme efficiencies are constant (i.e. completely independent of growth rate and metabolite concentrations), Basan et al. [10] were able to show how overflow metabolism in *E. coli* corresponds to the optimal allocation of enzymes. For our model, we chose to use the Michaelis-Menten approximation, so that metabolite concentrations (as extra variables) can be adjusted and become part of the optimization problem. We then apply the formulae derived in the previous sections of this paper to find the optimal allocation of enzymes and thereby maximize the growth rate of the cell. Importantly, all calculations are completely based on analytic expressions.

In section 2.3.1, we derived formula for the optimal allocation and maximal flux in the case where all metabolite levels are free variables (including s_0, which is denoted here by s_{sugar}). However, since in this cell model s_{sugar} represents the concentration of an external nutrient that is not subject to optimization, we would like to treat it as a constant system parameter (and later show how the optimum responds to changes in it). Fortunately, adding this assumptions changes the optimal solution only slightly, as described in Appendix D.1.4, and thus the optimal growth rate as a function of s_{sugar} can be written in the following simple form:

$$J^* = \mu^{\max} \cdot \frac{s_{\mathrm{sugar}}}{s_{\mathrm{sugar}} + K_{\mathrm{Monod}}} \tag{35}$$

where we define

$$\begin{aligned} \mu^{\max} &\equiv \frac{\varepsilon_{\mathrm{tot}}}{1/k_{\mathrm{t}}^{\mathrm{cat}} + 1/k_{\mathrm{m}}^{\mathrm{cat}} + 1/k_{\mathrm{r}}^{\mathrm{cat}} + \left(\sqrt{K_{\mathrm{M;m}}/k_{\mathrm{m}}^{\mathrm{cat}}} + \sqrt{K_{\mathrm{M;r}}/k_{\mathrm{r}}^{\mathrm{cat}}}\right)^2 / s_{\mathrm{tot}}} \\ K_{\mathrm{Monod}} &\equiv \frac{K_{\mathrm{M;t}}/k_{\mathrm{t}}^{\mathrm{cat}}}{1/k_{\mathrm{t}}^{\mathrm{cat}} + 1/k_{\mathrm{m}}^{\mathrm{cat}} + 1/k_{\mathrm{r}}^{\mathrm{cat}} + \left(\sqrt{K_{\mathrm{M;m}}/k_{\mathrm{m}}^{\mathrm{cat}}} + \sqrt{K_{\mathrm{M;r}}/k_{\mathrm{r}}^{\mathrm{cat}}}\right)^2 / s_{\mathrm{tot}}} \,. \end{aligned} \tag{36}$$

As implied by the symbol for K_{Monod}, this form corresponds to empirical observations by Monod [19] (and further followed up by others [28]) which stated that growth rate increases with the nutrient concentration until reaching a saturation level where growth is fastest. Interestingly, the value of K_{Monod} (e.g. the level of s_{sugar} for which growth rate is half of its maximum) is not determined solely by the kinetics of the transporter.

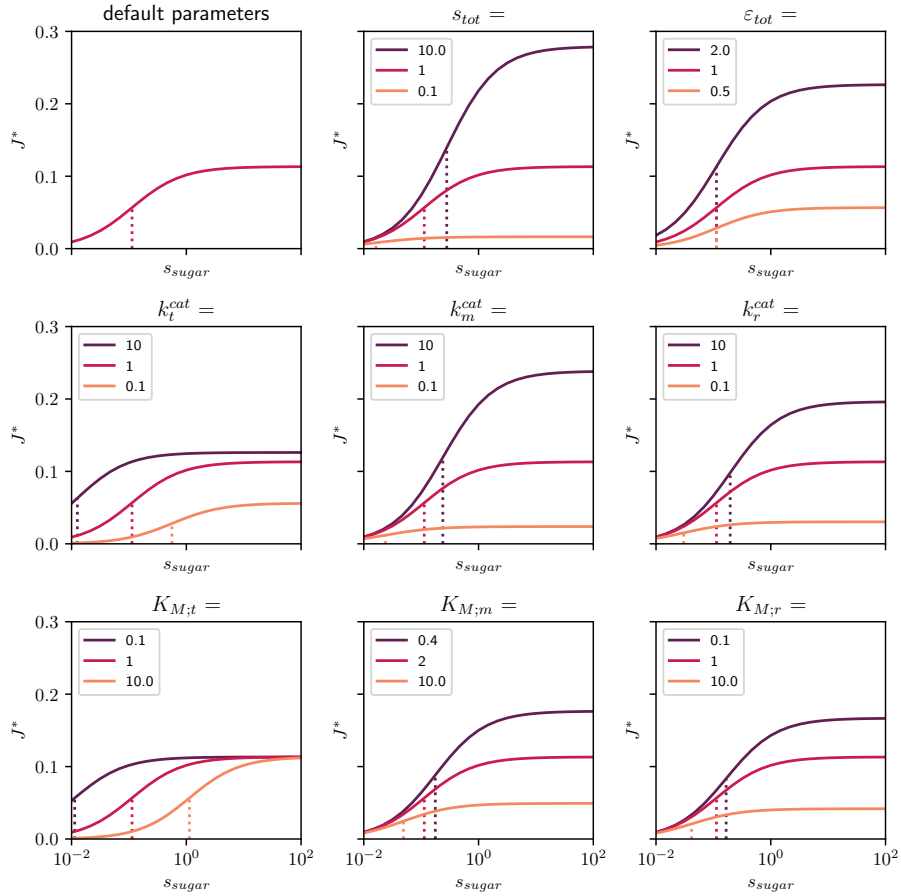


Figure 6: The optimal achievable flux (J^*) for a given total enzyme ε_{tot}. The model contains three irreversible steps based on Michaelis-Menten kinetics. The default parameters are $k_t^{\text{cat}} = k_m^{\text{cat}} = k_r^{\text{cat}} = K_{\text{M;t}} = K_{\text{M;r}} = 1$, $K_{\text{M;m}} = 2$, and the upper bounds are set to $s_{\text{tot}} = 1$, $\varepsilon_{\text{tot}} = 1$. The panel on the upper-left corner plots the maximal flux as a function of s_{sugar} for the default parameters. In each other panel, only one of the parameters is changed. The vertical dotted lines mark the K_{Monod} (i.e. the concentration of s_{sugar} where the flux is 50% of the maximum). Note that the x-axis is plotted in log-scale, and therefore the curves have a sigmoid-shape (rather than the familiar Monod-style). See Figure C for plots of the enzyme demands.

To better understand how changes in the model parameters affect the growth rate, we constructed a toy example, where all constants are set to a default value of 1 (except for $K_{\text{M;m}} = 2$). In Figure 6, we plot the value of J^* as a function of s_{sugar} (based on Eq. (35-36)), each time changing one of the parameters. In almost all cases, we find a trade-off between growth and affinity, namely that improving the kinetics (or relaxing a constraint) improves the maximal growth rate, while increasing K_{Monod} (i.e. making it worse). The three exceptions are the response to changes in ε_{tot} which only affects $\mu^{\max}$, in K_{t}, which only affects the K_{Monod}, and in $k_{\text{t}}^{\text{cat}}$, which can improve both growth parameters at the same time. Indeed, by observing the formulae in Eq. (36), one can see that all parameters that are only in the denominator should affect $\mu^{\max}$ and K_{Monod} in the same fashion (which leads to a trade-off since higher K_{Monod} is worse for the growth), while the three parameters in the numerators have each their own unique effect.

3 Discussion

This study is an attempt to address the enzyme allocation problem analytically by considering several different rate-law approximations. Along the way, we learned that each approximation comes with its own idiosyncrasies. For instance, the irreversible Michaelis-Menten chain leads to inherently uncontrollable states and essentially cannot be analyzed using metabolic control analysis. in this case, formulating a meaningful allocation problem requires adding some type of upper bound on metabolite levels (here, we bounded the sum of all their concentrations).

For historical reasons, we solve the optimality problem under the assumption that there is a bound on the sum

of enzyme abundances – $\sum_i \varepsilon_i \leq \varepsilon_{\text{tot}}$ – without explicitly specifying the units. The common interpretation would be that ε_i are *molar* concentrations (and where the symbol k_i^{cat} represent the turnover numbers in units of 1 over time). However, the total *mass* concentration of all pathway enzymes should be a better proxy for the cost since crowding effects in the cytoplasm are often limiting gene expression. Furthermore, fast growing cells are often limited by the rate of protein elongation, and the molecular mass is nearly proportional to the gene length. Therefore, we usually think of ε_i as *mass* concentrations (e.g. in $\text{g} \times \text{m}^{-3}$) and of k_i^{cat} as specific activities (e.g. in $\text{mol} \times \text{s}^{-1} \times \text{g}^{-1}$). Nevertheless, the analytic derivation and solutions provided here are agnostic to the choice of units and are equally valid for both these interpretations. Moreover, one could imagine a completely different set of linear weights for the enzyme cost function (i.e. $\sum_i w_i \ \varepsilon_i \leq \varepsilon_{\text{tot}}$, as in Figure 5) which essentially can be thought of as scaling factors for the k^{cat} values. Therefore, the provided solutions will still hold (while making use of the new "effective" k^{cat} values).

Previous studies focused on the "mass-action" rate law, justifying it by saying that the general reversible form derived by Haldane can be approximated at the limit of low concentrations. However, it is quite rare to have all the substrates and products of an enzyme at concentrations that are way below their K_{M} values [29]. Furthermore, the limit of all reactants concentrations going to zero is not very meaningful because, in the first place, Haldane derived his rate law assuming enzymes are much less abundant than metabolites [30]. Interestingly, just being close-to-equilibrium is not enough for this approximation, since the product can still affect the rate non-linearly via η^{kin} (see Figure 2). Here we tried to more comprehensively consider all the different approximations that yield an analytic solution to optimal enzyme allocation in unbranched pathways (see Table 1).

One of these approximations is the Michaelis-Menten rate law, which is a widely used in enzymatic assays and metabolic modeling of irreversible reactions. Curiously, using it for the simple optimality problem with linear chains leads to paradoxical results: the metabolite concentrations go to infinity, the elasticities vanish, and flux control coefficients are not defined. For solving this problem, we introduced an new upper bound on the total metabolite concentration in order to obtain realistic results and derive analytic solutions based on this rate law. Although it is very reasonable to assume that concentrations of small molecules (and not just enzymes) are restricted in cells, this fact is often ignored in metabolic models. One of the rare cases where this constraint was taken into account is the work of Dourado et al. [31], who found empirical evidence to the fact that there is a balance between enzymes and substrates when minimizing the total mass concentration. In addition to an analytic solution, we also found a new enzyme-control rule for models with a constraint and enzyme and metabolite concentrations: in this case, the enzyme amounts do not only reflect the flux control coefficients, but a sum of flux and concentration control coefficients.

Besides "mass-action" and Michaelis-Menten kinetics, we discussed a solution for one other rate law which we call *thermodynamic*, as the rate is only affected by metabolite levels through the thermodynamic driving force (i.e. ignoring any saturation effects). One advantage of this approach is that it does not require knowing the K_{M} values (which are often difficult to come by). The thermodynamic-only approximation was also used for deriving the Max-min Driving Force (MDF) method D.2.8, which similarly aimed to quantify the efficiency of metabolic pathways [32]. But, unlike the solution presented here, MDF does not explicitly optimize a simplified version of a kinetic rate law, but rather applies a heuristic based on the assumption that higher driving forces bring diminishing returns and thus should be distributed as evenly as possible.

All throughout this paper, we only considered pathways with uni-uni reactions (one substrate, one product, with stoichiometric coefficients of 1). In reality, many reactions involve co-factor pairs or other substrates or products. Instead of deriving results for this general case, we here assume that these extra reactants may exist, but with fixed concentrations. In this case, the rate laws contain extra terms, but these terms can be rearranged to yield simple formulae that are equivalent to the uni-uni case with effective kinetic constants. We demonstrate this for the case of two substrates and two products following convenience kinetics [33] in appendix D.5. Notably, the same logic also applies for more than two substrates and products as well as to enzyme activation or inhibition with constant activator or inhibitor levels.

This paper can be seen as an exercise in solving the enzyme allocation problem and describing the optimal states analytically using the MCA approach. Although some might argue that the required approximations are not realistic, they do represent a step forward compared to the very common approach of assuming metabolites have no effect on enzyme efficiency at all. On the other hand, adding metabolite concentrations as extra variables greatly increases the complexity of models and typically renders them unsolvable. Therefore, the solutions provided here might be handy, as the assumption of metabolite steady-state combined with the optimality argument give us analytic expressions that only depend on the initial and final metabolites in the pathway. We demonstrate this result using a toy example for a course-grained model of cell growth, and show how the analytic solutions provide valuable insights about the effects of changes in each parameter – all this without the need to simulate the metabolic network or use non-linear solvers. We hope that future studies will continue to use this approach in other, more

complex models.

