Flux cost functions and the choice of metabolic fluxes

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Abstract

Metabolic fluxes in cells are governed by physical, biochemical, physiological, and economic principles. Cells may show "economical" behaviour, trading metabolic performance against the costly side-effects of high enzyme or metabolite concentrations. Some constraint-based flux prediction methods score fluxes by heuristic flux costs as proxies of enzyme investments. However, linear cost functions ignore enzyme kinetics and the tight coupling between fluxes, metabolite levels and enzyme levels. To derive more realistic cost functions, I define an apparent "enzymatic flux cost" as the minimal enzyme cost at which the fluxes can be realised in a given kinetic model, and a "kinetic flux cost", which includes metabolite cost. I discuss the mathematical properties of such flux cost functions, their usage for flux prediction, and their importance for cells' metabolic strategies. The enzymatic flux cost scales linearly with the fluxes and is a concave function on the flux polytope. The costs of two flows are usually not additive, due to an additional "compromise cost". Between flux polytopes, where fluxes change their directions, the enzymatic cost shows a jump. With strictly concave flux cost functions, cells can reduce their enzymatic cost by running different fluxes in different cell compartments or at different moments in time. The enzymatic flux cost can be translated into an approximated cell growth rate, a convex function on the flux polytope. Growth-maximising metabolic states can be predicted by Flux Cost Minimisation (FCM), a variant of FBA based on general flux cost functions. The solutions are flux distributions in corners of the flux polytope, i.e. typically elementary flux modes. Enzymatic flux costs can be linearly or nonlinearly approximated, providing model parameters for linear FBA based on kinetic parameters and extracellular concentrations, and justified by a kinetic model.

Keywords: Flux balance analysis, enzyme cost, metabolite cost, concave function, elementary flux mode.

Abbreviations: CM: common modular rate law

1 Introduction

The metabolic state of cells, defined by enzyme activities, metabolite levels, and metabolic fluxes, is constantly adapted to the cells’ external conditions and internal demands. Since the main task of metabolism is substance conversion, metabolic fluxes can be seen as target variables to be optimised. What metabolic pathways should a cell use in a given situation? How should fluxes be adapted to external conditions and perturbations such as gene knock-outs (e.g. when should a pathway be switched on or off, by changing the enzyme levels)? And how (through what enzyme and metabolite profiles) should these fluxes be realised? To answer such questions, we first need to see what fluxes are physically possible. In flux balance analysis (FBA), a set of possible stationary flux distributions (or "metabolic flows", as I call them here) is defined by flux bounds, the assumption of stationary fluxes (i.e. mass-balanced internal metabolites), and thermodynamic laws. Geometrically, each flow can be presented as a point in flux space. The pattern of active reactions and flux directions in a network is called a flow pattern, and for each such flow pattern, the flows with this pattern form a convex polytope in flux space.
Figure 1: Enzymatic flux cost and the choice of a metabolic flow. The enzymatic flux cost of a given flow is defined as the minimum enzyme cost at which this flow can be realised in a given kinetic model. (a) Flows in central metabolism. Two basic flows (fermentation and respiration) and a convex combination (respiro-fermentation) are shown. We use ATP production (circles) as a benefit function and normalise all flows to a fixed benefit value (i.e., ATP production rate). (b) Flows as points in flux space (projection on the plane of \( v_1 \) and \( v_2 \)). The set of feasible flows with a given flux pattern (i.e., given flux directions) is a convex polytope \( P_S \) (called signed flux polytope). At the same time, each flow pattern also defines a set of feasible metabolite profiles, which form a convex polytope in log-metabolite space (called M-polytope). Given a desired flow, each metabolite profile will require a particular enzyme profile with a particular cost. These enzymatic costs, for a given flow \( v \), form an effective metabolite cost function on the M-polytope. Minimising this function yields optimal metabolite and enzyme profiles and an optimal cost value, the enzymatic flux cost. (c) Enzymatic flux cost as a function on the flux polytope. The flows that realise the desired ATP production rate lie on a diagonal line (called B-polytope). The flow with the smallest cost on the B-polytope is assumed to be optimal. In the example, the respiration rate is limited by a flux bound (dashed line), and respiro-fermentation turns out to be the optimal strategy.