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A Revisiting the enzyme-control rule

A.1 Why the enzyme-control rule fails with Michaelis-Menten kinetics

Curiously, we will see that the enzyme-control rule fails in a very simple case, the unbranched metabolic pathway with Michaelis-Menten rate laws. Since the rate law is irreversible, the rate in each reaction depends only on the substrate and enzyme level in this reaction (and not on anything that happens downstream). In steady state, the rate in every reaction is fixed by the stationary flux. Taken together, this means that the flux depends only on the pathway substrate and the level of the first enzyme, which suggests that this enzyme has full flux control and all other enzymes have zero control. However, according to the enzyme-control rule, this also means that the first enzyme is the only enzyme that should be expressed. This is obviously, because without the other enzymes, no steady flux would be possible. So how can this be?

In fact, an optimal steady state with irreversible rate laws has some pathological properties. To maximize enzyme efficiency, all metabolite levels must go to infinity, the enzymes are completely saturated, and the metabolite elasticities are all zero. As a result, the flux in every reaction is given by $v_i = \varepsilon_i \; k_i^{\mathrm{cat}}$, so the stationary flux completely determines all enzyme levels. If an enzyme level deviates from its value, its reaction rate will no longer match the other rates and the steady state breaks down[2]. Now we can see the catch: in the optimal state, any small change of an enzyme level will not just make the state non-optimal, but will break the steady state; this means that the control coefficients are not even defined, and the enzyme-control rule does not apply. For the enzyme-control rule to hold, control coefficients must be defined; if a variation of an enzyme level does not lead to a new steady state, this is not the case[3].

In the case of the Michaelis-Mention rate law, the practical solution to this problem is simple. Since the optimal state requires infinite metabolite concentrations, and since this is not realistic, we need to change our assumptions. We could either consider slightly reversible rate laws (which would penalize high product concentrations) or we could introduce an extra density constraint that penalizes high metabolite levels explicitly. We discuss a new enzyme-control rule with this extra constraint in section D.6.

A.2 A sufficient condition for stable states in unbranched pathways

Hence, before we can apply the enzyme-control rule, we need to check whether control coefficients even exist. A metabolic steady state can be stable or unstable. In an unstable state, the systems would not be able to remain close to its original state even under small perturbations. Usually, in MCA we are only interested in stable states, because unstable states are not able to persist even under small (for example chemical) noise, and mathematically, control coefficients for unstable states are not even defined. Mathematically a metabolic state is asymptotically stable if the Jacobian $\mathbf{N}\,\mathbf{E}_{\mathrm{c}}$ in this state has only negative eigenvalues. But what does this mean in practice? In Appendix D.7, we derive a sufficient (but not necessary) condition for stable steady states in unbranched metabolic pathways, based on Gershgorin's theorem: A steady state in an unbranched metabolic pathway is stable if (but not only if) the first reaction 1 is not completely irreversible (i.e. it has a non-zero product elasticity), the last reaction is not completely saturated (i.e. it has a non-zero substrate elasticity), and in all reactions in between, the substrate elasticity is larger than the (absolute) product elasticity. For a reversible mass-action rate law, the latter condition is satisfied if $K_i^{\mathrm{eq}} > 1$, and the first two conditions are generally satisfied.

A.3 An enzyme-control rule for models with general density constraints

A way to obtain meaningful optimal states, even with Michaelis-Menten kinetics, is to constrain the metabolite concentrations. This change in the optimality problem leads to different solutions, which must satisfy different enzyme-control rules.

High metabolite concentrations along with high enzyme levels may put an extra burden on cells. In our optimality problems, a constraint on metabolite concentrations will change the optimal solutions. Setting up such optimality problems raises a couple of questions: should we use a single density constraint or rather separate constraints

[2] In fact, there are a number of similar arguments why the enzyme-control rules fails in this case: first, the state with infinite concentrations does not exist mathematically, so formally we cannot even refer to it as a metabolic state; second, even if this state existed, all elasticities would vanish, and the Jacobian would not be invertible, so the control coefficient would not be defined; third, even if we argued that, logically, the first enzyme must have full flux control, the same argument would also apply to all other enzymes: each of the enzymes would have full flux control, thus violating the summation theorem.

[3] In in fact, this does not only occur with the Michaelis-Menten rate law, but with any irreversible, saturable rate law.

on enzyme levels and on metabolite concentrations? How should the different compounds be weighted? In fact, the resulting problems are not very different, and we demonstrate here one specific approach: we describe all compounds (enzymes and metablites) by concentrations and consider a joint bound on their weighted sum (where weights may have different meanings, for example molecular masses if the constraint concerns total cellular mass density). With a general cost function $h(\boldsymbol{\varepsilon}$, the weights in the formulae would be given by the enzyme prices $\partial h/\partial \varepsilon_i$.

We thus consider the optimality problem

$$\text{Maximize } \mathbf{z}\cdot\mathbf{v} \quad \text{s.t.} \quad \mathbf{a}\cdot\boldsymbol{\varepsilon}+\mathbf{b}\cdot\mathbf{s}\leq\rho \tag{37}$$

where $\mathbf{v}$, $\mathbf{s}$, and $\boldsymbol{\varepsilon}$ denote reaction fluxes, metabolite concentrations, and enzyme levels, and stationarity (of $\mathbf{v}$) and rate laws (between $\mathbf{v}$, $\mathbf{s}$, and $\boldsymbol{\varepsilon}$ must be satisfied as constraints. The maximization objective is a linear benefit function scoring the fluxes, $\mathbf{z}\cdot\mathbf{v}$ with linear weights z_i, while the constrained density is linear in all the concentrations, with linear weights[4] a_l (for enzymes) and b_i (for metabolites), e.g. representing effective[5] molecule sizes. To derive the enzyme-control rule, we now reformulate the optimality problem with enzyme levels as the only free variables, and steady-state fluxes and concentrations depending on them (satisfying stationary and the rate laws). We obtain

$$\text{Maximize } \mathbf{z}\cdot\mathbf{v}^{\text{steady}}(\boldsymbol{\varepsilon}) \quad \text{s.t.} \quad \mathbf{a}\cdot\boldsymbol{\varepsilon}+\mathbf{b}\cdot\mathbf{s}^{\text{steady}}(\boldsymbol{\varepsilon})\leq\rho \tag{38}$$

This optimality problem leads to a new enzyme-control rule (proof in Appendix D.6)

$$\boldsymbol{\varepsilon}^{*\top}\ \mathrm{diag}(\mathbf{a})\ \mathrm{diag}(\mathbf{v})^{-1}=\left[\frac{1}{\lambda}\ \mathbf{z}^\top\mathbf{C}^{\mathbf{v}*}-\mathbf{b}^\top\mathbf{C}^{\mathrm{s}*}\right]. \tag{39}$$

In a simple linear chain, we have same steady-state flux J in all the reactions, a benefit function given by J, and a density constraint with equal weights a for all enzymes, and equal weight b for all the metabolites. In this case, Eq. (39) simplifies to

$$\varepsilon_l^*\propto C^{J_l*}-\lambda\ \frac{a\ b}{v}\ \sum_i C_l^{c_i*} \tag{40}$$

which replaces the enzyme control rule (8). Given the sum of enyzme levels in the optimal state, called $\varepsilon_{\text{tot}}^*$, we can also write this as

$$\varepsilon_l^*=\varepsilon_{\text{tot}}^*\ C_l^{J*}-\frac{b}{a}\ J^*\ \sum_i C_l^{s_i*}. \tag{41}$$

Since the Lagrange multiplier λ arises from an upper bound in a maximization problem, it is negative in the optimal state, the second term including the minus sign will be positive.

Compared to the enzyme-control rule (8), the right hand side now contains an extra term: it is given by the metabolite control coefficients, summed over all metabolites, and with an unknown prefactor (a product of the shadow price from the density constraint and the protein and metabolite weights, divided by the flux). If either the shadow price or one of the weights is small (compared to the flux), the second term can be neglected and we obtain again our original enzyme-control rule.

In the general case (network with non-uniform flux distribution $\mathbf{v}$, a more complicated flux benefit function with derivatives $\frac{\partial z}{\partial v_i}$, and individual weights a_l and b_i for all enzymes and metabolites, all these vectors enter the formula as weights.

B The enzyme-elasticity rule

The connectivity theorem for flux control coefficients

$$\forall i \quad \sum_j C_j^J\cdot E_{j,i}=0 \tag{42}$$

[4]Instead of a linear flux objective, a nonlinear function $z(\mathbf{v})$ could be used. In this case, the end result remains the same, but the vector weight $\mathbf{z}$ is replaced by the gradient $\nabla_v z(\mathbf{v})$. Likewise, in a model with separate density constraints $\mathbf{a}\cdot\boldsymbol{\varepsilon}\leq\rho_\varepsilon$ and $\mathbf{b}\cdot\mathbf{s}\leq\rho_c$, we obtain again the same formulae, but with separate Lagrange multipliers for the two constraints, which also appear in the resulting formulae.

[5]In the simple case of a constraint on the total mass, the weights would be simply molecular weights. This could be a proxy for excluded volume (which would still ignore, for example, hydration shells). However, a "density constraint" does not necessarily represent space demand; it may also be related to osmotic effects or other opportunity costs (e.g. energy demand for production of compounds in growing cells. Therefore the meaning of the weights in this constraint may differ from model to model.

relates the flux control coefficients C_j^J (between enzyme ε_j and the pathway flux) to the unscaled elasticities $E_{j,i} = \frac{\partial v_j}{\partial s_i}$ (between metabolite s_i and reaction rate v_j). In optimal state, the enzyme-control rule tells us that control coefficients and enzyme levels must be proportional. Therefore we can replace C_j^J with ε_j in the equation above. For each metabolite i, we obtain an equation

$$\sum_j \varepsilon_j \cdot E_{j,i} = 0\,. \tag{43}$$

For a linear chain, where each metabolite has only one producing and one consuming reaction (with indices i and $i+1$, we obtain the equality

$$\frac{\varepsilon_{i+1}}{\varepsilon_i} = -\frac{E_{s_i}^{v_i}}{E_{s_i}^{v_{i+1}}} = \frac{|E_{s_i}^{v_i}|}{E_{s_i}^{v_{i+1}}}. \tag{44}$$

In the last step, we used the fact that the backward (product) elasticity $E_{s_i}^{v_i}$ is negative and the forward (substrate) elasticity $E_{s_i}^{v_{i+1}}$ is positive.

Example: mass-action rate law In a linear pathway, there will only be two non-zero derivatives in the sum (for $j = i$ and $j = i+1$). Specifically, with the mass-action kinetic rate law (see Appendix D.3), namely $v_j = \varepsilon_j \cdot (k_j s_{j-1} - k_{-j} s_j)$, Eq. (45) will become:

$$\forall i \quad 0 = \varepsilon_i \cdot \frac{\partial v_i}{\partial s_i} + \varepsilon_{i-1} \cdot \frac{\partial v_{i-1}}{\partial s_i} = \varepsilon_i \cdot (-\varepsilon_i k_{-i}) + \varepsilon_{i+1} \cdot (\varepsilon_{i+1} k_{i+1}) \tag{45}$$

which by rearranging leads us to the following relationship:

$$\forall i \quad \frac{\varepsilon_{i+1}}{\varepsilon_i} = \sqrt{\frac{k_{-i}}{k_{i+1}}} = \sqrt{\frac{a_{i+1}}{a_i}} \tag{46}$$

(where we remind ourselves of the definition $a_i \equiv k_{-i}^{-1} \prod_{j=1}^{i} k_{-j}/k_j$). Applying this equation recursively, we can see that $\varepsilon_i/\varepsilon_1 = \sqrt{a_i/a_1}$, which means that ε_i is proportional to $\sqrt{a_i}$. Since we know that the sum of all enzyme demands gives ε_{tot}, we can obtain the explicit formula for ε_i:

$$\varepsilon_i = \varepsilon_{\text{tot}} \frac{\sqrt{a_i}}{\sum_j \sqrt{a_j}} = \varepsilon_{\text{tot}} \sqrt{\frac{a_i}{||\boldsymbol{a}||_{\frac{1}{2}}}}\,. \tag{47}$$

This is the exact same result we got by directly optimizing the pathway flux in Appendix D.3 (see Equation 102).

C A cell model with enzyme kinetics and metabolite constraints

The model is described by three irreversible reactions:

$$\mathrm{S_{sugar}} \overset{\varepsilon_t}{\rightleftharpoons} \mathrm{S_1} \overset{\varepsilon_m}{\rightleftharpoons} \mathrm{S_2} \overset{\varepsilon_r}{\rightleftharpoons} \mathrm{S_3} \tag{48}$$

The optimal achievable flux (J^*) for a given total enzyme ε_{tot} is given in Eq. (35-36). Figure C plots the individual enzyme demands for the three steps, as a function of the external sugar concentration and J^*:

$$\begin{aligned}
{\varepsilon_{\mathrm{t}}}^* &= J^* \cdot \left(1/k_{\mathrm{t}}^{\mathrm{cat}} + K_{\mathrm{M;t}}/k_{\mathrm{t}}^{\mathrm{cat}} \cdot (1/s_{\mathrm{sugar}})\right) \\
{\varepsilon_{\mathrm{m}}}^* &= J^* \cdot \left(1/k_{\mathrm{m}}^{\mathrm{cat}} + \sqrt{K_{\mathrm{M;m}}/k_{\mathrm{m}}^{\mathrm{cat}}} \left(\sqrt{K_{\mathrm{M;m}}/k_{\mathrm{m}}^{\mathrm{cat}}} + \sqrt{K_{\mathrm{M;r}}/k_{\mathrm{r}}^{\mathrm{cat}}}\right) \Big/ s_{\mathrm{tot}}\right) \\
{\varepsilon_{\mathrm{r}}}^* &= J^* \cdot \left(1/k_{\mathrm{r}}^{\mathrm{cat}} + \sqrt{K_{\mathrm{M;r}}/k_{\mathrm{r}}^{\mathrm{cat}}} \left(\sqrt{K_{\mathrm{M;m}}/k_{\mathrm{m}}^{\mathrm{cat}}} + \sqrt{K_{\mathrm{M;r}}/k_{\mathrm{r}}^{\mathrm{cat}}}\right) \Big/ s_{\mathrm{tot}}\right)\,.
\end{aligned} \tag{49}$$

Note, that also ${\varepsilon_{\mathrm{m}}}^*$ and ${\varepsilon_{\mathrm{r}}}^*$ change with s_{sugar} even though it does not explicitly appear in the equations, because J^* itself is a function of s_{sugar}.

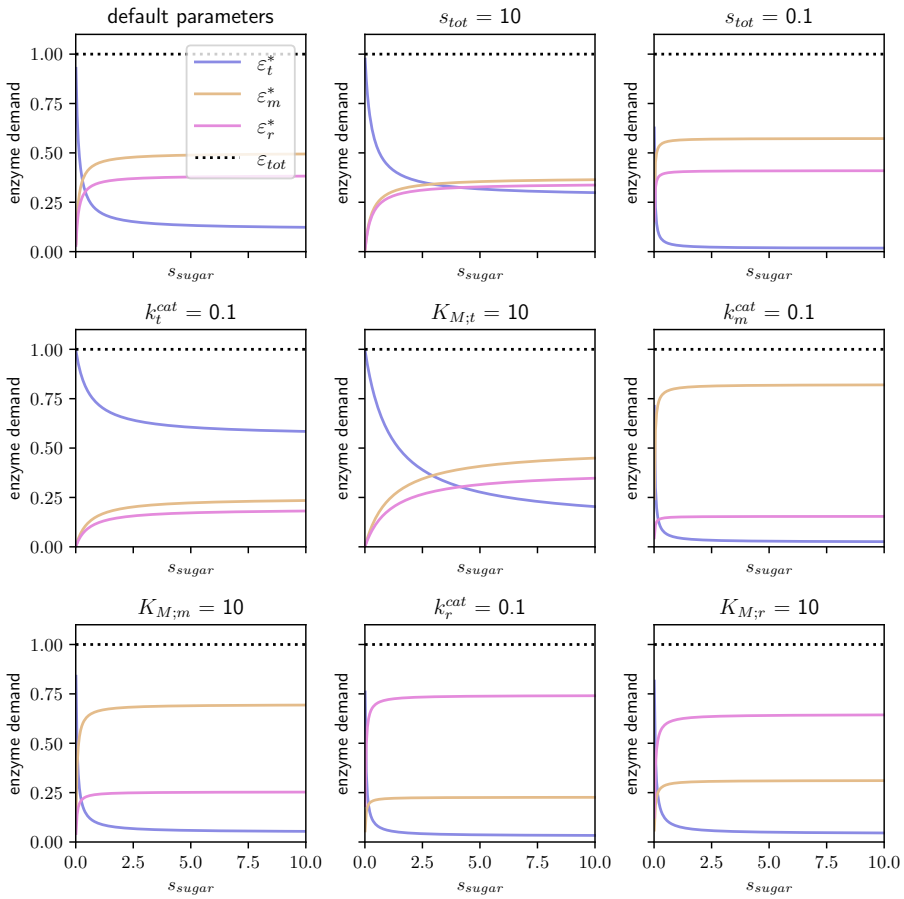

Figure S1: The default parameters are $k_t^{\text{cat}} = k_m^{\text{cat}} = k_r^{\text{cat}} = 1$, $K_{\text{M;t}} = K_{\text{M;r}} = 1$, $K_{\text{M;m}} = 2$ (we chose another value in order prevent the curves for ε_{m} and ε_{r} from overlapping), and the upper bounds are set to $s_{\text{tot}} = 1$, $\varepsilon_{\text{tot}} = 1$. In each panel only one of these parameters is changed.

D Mathematical proofs and derivations

This appendix contains many of the detailed mathematical proofs and derivations used in the manuscript for metabolic steady-states, minimum enzyme demand / maximal flux, connectivity theorem, and enzyme control rules. The derivations are grouped by the corresponding rate law in the following order: (1) Michaelis-Menten; (2) Thermodynamic; (3) Reversible mass-action; (4) Haldane. An overview of useful equations is given in Table D.

D.1 Michaelis-Menten kinetics

The Michaelis-Menten rate law is an approximation of the Haldane rate law for irreversible reactions:

$$v_i = \varepsilon_i \; k_i^{\text{cat}} \; \frac{s_{i-1}}{s_{i-1} + K_{\text{M},i}} \,. \tag{50}$$

with reactant elasticities

$$\frac{\partial v_i}{\partial s_j} \;\; = \delta_{j,i-1} \; \varepsilon_i \; k_i^{\text{cat}} \; \frac{K_{\text{M},i}}{(s_j + K_{\text{M},i})^2} \,. \tag{51}$$

	$s_i^{\text{steady}}(s_0, s_n, \boldsymbol{\varepsilon})$	$J(s_0, s_n, \boldsymbol{\varepsilon})$	$s_i(s_0, J, \boldsymbol{\varepsilon})$	Elasticities	$\mathbf{C}^{\text{s}}$	$\mathbf{C}^J$
Michaelis-Menten	Eq. (52)	$v_1(s_0)$	Eq. (53)	Eq. (51)		Eq. (54)
Thermodynamic (const η^{kin})	Eq. (32)		Eq. (32)		Eq. (90)	
Thermodynamic (substrate sat)						sec D.2.5
Mass-action	Eq. (25)	Eq. (99)		Eq. (107)		sec D.3.3
Haldane	-	-	Eq. (19) in [17]	e.g. [34]	-	-

Table S1: Overview of formulae for steady state variables and control coefficients. This table summarizes formulae for unbranched metabolic pathways with different rate laws (rows). The columns refer to the steady-state concentrations and flux (as a function enzyme levels and metabolite concentrations); the steady-state concentrations as a function of enzyme levels, pathway substrate concentration, and steady-state flux (but not of the product concentration); the reaction elasticities; and the concentration and flux control coefficients.

D.1.1 Steady-state concentrations

Computing the steady-state metabolite concentrations in a pathway with Michaelis-Menten kinetics is possible by inverting Eq. (50).

$$\begin{aligned} s_{i-1} &= \frac{v_i}{\varepsilon_i\ k_i^{\text{cat}}}(s_{i-1} + K_{\text{M},i}) \\ \Rightarrow s_{i-1}\left(1 - \frac{v_i}{\varepsilon_i\ k_i^{\text{cat}}}\right) &= \frac{v_i}{\varepsilon_i\ k_i^{\text{cat}}} K_{\text{M},i} \\ \Rightarrow s_{i-1} &= \frac{v_i K_{\text{M},i}}{\varepsilon_i\ k_i^{\text{cat}}(1 - \frac{v_i}{\varepsilon_i\ k_i^{\text{cat}}})} = \frac{K_{\text{M},i}}{\varepsilon_i\ k_i^{\text{cat}}/v_i - 1}\,. \end{aligned} \tag{52}$$

The steady-state concentration as a function of the pathway flux is given by replacing v_i with J for all reactions. Therefore, for any two reactions (i and j), we can relate between their substrate levels by replacing v_i in Eq. (52) with J and using the rate law for $J = v_j$ from Eq. (50)

$$s_{i-1} = \frac{K_{\text{M},i}}{\varepsilon_i\ k_i^{\text{cat}}/J - 1} = \frac{K_{\text{M},i}}{\varepsilon_i\ k_i^{\text{cat}}/\left(\varepsilon_j\ k_j^{\text{cat}}\ \frac{s_{j-1}}{s_{j-1}+K_{\text{M},j}}\right) - 1} = \frac{K_{\text{M},i}}{\frac{\varepsilon_i k_i^{\text{cat}}}{\varepsilon_j k_j^{\text{cat}}}\left(1 + \frac{K_{\text{M},j}}{s_{j-1}}\right) - 1}. \tag{53}$$

In fact, applying this condition to all metabolites is a sufficient condition for having a steady-state. Specifically, if we start with a given concentration s_0 and enzyme abundances $\boldsymbol{\varepsilon}$, there is only one solution for the steady-state flux and for the metabolite levels ($\mathbf{s}$) which support it.

D.1.2 Flux control coefficients

We can compute the flux control coefficients directly by taking derivatives: given that $J = v_1$ and that v_1 depends only on the external substrate, described by a fixed parameter and therefore not dependent on $\boldsymbol{\varepsilon}$, the flux control coefficients read

$$C_l^J = \frac{\partial J}{\partial E_l} \cdot \frac{\varepsilon_l}{J} = \frac{\partial v_1}{\partial \varepsilon_l}\frac{\varepsilon_l}{v_1} = \delta_{l1}. \tag{54}$$

As expected, due to the irreversible rate laws, only the first enzyme can have control (except for the pathological case where metabolites concentrations become infinite and flux control is not defined).

D.1.3 Minimum enzyme demand / maximal flux

We can also use Eq .(50) at steady-state (with flux J) to express the enzyme demands:

$$\varepsilon_i = \frac{J}{k_i^{\text{cat}}} \cdot \left(1 + \frac{K_{\text{M},i}}{s_{i-1}}\right) = J\left(\alpha_i + \frac{\beta_i}{s_{i-1}}\right) \tag{55}$$

where we define $\alpha_i \equiv 1/k_i^{\text{cat}}$ and $\beta_i \equiv K_{\text{M},i}/k_i^{\text{cat}}$. Now we can take the sum of all enzyme demands and equate it to ε_{tot}:

$$\varepsilon_{\text{tot}} = J\left(\sum_{i=1}^{n}\alpha_i + \sum_{i=1}^{n}\frac{\beta_i}{s_{i-1}}\right) = J\left(||\boldsymbol{\alpha}||_1 + \sum_{i=1}^{n}\frac{\beta_i}{s_{i-1}}\right)\,. \tag{56}$$

This inverse relationship between ε_{tot} and the metabolite concentrations means that in order to minimize ε_{tot} for a given flux, all s_i should be as large as possible. Obviously, infinite concentrations are not feasible. To avoid non-physical solutions, we can simply add a constraint on the total concentration of metabolites $\sum_{i=0}^{n-1} s_i \leq s_{\text{tot}}$. Now, using Lagrange's method, we define $\mathcal{L} = J\left(||\boldsymbol{\alpha}||_1 + \sum_i \beta_i / s_{i-1}\right) + \lambda\left(s_{\text{tot}} - \sum_i s_i\right)$ and derive it:

$$\begin{aligned} 0 = \frac{\partial \mathcal{L}}{\partial s_{i-1}} &= \frac{\beta_i J^*}{{s_{i-1}}^*{}^2} - \lambda \\ {s_{i-1}}^* &= \sqrt{\frac{J^*}{\lambda}}\sqrt{\beta_i}. \end{aligned} \tag{57}$$

To find λ, we can now use the constraint on the sum of all metabolites (assuming the upper bound is realized):

$$s_{\text{tot}} = \sum_{i=0}^{n-1} {s_i}^* = \sqrt{\frac{J^*}{\lambda}}\left(\sum_{i=0}^{n-1}\sqrt{\beta_i}\right) = \sqrt{\frac{J^*}{\lambda}}\sqrt{||\boldsymbol{\beta}||_{\frac{1}{2}}} \tag{58}$$

where $\boldsymbol{\beta}$ is the vector of all β_i, and $||\cdot||_{\frac{1}{2}}$ is the $l_{1/2}$ norm. Therefore, we can replace $\sqrt{J^*/\lambda}$ with $s_{\text{tot}}/\sqrt{||\boldsymbol{\beta}||_{\frac{1}{2}}}$ in Eq. (57) and find an explicit expression for ${s_{i-1}}^*$:

$${s_{i-1}}^* = \frac{s_{\text{tot}}}{\sqrt{||\boldsymbol{\beta}||_{\frac{1}{2}} \cdot \beta_i}}. \tag{59}$$

Using the results from Eqs (56) and (59), we can see that:

$$J^* = \frac{\varepsilon_{\text{tot}}}{||\boldsymbol{\alpha}||_1 + \sum_i \frac{\beta_i}{{s_{i-1}}^*}} = \frac{\varepsilon_{\text{tot}}}{||\boldsymbol{\alpha}||_1 + \sum_i \frac{\sqrt{\beta_i \cdot ||\boldsymbol{\beta}||_{\frac{1}{2}}}}{s_{\text{tot}}}} = \frac{\varepsilon_{\text{tot}}}{||\boldsymbol{\alpha}||_1 + \frac{||\boldsymbol{\beta}||_{\frac{1}{2}}}{s_{\text{tot}}}} \tag{60}$$

and similarly we can also find the optimal enzyme levels:

$${\varepsilon_i}^* = J^*\left(\alpha_i + \frac{\beta_i}{{s_{i-1}}^*}\right) = \varepsilon_{\text{tot}}\,\frac{\alpha_i + \sqrt{\beta_i\,||\boldsymbol{\beta}||_{\frac{1}{2}}}/s_{\text{tot}}}{||\boldsymbol{\alpha}||_1 + ||\boldsymbol{\beta}||_{\frac{1}{2}}/s_{\text{tot}}}. \tag{61}$$

D.1.4 Case where the first substrate has fixed concentration.