But which flows will actually be realised by cells, and how do these choices depend on enzyme kinetics and environmental conditions? Flux prediction can be based on mechanistic or on functional assumptions. In kinetic models, metabolic fluxes are explained by enzymatic rate laws and by enzyme and metabolite levels, and a metabolic flow can be computed by dynamical simulation. However, even if the rate laws were precisely, these predictions would still be uncertain because they depend on enzyme activities, which are variable and hard to predict from mechanistic models. This problem can be avoided by “economic” models: in such models, we assume that any physically feasible flow can be realised biochemically, and we ask which flows are most profitable, e.g., providing the best compromise between a benefit (e.g., biomass production rate) and a cost (e.g., enzyme burden).

Minimising cost at a fixed benefit is a common approach in microeconomics and is also used by constraint-based models (CBM) to predict metabolic fluxes. Constraint-based modelling approaches such as Flux Balance Analysis [1] or Resource Balance Analysis [2] constrain fluxes by a stationarity assumption and other, typically linear
constraints, but they do not consider enzyme kinetics. Since cost functions and flux constraints are only loosely inspired by kinetics, these methods lead to less good flux predictions [3]. Classical FBA predicts metabolic flows by assuming a linear benefit function \( b = b' \cdot v \) (e.g. biomass production rate) and maximising the benefit \( b \) on the flux polytope. Classical FBA does not suppress flux cycles, even thermodynamically infeasible ones. This leads to underdetermined, possibly unrealistic solutions. To fix this problem, one may penalise fluxes by a cost function, and there are different ways to implement this. FBA with molecular crowding [4] puts a bound on a weighted sum of all fluxes, assuming that each flow entails some total enzyme concentration, and that this concentration needs to be bounded to account for the limited space in a cell or on membranes. A linear relationship between fluxes and enzyme levels is used as an approximation. Minimal-flux FBA [5] works similarly: here, one predefines a flux benefit \( \sum b'_{vl} v_l \) and minimises a sum \( \sum |v_l| \) or a weighted sum \( \sum a'_{vl} |v_l| \) of fluxes with cost weights \( a'_{vl} \) (where flux signs are ignored). This procedure favours sparse flows; many reaction fluxes will vanish, and specific pathways will be selected.

To justify these methods, one may claim that a bigger flux requires a proportional increase of enzyme levels. However, in reality, stationary fluxes are not proportional to enzyme levels. A proportionality would only hold if the metabolite levels were fixed – which is usually not the case when enzyme levels are varied. This is why linear cost functions are not very realistic, and this is also the problem I address in this paper: in reality, different flux modes in cells require different metabolite levels, and this results in nonlinear relationships between fluxes and enzyme demand. To compute the enzyme cost of a metabolic flow, I combine kinetic and constraint-based models: I use a kinetic model and assume that fluxes must be kinetically realised (i.e. by metabolite and enzyme levels) in such a way that their overall cost is minimised. The calculations are based on a principle of minimal protein cost [6, 7] as implemented in enzyme cost minimisation (ECM) [8]. ECM determines flux-specific enzyme investments by a convex (and therefore computationally tractable) optimality problem. An optimisation over all possible metabolite profiles (see Box 1) yields nonlinear flux cost functions, which represent realistic enzyme and metabolite costs and can be used for constraint-based modelling. Below, I show under what conditions such functions are strictly convex; Using such flux cost functions, I propose flux cost minimisation (FCM), a generalised version of minimal-flux FBA that accounts for quantitative enzyme and metabolite costs in cells, and that leads to concave optimality problems [9, 10].

Flux cost minimisation resembles some other optimality problems that have been studied before (e.g. maximising a linear flux benefit at a limited sum of enzyme levels) [9, 11]. For these problems, it has been shown that the enzyme cost per flux is a concave function in flux space, and it has been claimed that optimal flows must be elementary flux modes (EFMs), no matter what underlying kinetic model is assumed. Here I extend these results: I show that the enzyme demand per flux benefit is not only concave, but often strictly concave, and I clarify the mathematical conditions for this. As a consequence, optimal flows must be vertices of the flux polytope (while for non-strict convexity, which always holds, there can be additional optimal flows that are non-vertex points). Optimal flows need not be EFMs, but can also be other vertices caused by flux bounds. Below, I illustrate this with the simple example of a branch point model that describes, in a simplified way, the choice between fermentation and respiration. Of course, all methods also apply to complex networks. The relation to cell growth rate, “bacterial growth laws” [12], and cell growth maximisation in whole-cell models is also discussed.