If our pathway model represents a cell growing on an external substrate (S_0), then it would be unrealistic to assume that the concentration s_0 is subject to optimization. Instead, we can assume it is fixed (e.g. based on the environmental conditions) and that the cell can optimize only the internal concentrations of enzymes and metabolites.

From Eq. (60) we can see that:

$$J = \frac{\varepsilon_{\text{tot}}}{||\boldsymbol{\alpha}||_1 + \sum_i \beta_i / s_{i-1}} = \frac{\varepsilon_{\text{tot}}}{||\boldsymbol{\alpha}||_1 + \beta_1/s_0 + \sum_{i=2}^{n} \beta_i / s_{i-1}}. \tag{62}$$

Since s_0 is fixed, we consider the term β_1/s_0 to be part of the constant part of the denominator (usually just $\sum 1/k_i^{\text{cat}}$). Furthermore, we can redefine $||\boldsymbol{\beta}||_{\frac{1}{2}}$ to exclude the value for the first enzyme (β_1) and that the upper bound s_{tot} is only imposed on the sum of the internal metabolites. In this case, the solution we got in Eqs (57)-(58) will still be applicable, and therefore we can write:

$$J^* = \frac{\varepsilon_{\text{tot}}}{\beta_1/s_0 + ||\boldsymbol{\alpha}||_1 + ||\boldsymbol{\beta}||_{\frac{1}{2}}/s_{\text{tot}}} = \mu^{\max} \cdot \frac{s_0}{s_0 + K_{\text{Monod}}} \tag{63}$$

where we define

$$\begin{aligned} \mu^{\max} &\equiv \frac{\varepsilon_{\text{tot}}}{||\boldsymbol{\alpha}||_1 + ||\boldsymbol{\beta}||_{\frac{1}{2}}/s_{\text{tot}}} \\ K_{\text{Monod}} &\equiv \frac{\beta_1}{||\boldsymbol{\alpha}||_1 + ||\boldsymbol{\beta}||_{\frac{1}{2}}/s_{\text{tot}}} \end{aligned} \tag{64}$$

As we can see, the solution provides a prediction for the Monod constant – i.e., the concentration of substrate (s_0) where the growth rate is half of its maximum. If the transporter turnover rate (k_1^{cat}) is indeed much slower

than all other enzymes (and therefore its control coefficient is 1), we will get $K_{\text{Monod}} = K_{\text{M},1}$, which is the naïve assumption. However, if the control is distributed along the pathway, the Monod constant can become significantly smaller than $K_{\text{M},1}$.

We can also write expressions for the individual enzyme levels, using Eq. (61). Because s_0 is fixed, the demand for the first enzyme is can be simply expressed as:

$$\varepsilon_i{}^* = J^* \left(\alpha_i + \frac{\beta_i}{s_{i-1}{}^*} \right) \tag{65}$$

For the first enzyme, the substrate (s_0) is fixed so the expression is simply $\varepsilon_1{}^* = J^* (\alpha_1 + \beta_1/s_0)$, while for all other enzymes ($\forall i > 0$) we can use the solution for $s_{i-1}{}^*$ from Eq. (59), i.e. $\varepsilon_i{}^* = J^*(\alpha_i + \sqrt{\beta_i \; ||\boldsymbol{\beta}||_{\frac{1}{2}}}/s_{\text{tot}})$.

This result might have implications for the design of optimal enzyme regulation. We can imagine that ε_1 is a membrane transporter that imports s_0 into the cell (e.g., a glucose transporter), and that all downstream cytoplasmic enzymes are part of a catabolic pathway (glycolysis, for instance). Now consider a shift from an environment with low glucose to high glucose. If the cell indeed has a constant amount of total enzyme resource, it would make sense for it to "spend" less on the transporter and shift the freed resources to the glycolysis enzymes. What we showed here, is that the optimal proportions within the pathway are constant no matter what the level of glucose is outside. Only the ratio between the transporter and the rest of the enzymes will change.

In fact, the design principle described above can be shown to be true in a much more general case. As long as one section of the metabolic network is "isolated" from the rest (i.e., connected only by irreversible steps), changes in the upstream parameters will affect the incoming flux but would not change the optimal allocation of enzymes (and the absolute metabolite concentrations).

D.2 Thermodynamic rate law with fixed kinetic efficiency

The thermodynamic rate law with fixed kinetic efficiency reads:

$$v_i = \varepsilon_i \; k_i^{\text{cat}} \left(1 - e^{-\theta_i}\right) = \varepsilon_i \; k_i^{\text{cat}} \left(1 - \frac{s_i}{s_{i-1} \; K_i^{\text{eq}}}\right) . \tag{66}$$

where $\theta_i \equiv \ln\left(\frac{s_{i-1} K_i^{\text{eq}}}{s_i}\right)$

D.2.1 Steady-state concentrations

Assuming steady-state (i.e. all fluxes are equal to J), we can find a simple recursion formula for the concentration of s_i:

$$s_i = s_{i-1} K_i^{\text{eq}} (1 - J \cdot (\varepsilon_i k_i^{\text{cat}})^{-1}) . \tag{67}$$

Solving the recursion for s_n we get:

$$s_n = s_{n-1} K_n^{\text{eq}} (1 - J \cdot (\varepsilon_n k_n^{\text{cat}})^{-1}) = \ldots = s_0 \prod_{i=1}^{n} K_i^{\text{eq}} (1 - J \cdot (\varepsilon_i k_i^{\text{cat}})^{-1}) = s_0 K_{\text{tot}}^{\text{eq}} \prod_{i=1}^{n} (1 - J \cdot (\varepsilon_i k_i^{\text{cat}})^{-1}) , \tag{68}$$

where we used the fact that $K_{\text{tot}}^{\text{eq}} = \prod_{j=1}^{i} K_i^{\text{eq}}$.

The driving force of the entire pathway, which we denote by θ_{tot}, is the sum of all the individual driving forces:

$$\theta_{\text{tot}} = \sum_{i=1}^{n} \theta_i = \sum_{i=1}^{n} \ln\left(\frac{s_{i-1} K_i^{\text{eq}}}{s_i}\right) = \ln\left(\prod_{i=1}^{n} s_{i-1}/s_i\right) + \ln\left(\prod_{i=1}^{n} K_i^{\text{eq}}\right) = \ln\left(s_0/s_n\right) + \ln\left(K_{\text{tot}}^{\text{eq}}\right) . \tag{69}$$

Since θ_{tot} depends only on $K_{\text{tot}}^{\text{eq}}$, s_0, and s_n – all of which are constant – it is a constant as well. Therefore, we can use this result to rewrite Equation (68) as:

$$\theta_{\text{tot}} = -\sum_{i=1}^{n} \ln\left(1 - J \cdot (\varepsilon_i k_i^{\text{cat}})^{-1}\right) . \tag{70}$$

D.2.2 Minimum enzyme demand / maximal flux

Directly solving the flux maximization problem using Equation (70) would be difficult, because it cannot be solved analytically to find an expression for J as a function of enzyme levels. However, it is rather simple to do the opposite and express the enzyme levels as functions of J and the other parameters, sum them up and compare to the total (ε_{tot}):

$$\begin{aligned} \varepsilon_i &= \frac{J}{k_i^{\text{cat}}\left(1-e^{-\theta_i}\right)} = J\,\frac{\alpha_i}{1-e^{-\theta_i}} \\ \frac{\varepsilon_{\text{tot}}}{J} &\geq \frac{1}{J}\sum_i \varepsilon_i = \sum_i \alpha_i \left(1-e^{-\theta_i}\right)^{-1} \end{aligned} \tag{71}$$

where we define $\alpha_i \equiv 1/k_i^{\text{cat}}$ for simplicity.

Minimizing $\varepsilon_{\text{tot}}/J$ is an optimization problem that can be solved directly using the s_i as variables, but it can be easier to solve if we consider the θ_i to be the independent variables instead. This variable switch is justified because there is a linear homomorphism between the two sets $\{\ln s_i\}_{i=1}^{n-1}$ and $\{\theta_i\}_{i=1}^{n}$, given fixed s_0 and s_n and under the constraint that $\sum \theta_i = \theta$.

Now, we want to find an optimal set of values for the driving forces, denoted ${\theta_i}^*$, that sum up to θ and maximize J (similarly, J^* and ${\varepsilon_i}^*$ would be the corresponding values of J and ε_i at the optimum).

Lemma D.1. *The values of x_i that minimize the function $\sum_i \alpha_i \left(1-e^{-\theta_i}\right)^{-1}$ under the constraint $\sum_i x_i = x_{\text{tot}}$, satisfy*

$$x_i = 2\,\sinh^{-1}\left(\sqrt{\Psi\alpha_i}\right) \tag{72}$$

for some $\Psi \in \mathbb{R}$.

Proof. Using Lagrange's method, we first define $\mathcal{L} = \sum_i \frac{\alpha_i}{1-e^{-x_i}} + \lambda\left(\sum_i x_i - x_{\text{tot}}\right)$

$$\begin{aligned} 0 = \frac{\partial \mathcal{L}}{\partial x_i} &= \frac{\alpha_i e^{-x_i}}{(1-e^{-x_i})^2} + \lambda = \alpha_i \left(e^{x_i/2} - e^{-x_i/2}\right)^{-2} + \lambda \\ \sqrt{-\frac{\alpha_i}{\lambda}} &= e^{x_i/2} - e^{-x_i/2} = 2\,\sinh(x_i/2) \\ x_i &= 2\,\sinh^{-1}\left(\sqrt{\frac{\alpha_i}{-4\lambda}}\right) \end{aligned} \tag{73}$$

and if we define $\Psi = -\frac{1}{4\lambda}$, we can see that it proves the lemma. □

Using Lemma (D.1), one can see that the optimal distribution of driving forces satisfies the following relationship, for some value of Ψ

$${\theta_i}^* = 2\,\sinh^{-1}\left(\sqrt{\Psi\alpha_i}\right) \tag{74}$$

where the exact value of Ψ can be determined by applying the constraint on the sum of θ_i from Eq. (69):

$$\ln\left(\frac{s_0}{s_n}K_{\text{tot}}^{\text{eq}}\right) = \theta_{\text{tot}} = \sum_i {\theta_i}^* = 2\sum_i \sinh^{-1}\left(\sqrt{\Psi\alpha_i}\right) = 2\sum_i \ln\left(\sqrt{\Psi\alpha_i} + \sqrt{1+\Psi\alpha_i}\right). \tag{75}$$

Unfortunately, there is no analytical solution to this equation. Nevertheless, the function on the left-hand side is strictly monotonically increasing with Ψ (in the entire range $\Psi \in \mathbb{R}$), so solving it numerically should be straightforward. Nevertheless, we can still express $\left(1-e^{-\theta_i}\right)^{-1}$ as a function of Ψ:

$$\left(1-e^{-\theta_i}\right)^{-1} = \left(1-e^{-2\cdot\sinh^{-1}(\sqrt{\Psi\alpha_i})}\right)^{-1} = \frac{1}{2} + \frac{1}{2}\sqrt{1+(\Psi\alpha_i)^{-1}}, \tag{76}$$

where we use the fact that $1-e^{-2\cdot\sinh^{-1}(x)} = 2/(1+\sqrt{1+x^{-2}})$. Therefore, the solutions for the maximal flux and individual enzyme allocations would be:

$$\begin{aligned} J^* &= \frac{\varepsilon_{\text{tot}}}{\sum_i \alpha_i \left(1-e^{-\theta_i}\right)^{-1}} = \frac{\varepsilon_{\text{tot}}}{\sum_i \alpha_i \left(\frac{1}{2} + \frac{1}{2}\sqrt{1+(\Psi\alpha_i)^{-1}}\right)} \\ {\varepsilon_i}^* &= J^*\alpha_i \left(\frac{1}{2} + \frac{1}{2}\sqrt{1+(\Psi\alpha_i)^{-1}}\right). \end{aligned} \tag{77}$$

D.2.3 Limit of small driving forces

If we further assume that the total driving force θ_{tot} is small (and therefore also each one of the reaction driving forces θ_i is even smaller), then we can write:

$$J \leq \varepsilon_{\text{tot}} \cdot \left(\sum_i \frac{1}{k_i^{\text{cat}}} \cdot \frac{1}{1 - e^{-\theta_i}} \right)^{-1} \approx \varepsilon_{\text{tot}} \cdot \left(\sum_i \frac{1}{k_i^{\text{cat}}\ \theta_i} \right)^{-1} \tag{78}$$

As we did before, the solution for J^* can be found by minimizing $\varepsilon_{\text{tot}}/J$ under the constraint $\sum_i \theta_i = \theta_{\text{tot}}$, using the Lagrange method: $\mathcal{L} = \sum_i \frac{1}{k_i^{\text{cat}}\ \theta_i} + \lambda(\sum_i \theta_i - \theta_{\text{tot}})$.

$$0 = \frac{\partial \mathcal{L}}{\partial \theta_i} = -\frac{1}{k_i^{\text{cat}} \cdot (\theta_i{}^*)^2} + \lambda \tag{79}$$

$$\theta_i{}^* = \frac{1}{\sqrt{\lambda k_i^{\text{cat}}}}\,. \tag{80}$$

By applying the constraint $\theta_{\text{tot}} = \sum_i \theta_i{}^* = \lambda^{-1/2} \sum_i \sqrt{1/k_i^{\text{cat}}} \Rightarrow \sqrt{\lambda} = \theta_{\text{tot}}^{-1} \cdot (\sum_i \sqrt{1/k_i^{\text{cat}}})$, we can write:

$$\begin{aligned} \theta_i{}^* &= \theta_{\text{tot}} \frac{\sqrt{1/k_i^{\text{cat}}}}{\sum_i \sqrt{1/k_i^{\text{cat}}}} \\ J^* &= \varepsilon_{\text{tot}} \cdot \left(\sum_i \frac{1}{k_i^{\text{cat}}\ \theta_i{}^*} \right)^{-1} = \frac{\theta_{\text{tot}}\ \varepsilon_{\text{tot}}}{\left(\sum_i \sqrt{1/k_i^{\text{cat}}} \right)^2} = \frac{\theta_{\text{tot}}\ \varepsilon_{\text{tot}}}{||\boldsymbol{\alpha}||_{\frac{1}{2}}} \end{aligned} \tag{81}$$

and furthermore, we can see that:

$$\varepsilon_i{}^* = \frac{J^*}{k_i^{\text{cat}}\ \theta_i{}^*} \propto \frac{1}{k_i^{\text{cat}}\ \theta_i{}^*} \propto \frac{1}{k_i^{\text{cat}}\ \sqrt{1/k_i^{\text{cat}}}} = \sqrt{1/k_i^{\text{cat}}} = \sqrt{\alpha_i}\,. \tag{82}$$

D.2.4 Connectivity theorem

The unscaled metabolite elasticities for the thermodynamic rate law read

$$E_{s_i}^{v_l} \equiv \frac{\partial v_l}{\partial s_i} = \frac{\partial}{\partial s_i} \varepsilon_l k_l^{\text{cat}} \left(1 - e^{-\theta_l(\mathbf{s})}\right) = \varepsilon_l\ k_l^{\text{cat}}\ e^{-\theta_l}\ \frac{\partial \theta_l}{\partial s_i} = \left(\varepsilon_l\ k_l^{\text{cat}} - J\right) \frac{\partial \theta_l}{\partial s_i}. \tag{83}$$

In the last step we used the fact that $\varepsilon_l\ k_l^{\text{cat}}\ e^{-\theta_l} = \varepsilon_l\ k_l^{\text{cat}} - \varepsilon_l\ k_l^{\text{cat}}(1 - e^{-\theta_l}) = \varepsilon_l\ k_l^{\text{cat}} - J$. With flux control coefficients proportional to $(\varepsilon_l\ k_l^{\text{cat}} - J)^{-1}$, we can now derive the connectivity theorem for each metabolite s_i,

$$\sum_l C_l^J\ E_{s_i}^{J_l} \propto \sum_l (\varepsilon_l\ k_l^{\text{cat}} - J)^{-1} (\varepsilon_l\ k_l^{\text{cat}} - J)\ \frac{\partial \theta_l}{\partial s_i} = \sum_l \frac{\partial \theta_l}{\partial s_i} = \sum_l \frac{n_{il}}{s_i} = 0. \tag{84}$$

Here we used $\theta_l = \theta_l^\circ + \sum_i n_{il} \ln s_i$ with derivative $\frac{\partial \theta_l}{\partial s_i} = \frac{n_{il}}{s_i}$ (in the second but last step), and the fact that our reactions are uni-molecular, with stoichiometric coefficients of 1 for substrate and products (in the last step).

D.2.5 Flux control coefficients

Turning Eq. (69) to an equality constraint:

$$\mathcal{Y} \equiv \prod_{i=1}^{n} \left(1 - J(\varepsilon_i\ k_i^{\text{cat}})^{-1}\right) - \frac{s_n}{s_0} \frac{1}{K_{\text{tot}}^{\text{eq}}} = 0. \tag{85}$$

This equation implicitly determines the flux J (given the enzyme levels ε_i and the external concentrations s_0 and s_n). Since $J \leq \varepsilon_i\ k_i^{\text{cat}}$, all product terms are positive and decreasing in J, and so the entire product is a decreasing function of J. This means that there can be only one solution with a positive flux. While Eq. (85) cannot be solved for J directly, it determines J implicitly and is sufficient for computing the flux response coefficients. For doing this we use the following lemma:

Lemma D.2. *We consider two variables x and y, constrained by an equality $f(x,y)=0$. We assume that for each given value of x, the constraint is satisfied by a single value of y, which we call $y=g(x)$. To compute the derivative $\partial g(x)/\partial x$ directly $f(x,y)$, without an explicit expression for $g(x)$, we insert $g(x)$ and write the constraint as $h(x)=f(x,g(x))=0$. With the chain rule, we can write this as $0=\frac{\partial h(x)}{\partial x}=\frac{\partial f(x,y)}{\partial x}|_{y=g(x)}+\frac{\partial f(x,y)}{\partial y}|_{y=g(x)}\ \frac{\partial g(x)}{\partial x}$, which yields*

$$\frac{\partial g(x)}{\partial x}=-\frac{\partial f(x,y)/\partial x}{\partial f(x,y)/\partial y}|_{y=g(x)} \tag{86}$$

To compute the flux control coefficients, we read Eq. (85) as a constraint between ε_i and J, given all other enzyme levels, which is solved by a function $J(\varepsilon_i)$. To compute the derivative $\partial J/\partial\varepsilon_i$ (assuming all other enzyme levels are constant), we set $x\to\varepsilon_i$; $y\to J$; $g(x)\to J(\varepsilon_i)$; and $f(x,y)\to\mathcal{Y}(\varepsilon_i,J)$, and obtain:

$$\begin{aligned}
R^J_{\varepsilon_l}&=\frac{\partial J}{\partial\varepsilon_l}=-\frac{\partial\mathcal{Y}}{\partial\varepsilon_l}\left(\frac{\partial\mathcal{Y}}{\partial J}\right)^{-1}\\
&=-\frac{\prod_{i\neq l}(1-J\cdot(\varepsilon_i\ k_i^{\mathrm{cat}})^{-1})}{\prod_{i=1}^n(1-J\cdot(\varepsilon_i\ k_i^{\mathrm{cat}})^{-1})}\cdot\frac{-J\cdot(k_l^{\mathrm{cat}})^{-1}\cdot\varepsilon_l^{-2}}{\sum_{i=1}^n\frac{-(\varepsilon_i\ k_i^{\mathrm{cat}})^{-1}}{1-J\cdot(\varepsilon_i\ k_i^{\mathrm{cat}})^{-1}}}\\
&=-\frac{1}{1-J\cdot(\varepsilon_l\ k_l^{\mathrm{cat}})^{-1}}\cdot\frac{J\cdot(k_l^{\mathrm{cat}})^{-1}\cdot\varepsilon_l^{-2}}{\sum_{i=1}^n(\varepsilon_i\ k_i^{\mathrm{cat}}-J)^{-1}}\\
&=-\frac{J}{\varepsilon_l}\cdot\underbrace{\frac{(\varepsilon_l\ k_l^{\mathrm{cat}}-J)^{-1}}{\sum_{i=1}^n(\varepsilon_i\ k_i^{\mathrm{cat}}-J)^{-1}}}_{C_l^J}\cdot
\end{aligned} \tag{87}$$

The result is simple: the control coefficients (C_l^J) are proportional to $(\varepsilon_l\ k_l^{\mathrm{cat}}-J)^{-1}$ and are normalized to a sum of 1 as required by the summation theorem.

D.2.6 Concentration control coefficients

Using Eq. (68), which we can solve for any s_j in the same way as for s_n, we obtain $s_j=s_0\prod_{i=1}^j K_i^{\mathrm{eq}}(1-J\cdot(\varepsilon_i k_i^{\mathrm{cat}})^{-1})$

$$R^{s_j}_{\varepsilon_l}=\frac{\partial s_j}{\partial\varepsilon_l}=\underbrace{s_0\prod_{i=1}^j K_i^{\mathrm{eq}}(1-J\cdot(\varepsilon_i k_i^{\mathrm{cat}})^{-1})}_{s_j}\cdot\underbrace{\frac{\frac{\partial}{\partial\varepsilon_l}(1-J\cdot(\varepsilon_l k_l^{\mathrm{cat}})^{-1})}{1-J\cdot(\varepsilon_l k_l^{\mathrm{cat}})^{-1}}}_{\mathcal{Z}} \tag{88}$$

if $l\leq j$ (otherwise 0). The first product term is simply s_j. The second product term can be rewritten as:

$$\begin{aligned}
\mathcal{Z}&=-\frac{\frac{\partial J}{\partial\varepsilon_l}\cdot(\varepsilon_l\ k_l^{\mathrm{cat}})^{-1}+J\cdot(k_l^{\mathrm{cat}})^{-1}\varepsilon_l^{-2}}{1-J\cdot(\varepsilon_l k_l^{\mathrm{cat}})^{-1}}=-\frac{R^{s_j}_{\varepsilon_l}+J\varepsilon_l^{-1}}{\varepsilon_l k_l^{\mathrm{cat}}-J}\\
&=\frac{\frac{J}{\varepsilon_l}\frac{(\varepsilon_l\ k_l^{\mathrm{cat}}-J)^{-1}}{\sum_{i=1}^n(\varepsilon_i\ k_i^{\mathrm{cat}}-J)^{-1}}-\frac{J}{\varepsilon_l}}{\varepsilon_l k_l^{\mathrm{cat}}-J}=\frac{J}{\varepsilon_l}\cdot\frac{\frac{(\varepsilon_l\ k_l^{\mathrm{cat}}-J)^{-1}}{\sum_{i=1}^n(\varepsilon_i\ k_i^{\mathrm{cat}}-J)^{-1}}-1}{\varepsilon_l k_l^{\mathrm{cat}}-J}=\frac{J}{\varepsilon_l}\cdot\frac{\frac{1}{\sum_{i=1}^n(\varepsilon_i\ k_i^{\mathrm{cat}}-J)^{-1}}-\varepsilon_l\ k_l^{\mathrm{cat}}+J}{(\varepsilon_l\ k_l^{\mathrm{cat}}-J)^{-2}}
\end{aligned} \tag{89}$$

Putting all this together, we obtain the concentration control coefficients

$$C_l^{s_j}=\frac{R^{s_j}_{\varepsilon_l}}{J/\varepsilon_l}=s_j\left[\frac{\frac{1}{\sum_{i=1}^n(\varepsilon_i\ k_i^{\mathrm{cat}}-J)^{-1}}-\varepsilon_l\ k_l^{\mathrm{cat}}+J}{(\varepsilon_l\ k_l^{\mathrm{cat}}-J)^{-2}}\right]. \tag{90}$$

Again, this holds only for $l\leq j$ (otherwise $C_l^{s_j}=0$).

D.2.7 Enzyme-control rule

To show that the control coefficients Eq. (33) satisfy the enzyme-control rule, we assume that the rule is correct, insert our formula for control coefficients, derive an expression for the enzyme levels, and compare the result to our known formula Eq. (77). We do this now step by step. Starting from the proportionality

$$C_l^J\propto\frac{1}{\varepsilon_l\ k_l^{\mathrm{cat}}-J}\propto\varepsilon_l \tag{91}$$

we obtain

$$\begin{aligned}
&\Rightarrow \frac{1}{\varepsilon_l^2\ k_l^{\mathrm{cat}} - J\ \varepsilon_l} = \mathrm{const} = 1/a \\
&\Rightarrow \varepsilon_l^2\ k_l^{\mathrm{cat}} - J\ \varepsilon_l - a = 0 \\
&\Rightarrow \varepsilon_l^2 - \frac{J}{k_l^{\mathrm{cat}}}\ \varepsilon_l - \frac{a}{k_l^{\mathrm{cat}}} = 0 \\
&\Rightarrow \varepsilon_l = \frac{J}{2\ k_l^{\mathrm{cat}}} \pm \sqrt{\left(\frac{J}{2\ k_l^{\mathrm{cat}}}\right)^2 + \frac{a}{k_l^{\mathrm{cat}}}} = \frac{J}{k_l^{\mathrm{cat}}}\left(\frac{1}{2} \pm \frac{1}{2}\sqrt{1 + k_l^{\mathrm{cat}}\ \frac{4\ a}{J^2}}\right)
\end{aligned} \tag{92}$$

The solution with a minus sign is physically meaningless and can be discarded. Remember that we defined $\alpha_i \equiv 1/k_i^{\mathrm{cat}}$, so we can rewrite Eq. (92) as

$$\varepsilon_i = J\alpha_i \left(\frac{1}{2} + \frac{1}{2}\sqrt{1 + (\Psi\alpha_i)^{-1}}\right) \tag{93}$$

Where we also replaced $J^2/(4a)$ with Ψ by comparing this solution to Eq. (77) (and due to the fact that the value of this constant is uniquely determined by the constraint $\sum_i \varepsilon_i = \varepsilon_{\mathrm{tot}}$).

D.2.8 A note on Max-min Driving Force

The Max-Min Driving Force method (MDF) is a heuristics for finding realistic metabolite concentrations in a metabolic model with known flux directions. It relies on the fact that very small driving forces lead to very large enzyme demands and should be avoided. This heuristics can be seen as a way to reduce enzyme cost. To see this, we start from the thermodynamic rate law and compute the enzyme demand:

$$\varepsilon_l = \frac{v_l}{k_l^{\mathrm{cat}}} \frac{1}{1 - \mathrm{e}^{-\theta_l}} \geq \frac{v_l}{k_l^{\mathrm{cat}}}\ \max(1,\ \frac{1}{\theta_l}). \tag{94}$$

where we use the fact that $e^{-x} \geq \max(0, 1-x)$. Let us now consider an unbranched pathway with a steady forward flux and a given overall driving force θ. In this case, we obtain the constraints $\theta_l \geq 0$ and $\sum_l \theta_l = \theta$. The forces θ_l are not free variables, but a function $\theta_l = \ln K_l^{\mathrm{eq}} - (\ln c_l - \ln c_{l-1})$ of the metabolite concentrations, which in turn are constrained by physiological ranges. Let us see how the MDF principle can be derived from our present optimality problem, flux maximization at a fixed enzyme budget. Since pathway flux and total enzyme demand scale proportionally at given metabolite concentrations, this flux maximization is equivalent to minimizing the total enzyme demand $\sum_l \varepsilon_l$ at a given flux. With Eq. (94), we can approximate this demand as

$$\sum_l \varepsilon_l \geq \sum_{l=1}^{n} \frac{v_l}{k_l^{\mathrm{cat}}} + \sum_{l:\theta_l<1} \frac{v_l}{k_l^{\mathrm{cat}}}\left(\frac{1}{\theta_l} - 1\right) \leq \sum_{l=1}^{n} \frac{v_l}{k_l^{\mathrm{cat}}} + \max_{l:\theta_l<1} \frac{v_l}{k_l^{\mathrm{cat}}}\left(\frac{1}{\theta_l} - 1\right). \tag{95}$$

The first term describes a constant enzyme demand, representing a hypothetical model in which all enzymes work at their maximal speed. The second term concerns reactions with driving forces smaller than 1 (if such reactions exist) and denotes the maximal value of $\frac{v_l}{k_l^{\mathrm{cat}}}\left(\frac{1}{\theta_l} - 1\right)$ among these reactions. To minimize the overall enzyme demand, we choose the heuristics of minimizing this term. This is equivalent to maximizing

$$\min_{l:\theta_l<1} \frac{k_l^{\mathrm{cat}}}{v_l}\theta_l. \tag{96}$$

where l runs over all reactions with a driving force smaller than 1. Thus, in the resulting state, the smallest driving force across the pathway (weighted by $\frac{k_l^{\mathrm{cat}}}{v_l}$), should be as large as possible. The original MDF driving force method employs some additional simplifications. First, we neglect the prefactor $\frac{k_l^{\mathrm{cat}}}{v_l}$ (assuming that nothing is known about enzyme kinetics. Second, if all the reactions have driving forces larger than 1, we let the index l run over all reactions, assuming that the smallest driving force will still cause the largest enzyme cost.

D.3 Reversible mass-action kinetics

D.3.1 Steady-state concentrations

Here we will show the full derivation of the optimal solution in the reversible mass-action case. We start by reminding ourselves that the rate law for each reaction is given by:

$$v_i = \varepsilon_i \ \beta_i^{-1} \ (s_{i-1} - s_i/K_i^{\mathrm{eq}}) \,. \tag{97}$$

and the pathway flux is thus (as in Eq. (25)):

$$J = \varepsilon_{\mathrm{tot}} \ \left(\sum_i \frac{\beta_i}{s_{i-1} - s_i/K_i^{\mathrm{eq}}} \right)^{-1} \,. \tag{98}$$

where we replaced the inequality by an equality since we are looking for the maximal flux.

While J can in theory be maximized by equating the gradient with respect to all metabolite levels to 0, the resulting system of equations would be difficult to solve. Instead, we will use a different approach that involves finding an expression for J as a function of the different enzyme levels (rather than metabolite levels).

First, we use Eq. (97) to generate a formula for s_i, and by equating $v_i = J$ and applying it recursively we get:

$$\begin{aligned}
s_1 &= K_1^{\mathrm{eq}} \left(s_0 - \frac{J\beta_1}{\varepsilon_1} \right) \\
s_2 &= K_2^{\mathrm{eq}} \left(s_1 - \frac{J\beta_2}{\varepsilon_2} \right) = K_2^{\mathrm{eq}} \left(K_1^{\mathrm{eq}} \left(s_0 - \frac{J\beta_1}{\varepsilon_1} \right) - \frac{J\beta_2}{\varepsilon_2} \right) \\
&= s_0 K_1^{\mathrm{eq}} K_2^{\mathrm{eq}} - J \left(\frac{\beta_1}{\varepsilon_1} K_1^{\mathrm{eq}} K_2^{\mathrm{eq}} + \frac{\beta_2}{\varepsilon_2} K_2^{\mathrm{eq}} \right) \\
s_3 &= s_0 K_1^{\mathrm{eq}} K_2^{\mathrm{eq}} K_3^{\mathrm{eq}} - J \left(\frac{\beta_1}{\varepsilon_1} K_1^{\mathrm{eq}} K_2^{\mathrm{eq}} K_3^{\mathrm{eq}} + \frac{\beta_2}{\varepsilon_2} K_2^{\mathrm{eq}} K_3^{\mathrm{eq}} + \frac{\beta_3}{\varepsilon_3} K_3^{\mathrm{eq}} \right) \\
&\vdots \\
s_n &= s_0 \prod_{i=1}^{n} K_i^{\mathrm{eq}} - J \sum_{j=1}^{n} \frac{\beta_j}{\varepsilon_j} \prod_{i=j}^{n} K_i^{\mathrm{eq}} = s_0 \ K_{\mathrm{tot}}^{\mathrm{eq}} - J \sum_{j=1}^{n} \frac{\gamma_j}{\varepsilon_j}
\end{aligned} \tag{99}$$

where we use the fact that $K^{\mathrm{eq}} = \prod_{i=1}^{n} K_i^{\mathrm{eq}}$ (i.e. the equilibrium constant of the pathway net reaction), and substituting $\gamma_j \equiv \beta_j \prod_{i=j}^{n} K_i^{\mathrm{eq}}$. Solving for J we get:

$$J = \frac{s_0 \ K_{\mathrm{tot}}^{\mathrm{eq}} \ - \ s_n}{\sum_{j=1}^{n} \gamma_j (\varepsilon_j)^{-1}} \,. \tag{100}$$

D.3.2 Minimum enzyme demand / maximal flux

Now, in order to maximize J we need to minimize the denominator (the numerator is a constant). The variables (ε_j) have to satisfy the constraint $\sum_j \varepsilon_j \leq \varepsilon_{\mathrm{tot}}$. To find the minimal value for the denominator, we can use the Lagrange method:

$$0 = \frac{\partial}{\partial \varepsilon_j} \left[\sum_{j=1}^{n} \gamma_j (\varepsilon_j)^{-1} - \lambda \left(\sum_j \varepsilon_j - \varepsilon_{\mathrm{tot}} \right) \right]_{\varepsilon_j{}^*} = -\gamma_j (\varepsilon_j{}^*)^{-2} - \lambda \tag{101}$$

which leads us to conclude that $\varepsilon_j{}^* \propto \sqrt{\gamma_j}$. Since we know the sum of all the enzyme levels, $\varepsilon_{\mathrm{tot}}$, we can also find the proper scaling factor, i.e.:

$$\varepsilon_j{}^* = \varepsilon_{\mathrm{tot}} \ \frac{\sqrt{\gamma_j}}{\sum_{j=1}^{n} \sqrt{\gamma_j}} = \varepsilon_{\mathrm{tot}} \ \sqrt{\frac{\gamma_j}{||\boldsymbol{\gamma}||_{\frac{1}{2}}}} \tag{102}$$

where $\boldsymbol{\gamma}$ is the vector of all γ_j, and $||\cdot||_{\frac{1}{2}}$ is the $\mathrm{l}_{1/2}$ norm.

Furthermore, we can calculate the maximal achievable flux J^* by plugging in the ${\varepsilon_j}^*$ values:

$$J^* = \frac{s_0\ K_{\text{tot}}^{\text{eq}} - s_n}{\sum_{j=1}^n \gamma_j ({\varepsilon_j}^*)^{-1}} = \frac{s_0\ K_{\text{tot}}^{\text{eq}} - s_n}{\sum_{j=1}^n \gamma_j \ \frac{1}{\varepsilon_{\text{tot}}} \sqrt{\frac{||\boldsymbol{\gamma}||_{\frac{1}{2}}}{\gamma_j}}} = \varepsilon_{\text{tot}} \frac{s_0\ K_{\text{tot}}^{\text{eq}} - s_n}{\sqrt{||\boldsymbol{\gamma}||_{\frac{1}{2}}} \sum_{j=1}^n \sqrt{\gamma_j}} = \varepsilon_{\text{tot}}\ \frac{s_0\ K_{\text{tot}}^{\text{eq}} - s_n}{||\boldsymbol{\gamma}||_{\frac{1}{2}}} \tag{103}$$

Another useful perspective that we can draw this optimization, is looking at optimal ratios between consecutive enzymes:

$$\frac{{\varepsilon_j}^*}{{\varepsilon_{j-1}}^*} = \sqrt{\frac{\gamma_j}{\gamma_{j-1}}} = \sqrt{\frac{\beta_j}{\beta_{j-1}\ K_j^{\text{eq}}}} = \sqrt{\frac{K_{\text{M},j}\ k_{j-1}^{\text{cat}}}{K_{\text{M},j-1}\ k_j^{\text{cat}}\ K_j^{\text{eq}}}}\,. \tag{104}$$

Interestingly, the ratio depends only on the K_{M} and k^{cat} values of the two reactions and is independent of all other enzymes in the system. This might be a design principle supporting for the existence of regulatory mechanisms that ensure these ratios are maintained even if the total expression level changes, or when some sub-pathways are re-used in different contexts of metabolism.

D.3.3 Flux control coefficients

The steady-state flux is given by Eq. (100). By taking the partial derivative with respect to enzyme levels, we obtain the response coefficients

$$R_{\varepsilon_l}^J = \frac{\partial}{\partial \varepsilon_l} \frac{s_0\ K_{\text{tot}}^{\text{eq}} - s_n}{\sum_{i=1}^n \gamma_i/\varepsilon_i} = -\frac{s_0\ K_{\text{tot}}^{\text{eq}} - s_n}{(\sum_{i=1}^n \gamma_i/\varepsilon_i)^2} \cdot \left(-\frac{\gamma_l}{\varepsilon_l^2}\right) = J\ \frac{\gamma_l/\varepsilon_l^2}{\sum_{i=1}^n \gamma_i/\varepsilon_i}. \tag{105}$$

After dividing by the enzyme elasticities $E_{\varepsilon_l}^{v_l} = J/\varepsilon_l$, we obtain the control coefficients

$$C^J = \frac{R_{\varepsilon_l}^J}{E_{\varepsilon_l}^{v_l}} = \frac{\gamma_l/\varepsilon_l}{\sum_{i=1}^n \gamma_i/\varepsilon_i}. \tag{106}$$

D.3.4 Connectivity theorem for flux control coefficients

The connectivity theorem for flux control coefficients reads $\mathbf{C}^J\ \mathbf{E}_c^v = 0$. Here we show this explicitly for an unbranched pathway with reversible mass-action kinetics. With the control coefficients Eq. (106) and the metabolite elasticities for the reversible mass action rate law

$$E_{c_i}^{v_l} = \varepsilon_l\ \beta_l^{-1}\ (\delta_{i,l-1} - \delta_{il}/K_l^{\text{eq}}). \tag{107}$$

we obtain

$$\begin{aligned}
(\mathbf{C}^J\ \mathbf{E}_c^v)_i &= \sum_l \frac{\gamma_l/\varepsilon_l}{\sum_j \gamma_j/\varepsilon_j}\ \varepsilon_l\ \beta_l^{-1}\ (\delta_{i,l-1} - \delta_{il}/K_l^{\text{eq}}) \propto \sum_l \gamma_l\ \beta_l^{-1}\ (\delta_{i,l-1} - \delta_{il}/K_l^{\text{eq}}) \\
&= \gamma_{i+1}\ \beta_{i+1}^{-1} - \gamma_i\ \beta_i^{-1}\ \cdot\ 1/K_i^{\text{eq}} \\
&= \prod_{j=i+1}^n K_j^{\text{eq}} - \prod_{j=i}^n K_j^{\text{eq}}\ \cdot\ 1/K_i^{\text{eq}} \\
&= \prod_{j=i+1}^n K_j^{\text{eq}} - \prod_{j=i+1}^n K_j^{\text{eq}} = 0\,.
\end{aligned} \tag{108}$$

D.3.5 Enzyme-control rule

The enzyme levels in optimal state Eq. (26) read

$$\varepsilon_l^* = \varepsilon_{\text{tot}} \sqrt{\frac{\gamma_l}{||\boldsymbol{\gamma}||_{\frac{1}{2}}}}. \tag{109}$$

By inserting this into the formula Eq. (106) for control coefficients, we obtain (where the constant denominator is a normalisation term)

$$C_l^{J^*} \equiv \frac{\gamma_l \left(\varepsilon_{\text{tot}} \sqrt{\frac{\gamma_l}{||\boldsymbol{\gamma}||_{\frac{1}{2}}}}\right)^{-1}}{\text{const}} \propto \sqrt{\gamma_l}, \tag{110}$$

which is proportional to the enzyme level ε_l^* itself.

D.4 Haldane rate law

D.4.1 Steady-state concentrations

For this derivation, we first use the notation in the original publication by Heinrich and Klipp [17]. For a unbranched chain at steady-state (with flux J in all reactions), the Haldane rate law dictates that:

$$J = \frac{v_{+l}^{\max}\ s_{l-1}/K_l^{\text{S}} - v_{-l}^{\max}\ s_l/K_l^{\text{P}}}{1 + s_{l-1}/K_l^{\text{S}} + s_l/K_l^{\text{P}}}\,. \tag{111}$$

Using this formula, we can solve for s_l given all other parameters (including s_{l-1}) and get the following recursion:

$$s_l = s_{l-1}\frac{K_l^{\text{P}}}{K_l^{\text{S}}}\frac{v_{+l}^{\max} - J}{v_{-l}^{\max} + J} - J\frac{K_l^{\text{P}}}{v_{-l}^{\max} + J} \tag{112}$$

which can be solved to give:

$$s_l^{\text{steady}} = s_0\left(\prod_{i=1}^{l}\frac{K_i^{\text{P}}}{K_i^{\text{S}}}\frac{v_{+i}^{\max} - J}{v_{-i}^{\max} + J}\right) - J\sum_{i=1}^{l}\frac{K_i^{\text{S}}}{v_{+i}^{\max} - J}\prod_{j=i}^{l}\frac{K_j^{\text{P}}}{K_j^{\text{S}}}\frac{(v_{+j}^{\max} - J)}{(v_{-j}^{\max} + J)} \tag{113}$$

From now on, we will replace $v_{\pm i}^{\max}$ with $\varepsilon_i \cdot k_{\pm i}^{\text{cat}}$ in order to better match the notation of this paper. If we use the solution for the steady-state concentration of s_n we can write:

$$f(J;\boldsymbol{\varepsilon}) \equiv s_0\left(\prod_{i=1}^{n}\frac{K_i^{\text{P}}}{K_i^{\text{S}}}\frac{\varepsilon_i k_{+i}^{\text{cat}} - J}{\varepsilon_i k_{-i}^{\text{cat}} + J}\right) - J\sum_{i=1}^{n}\frac{K_i^{\text{S}}}{\varepsilon_i k_{+i}^{\text{cat}} - J}\prod_{j=i}^{n}\frac{K_j^{\text{P}}}{K_j^{\text{S}}}\frac{(\varepsilon_i k_{+j}^{\text{cat}} - J)}{(\varepsilon_i k_{-j}^{\text{cat}} + J)} - s_n = 0\,. \tag{114}$$

Solving this equality (i.e., finding the roots of $f(J;\boldsymbol{\varepsilon})$) would give us an expression for $J(\boldsymbol{\varepsilon})$ – the flux as a function of the enzyme levels. This can be used in different ways. First, we can determine the flux response coefficients by implicit differentiation (Lemma D.2: $R_{\varepsilon_i}^J = -\frac{\partial f/\partial \varepsilon_i}{\partial f/\partial J}$), and by dividing by J/ε_i we obtain the flux control coefficients. Second, we can find an optimal metabolic state by minimizing $\sum_i \varepsilon_i$ at a given flux J, and requiring Eq. (114) as a constraint (with Lagrange multiplier λ). Optimization using the Lagrange method would lead to the optimality condition $\partial f/\partial \varepsilon_i = \lambda$. However, since the derivatives are complicated and J and λ are unknown, this is hard to solve.

The most efficient method we currently have for solving this optimization problem is, unfortunately, not analytical. First, we start by writing $\varepsilon_{\text{tot}}/J$ as a function of the metabolite concentrations:

$$\frac{\varepsilon_{\text{tot}}}{J} = \frac{\sum_i \varepsilon_i}{J} = \sum_i \frac{1 + s_{i-1}/K_i^{\text{S}} + s_i/K_i^{\text{P}}}{k_{+i}^{\text{cat}}\ s_{i-1}/K_i^{\text{S}} - k_{+i}^{\text{cat}}\ s_i/K_i^{\text{P}}}\,. \tag{115}$$

We showed previously that this function is convex with respect to the metabolite log-concentrations ($\ln \mathbf{s}$) and therefore has a single (global) minimum which is simple to find numerically [35, 36].

D.5 Reactions with several substrates and products: effective kinetic constants

The pathways we consider in this paper consist of uni-uni reactions S $\leftrightarrow$ P. Here we show how the results can be applied to reactions with several substrates and products, assuming that only one of the substrates and one of the products have variable concentrations, while all other concentrations are fixed. As an example, we consider a

reaction with stoichiometric coefficients of 1, substrates s (variable) and a (constant), and products p (variable) and b (constant). There are various ways to generalize the Haldane rate law to reactions with several substrates or products. One of them, the convenience kinetics [33], for this reaction reads

$$v = \varepsilon \frac{k_+^{\mathrm{cat}}\ s/K_{\mathrm{S}}\ a/K_{\mathrm{A}} - k_-^{\mathrm{cat}}\ p/K_{\mathrm{P}}\ b/K_{\mathrm{B}}}{(1+s/K_{\mathrm{S}})(1+a/K_{\mathrm{A}}) + (1+p/K_{\mathrm{P}})(1+b/K_{\mathrm{B}}) - 1}. \tag{116}$$

Setting $\sigma = a/K_{\mathrm{A}}$ and $\pi = b/K_{\mathrm{B}}$, we obtain

$$\begin{aligned} v &= \varepsilon \frac{k_+^{\mathrm{cat}}\ \sigma\ s/K_{\mathrm{S}} - k_-^{\mathrm{cat}}\ \pi\ p/K_{\mathrm{P}}}{(1+s/K_{\mathrm{S}})(1+\sigma) + (1+p/K_{\mathrm{P}})(1+\pi) - 1} \\ &= \varepsilon \frac{k_+^{\mathrm{cat}}\ \sigma\ s/K_{\mathrm{S}} - k_-^{\mathrm{cat}}\ \pi\ p/K_{\mathrm{P}}}{(1+\sigma) + (1+\pi) - 1 + (1+\sigma)s/K_{\mathrm{S}} + (1+\pi)p/K_{\mathrm{P}}} \\ &= \varepsilon \frac{\frac{\sigma}{1+\sigma+\pi}k_+^{\mathrm{cat}} s/K_{\mathrm{S}} - \frac{\pi}{1+\sigma+\pi}k_-^{\mathrm{cat}} p/K_{\mathrm{P}}}{1 + \frac{1+\sigma}{1+\sigma+\pi}\ s/K_{\mathrm{S}} + \frac{1+\pi}{1+\sigma+\pi}\ p/K_{\mathrm{P}}} \end{aligned} \tag{117}$$

and by defining

$$\begin{aligned} \widehat{K_{\mathrm{S}}} &= \frac{1+\sigma+\pi}{1+\sigma} K_{\mathrm{S}}, \qquad \widehat{K_{\mathrm{P}}} = \frac{1+\sigma+\pi}{1+\pi} K_{\mathrm{P}} \\ \widehat{k_+^{\mathrm{cat}}} &= \frac{\sigma}{1+\sigma} k_+^{\mathrm{cat}}, \qquad \widehat{k_-^{\mathrm{cat}}} = \frac{\pi}{1+\pi} k_-^{\mathrm{cat}} \end{aligned} \tag{118}$$

we can write this in the form of our uni-uni rate law:

$$v = \varepsilon \cdot \frac{\widehat{k_+^{\mathrm{cat}}}\ s/\widehat{K_{\mathrm{S}}} - \widehat{k_-^{\mathrm{cat}}}\ p/\widehat{K_{\mathrm{P}}}}{(1+s/\widehat{K_{\mathrm{S}}}) + (1+p/\widehat{K_{\mathrm{P}}}) - 1} \tag{119}$$

with effective ($\widehat{\cdot}$) parameters. From the Haldane relationship, we obtain the effective equilibrium constant:

$$\widehat{K^{\mathrm{eq}}} = \frac{\widehat{k_+^{\mathrm{cat}}}}{\widehat{k_-^{\mathrm{cat}}}} \cdot \frac{\widehat{K_{\mathrm{P}}}}{\widehat{K_{\mathrm{S}}}} = \frac{\sigma}{\pi} \frac{\cancel{(1+\pi)}}{\cancel{(1+\sigma)}} \frac{k_+^{\mathrm{cat}}}{k_-^{\mathrm{cat}}} \cdot \frac{\cancel{(1+\sigma+\pi)}}{\cancel{(1+\sigma+\pi)}} \frac{\cancel{(1+\sigma)}}{\cancel{(1+\pi)}} \frac{K_{\mathrm{P}}}{K_{\mathrm{S}}} = \frac{a}{b} \frac{k_+^{\mathrm{cat}}}{k_-^{\mathrm{cat}}} \frac{K_{\mathrm{P}} K_{\mathrm{B}}}{K_{\mathrm{S}} K_{\mathrm{A}}} = \frac{a}{b} K^{\mathrm{eq}}, \tag{120}$$

where we used the Haldane relationship for the original reaction: $K^{\mathrm{eq}} = \frac{k_+^{\mathrm{cat}}}{k_-^{\mathrm{cat}}} \frac{K_{\mathrm{P}} K_{\mathrm{B}}}{K_{\mathrm{S}} K_{\mathrm{A}}}$.

The driving force $\theta = \ln K^{\mathrm{eq}} - \ln \frac{p}{s}\frac{b}{a}$ of a reaction, as a thermodynamic quantity, cannot depend on how the rate law is written. With the original rate law, it depends on the (true) equilibrium constant as well as on all four reactant concentrations. But if we ignore A and B in the rate law and simply define our equilibrium constant as the ratio b^{eq}/a^{eq} in some imagined equilibrium state, the resulting driving force would be different from our original driving force and the term $1 - \mathrm{e}^{-\theta}$ will be wrong or, even worse, the new θ will have the opposite sign and be opposite to the flux direction. To avoid this problem, there are two possibilities: either we use the effective equilibrium constant Eq. (120), which contains the constant ignored concentrations, or we compute the driving force with an "external" extra term,

$$\theta = \ln K^{\mathrm{eq}} - \underbrace{\ln \frac{b}{a}}_{\theta_{\mathrm{ext}}} - \ln \frac{p}{s}. \tag{121}$$

If we compare the thermodynamic force to voltage in an electric circuit, A and B would act like an external voltage source.

D.6 Enzyme-control rule in models with constraints on enzyme and metabolite levels

We assume an unbranched metabolic pathway in steady state. The steady state fluxes $\mathbf{v} = \mathbf{v}^{\mathrm{steady}}(\boldsymbol{\varepsilon})$ and steady-state metabolite concentrations $\mathbf{s} = \mathbf{s}^{\mathrm{steady}}(\boldsymbol{\varepsilon})$, differentiated by ε_l, yield the response coefficients $R^{v_i}_{\varepsilon_l} = \partial v_i^{\mathrm{steady}}/\partial\varepsilon_l$ and $R^{s_i}_{\varepsilon_l} = \partial s_i^{\mathrm{steady}}/\partial\varepsilon_l$. We now consider the optimality problem (same as in 37):

$$\text{Maximize } \mathbf{z}\cdot\mathbf{v} \quad \text{s.t.} \quad \mathbf{a}\cdot\boldsymbol{\varepsilon} + \mathbf{b}\cdot\mathbf{s} \le \rho$$

where $\mathbf{v}$, $\mathbf{s}$, and $\boldsymbol{\varepsilon}$ denote reaction fluxes, metabolite concentrations, and enzyme levels. The maximization objective is a linear benefit function scoring the fluxes, $\mathbf{z}\cdot\mathbf{v}$ with linear weights z_i, while the constrained density is linear in all the concentrations, with linear weights a_l (for enzymes) and b_i (for metabolites), e.g. representing effective molecule sizes. Optimization with a Lagrange multiplier λ yields

$$\text{Maximize} \quad \mathcal{L}(\boldsymbol{\varepsilon}) = \mathbf{z}^\top \mathbf{v}^{\text{steady}}(\boldsymbol{\varepsilon}) - \lambda \, \left[\mathbf{a}^\top \boldsymbol{\varepsilon} + \mathbf{b}^\top \mathbf{s}^{\text{steady}}(\boldsymbol{\varepsilon}) - \rho\right], \tag{122}$$

where $\mathbf{z}$ is the flux weight vector, $\mathbf{a}$ and $\mathbf{b}$ are the molecular masses of the enzymes and metabolites (respectively), and ρ is the upper bound on the total density. The Lagrangian yields the optimality condition:

$$\begin{aligned} 0 = \frac{\partial \mathcal{L}}{\partial \boldsymbol{\varepsilon}} &= \mathbf{z}^\top \frac{\partial \mathbf{v}^{\text{steady}}}{\partial \boldsymbol{\varepsilon}} - \lambda \, \left[\mathbf{a}^\top + \mathbf{b}^\top \frac{\partial \mathbf{s}^{\text{steady}}}{\partial \boldsymbol{\varepsilon}}\right] \\ &= \mathbf{z}^\top \mathbf{R}^{\mathbf{v}}_{\boldsymbol{\varepsilon}} - \lambda \left[\mathbf{a}^\top + \mathbf{b}^\top \mathbf{R}^{\mathbf{s}}_{\boldsymbol{\varepsilon}}\right] \\ \Rightarrow \mathbf{a}^\top &= \frac{1}{\lambda} \mathbf{z}^\top \mathbf{R}^{\mathbf{v}}_{\boldsymbol{\varepsilon}} - \mathbf{b}^\top \mathbf{R}^{\mathbf{s}}_{\boldsymbol{\varepsilon}}. \end{aligned} \tag{123}$$

By splitting the response matrices into control and enzyme elasticity matrices, $\mathbf{R}^x_{\boldsymbol{\varepsilon}} = \mathbf{C}^x E^v_{\boldsymbol{\varepsilon}} = \mathbf{C}^x \, \text{diag}(\mathbf{v}) \, \text{diag}(\boldsymbol{\varepsilon})^{-1}$, we next obtain

$$\begin{aligned} \mathbf{a}^\top &= \left[\frac{1}{\lambda}\mathbf{z}^\top \, \mathbf{C}^{\mathbf{v}} - \mathbf{b}^\top \mathbf{C}^{\mathbf{s}}\right] \, \text{diag}(\mathbf{v}) \, \text{diag}(\boldsymbol{\varepsilon})^{-1} \\ \Rightarrow \boldsymbol{\varepsilon}^\top \, \text{diag}(\mathbf{a}) \, \text{diag}(\mathbf{v})^{-1} &= \left[\frac{1}{\lambda} \, \mathbf{z}^\top \mathbf{C}^{\mathbf{v}} - \mathbf{b}^\top \mathbf{C}^{\mathbf{s}}\right]. \end{aligned} \tag{124}$$

In the case of an unbranched pathway, i.e. with the same steady-state $v_i^{\text{steady}} = J$ in all reactions, we can arbitrarily chose a benefit function $\mathbf{z}^\top = (1, 0, \ldots, 0)$ which will give use $\mathbf{z}^\top \mathbf{v} = v_1 = J$. In this case $\mathbf{z}^\top \mathbf{C}^{\mathbf{v}}$ will simply be the first column of the $\mathbf{C}^{\mathbf{v}}$ matrix, which we will refer to as $\mathbf{C}^J$. In addition, we can chose density constraints with uniform weights for all enzymes and for all metabolites ($\mathbf{a} = a\mathbf{1}$, $\mathbf{b} = b\mathbf{1}$). Then, the enzyme profile becomes:

$$\boldsymbol{\varepsilon} = \frac{J}{a}\left[\frac{1}{\lambda} \, \mathbf{C}^J - b \, \mathbf{1}^\top \mathbf{C}^{\mathbf{s}}\right] \tag{125}$$

Our new enzyme-control rule thus reads, as a proportionality (and marking again the optimal state by *)

$$\varepsilon_l^* \propto C_l^{J*} - \lambda \, b \, \sum_i C_l^{s_i *}. \tag{126}$$

In our density constraint in Eq. (37), with a predefined overall density ρ, there is no explicit bound ε_{tot} on the sum of enzyme levels. However, given the sum of enzyme levels $\varepsilon^*_{\text{tot}}$ that emerges from the solution, we obtain an explicit enzyme-control rule with no unknown parameters. To derive it, we write Eq. (125) in the form

$$\varepsilon_l^* = \frac{J}{a \, \lambda} C_l^{J*} - \frac{bJ^*}{a} \, \sum_i C_l^{s_i *} \tag{127}$$

We can then sum over l and get:

$$\varepsilon^*_{\text{tot}} = \sum_l \varepsilon_l^* = \frac{J^*}{a \, \lambda} \sum_l C_l^{J*} - \frac{bJ^*}{a} \, \sum_i \sum_l C_l^{s_i *} = \frac{J^*}{a \, \lambda} \cdot 1 - \frac{bJ}{a} \cdot \sum_i 0, \tag{128}$$

where in the last step we used the summation theorems for fluxes and metabolite concentrations. Since $\lambda = J^* \, (a\varepsilon_{\text{tot}})^{-1}$, the optimal enzyme levels read

$$\varepsilon_l^* = \varepsilon^*_{\text{tot}} \, C_l^{J*} - \frac{b}{a} \, J^* \, \sum_i C_l^{s_i *}. \tag{129}$$

D.7 Sufficient condition for stable states in unbranched pathways

Here we show, for an unbranched metabolic pathway, that a steady state is stable if (but not only if) the first reaction 1 is reversible (i.e. has a non-zero product elasticity), if the last reaction is not completely saturated (i.e. has a non-zero substrate elasticity), and if in all other reactions the substrate elasticity is larger than the

(absolute) product elasticity. For a reversible mass-action rate law, the first two conditions are satisfied and the latter condition is satisfied if $k_{+i} > k_{-(i-1)}$.

Proof A state is asymptotically stable if all the Jacobian eigenvalues are negative (i.e. they have negative real parts). A sufficient (but not necessary) condition for stable metabolic states can be obtained from the Gershgorin's disc theorem, which we recall here:

Theorem D.3. *Let* $\mathbf{A}$ *be a square matrix. Each diagonal element* a_{ii} *is associated with a closed disc* $s_i = D_i(a_{ii}, \sum_j |a_{ij}|))$ *in the complex plane, with center* a_{ii} *and radius* $\sum_j |a_{ij}|$. *The theorem states that all eigenvalues of* $\mathbf{A}$ *must lie in the union of all these discs. Since the theorem applies both two* $\mathbf{A}$ *and to its transpose, each diagonal element gives rise to two discs with the same center, but different radii* $\sum_j |a_{ij}|$ *and* $\sum_j |a_{ji}|$.

For a real-valued matrix, this means: our system is stable if for all i, $a_{ii} + \sum_j |a_{ij}| < 0$ or if for all i, $a_{ii} + \sum_j |a_{ji}| < 0$.

As an example, we now consider an unbranched metabolic chain with 4 reactions and 3 internal metabolites A, B, C:

$$S \xrightarrow{1} A \xrightarrow{2} B \xrightarrow{3} C \xrightarrow{4} P \tag{130}$$

The system has no conservation relations. From the stoichiometric matrix

$$\mathbf{N} = \begin{pmatrix} 1 & -1 & 0 & 0 \\ 0 & 1 & -1 & 0 \\ 0 & 0 & 1 & -1 \end{pmatrix} \tag{131}$$

and the metabolite elasticity matrix

$$\mathbf{E}_\mathrm{c} = \begin{pmatrix} -g_A^1 & 0 & 0 \\ g_A^2 & -g_B^2 & 0 \\ 0 & g_B^3 & -g_C^3 \\ 0 & 0 & g_C^4 \end{pmatrix}, \tag{132}$$

where all g are positive, we obtain the Jacobian

$$\mathbf{A} = \mathbf{N}\ \mathbf{E}_\mathrm{c} \begin{pmatrix} -(g_A^1 + g_A^2) & g_B^2 & 0 \\ g_A^2 & -(g_B^2 + g_B^3) & g_C^3 \\ 0 & g_B^3 & -(g_C^3 + g_C^4) \end{pmatrix}. \tag{133}$$

From the condition for matrix columns[6], we obtain the sufficient stability conditions

$$\begin{aligned} -(g_A^1 + g_A^2) + g_A^2 &< 0 \\ -(g_B^2 + g_B^3) + (g_B^2 + g_C^3) &< 0 \\ -(g_C^3 + g_C^4) + g_C^3 &< 0. \end{aligned} \tag{135}$$

To guarantee a stable steady state, all three inequalities must be satisfied. The latter conditions are satisfied whenever $g_A^1 > 0$ (reaction 1 is not completely irreversible), $g_C^4 > 0$ (reaction 4 is not completely saturated), and $g_B^3 > g_C^3$ (the substrate elasticity of the reaction 3 is larger than its (absolute) product elasticity). For longer metabolic chains, we obtain the same type of conditions: the steady state will be stable if (but not only if) the first reaction is not completely irreversible, the last reaction is not completely saturated, and in every reaction in between, the substrate elasticity is larger than the (absolute) product elasticity.

[6] From the condition for rows, we would obtain another (alternative) set of sufficient conditions

$$\begin{aligned} -(g_A^1 + g_A^2) + g_B^2 &< 0 \\ -(g_B^2 + g_B^3) + (g_A^2 + g_C^3) &< 0 \\ -(g_C^3 + g_C^4) + g_B^3 &< 0. \end{aligned} \tag{134}$$

These conditions are satisfied, for example, if all elasticities are non-zero, if the product elasticity of the first reaction is equal or bigger than the product elasticity of the second reaction, if the substrate elasticity of the last reaction is equal or bigger that the substrate elasticity of the second but last reaction, and if $g_A^2 - g_B^2 < g_B^3 - g_C^3$. For a reversible mass-action kinetics $v = e[k_+ s - k_- p]$ the latter condition would mean: $\varepsilon_2[k_+^2 - k_-^2] < \varepsilon_3[k_+^3 - k_-^3]$ or $\frac{\varepsilon_3}{\varepsilon_2} > \frac{k_+^2 - k_-^2}{k_+^3 - k_-^3} = \frac{v_2^\mathrm{std}}{v_3^\mathrm{std}}$ where the "standard velocity" v_l^std denotes the rate that reaction l would show at unit metabolite and enzyme levels.