2 Enzymatic flux cost functions

2.1 Flux cost defined by optimised enzyme and metabolite cost

To realise a metabolic flow \( v \), a cell must provide suitable enzyme and metabolite levels. Typically, higher levels are costly: increased enzyme levels, for example, have been shown to impair cell growth [13, 14], and such growth defects, as “costs” will also arise when fluxes require such higher enzyme levels. In flux analysis, enzyme costs
Box 1: Screening all states of a kinetic metabolic model and finding optimal metabolic flows

**Set of all metabolic flows** In a kinetic model, each metabolic steady state is characterised by a choice of enzyme levels and the resulting stationary metabolite levels and fluxes. The set of all such states (enzyme profile $e$, metabolite profile $c$, flow $v$) can be parametrised by choosing a flow pattern, a flow and a metabolite profile that agree with the flow pattern, and the required enzyme levels.

**Screening the metabolic states** The set of metabolic states can be screened systematically (see graphics). First, we screen all feasible flow patterns and construct the corresponding $S$-polytopes and $M$-polytopes. Flow patterns can be excluded for thermodynamic reasons or because they corresponding orthants in flux space are not intersected by the hyperplane of stationary flows. Then, for each flow pattern, we screen each of the polytopes, and consider all possible combinations of flux profiles $v$ and metabolite profiles $c$. For each combination, we can easily compute the required enzyme profile $e$. This screening yields all possible steady states $(v, c, e)$. To select only stable steady states, the metabolic states need to be checked by inspecting their Jacobian matrices. The screening procedure allows us to parametrise all metabolic states of a kinetic model, and it also shows that the feasible $S$-polytopes, as a set, represent all feasible steady states of the model, projected to flux space.

**Computing the enzymatic cost of a flow** We have seen that the metabolic states of a kinetic model can be systematically enumerated. To find optimal states that realise a maximal flux benefit per enzyme investment, we consider a given flow pattern with predefined flux benefit, and minimise the enzyme cost by screening all possible states. We can do so in two ways. We may either screen the flows (restricted to a fixed benefit), optimise the metabolite profile (over the $M$-polytope – which is an ECM problem), and pick the flow with the lowest metabolite-optimised cost; or we screen the metabolite profiles, optimise for each of them the flow (restricted to a fixed benefit – which is a linear flux cost minimisation problem), and then pick the metabolite profile with the lowest flux-optimised cost.

**Flux cost minimisation step by step** The graphics on the left shows how metabolic states are screened to compute an enzymatic flux cost. The aim is to realise a given flow $v$ at a minimal enzyme cost. Flow pattern, equilibrium constants, concentration ranges, and given external metabolite levels together define an $M$-polytope. Given the flow $v$, shown as a point in the flux polytope, each metabolite profile $m$ of the $M$-polytope also defines an enzyme profile $e$ and represents a feasible state of the kinetic model. Using the enzyme cost as a cost for $m$, we obtain an optimality problem for metabolite profiles $m$ on the $M$-polytope. The resulting minimal value is then assigned to the flow as an enzymatic flux cost.

may be modelled by flux cost functions that increase proportionally to the fluxes. However, the assumption of linear cost functions and the specific choices of cost weight are often unjustified.

To derive more realistic flux cost functions, justified by detailed biochemical models, I propose to consider the minimal enzyme and metabolite costs at which a flux can be realised in a given kinetic model (Figure 1). Both kinds of cost have been considered before (enzyme costs in [15, 6, 16], additional metabolite costs in [17, 18]). To represent them as flux costs, we need to be able to quantify metabolite and enzyme costs of a given metabolic flow. To do so, we assume simple fitness terms for enzyme levels, metabolite levels, and fluxes: enzyme costs (see Box 2) are proportional to enzyme levels (i.e. penalising high enzyme levels), metabolite cost is a convex function of the logarithmic metabolite levels (e.g. penalising deviations from some ideal metabolite profile), and the flux benefit is a linear function of the fluxes. We then assume that cells adopt an optimal strategy by maximising
Box 2: Cost functions for enzymes, metabolites, and fluxes

(a) Cost and benefit terms in metabolism

(b) Effective cost functions

The enzyme cost \( h(e) \), originally a function of the enzyme levels, can be cast as an apparent cost function for metabolite levels (assuming predefined fluxes) or fluxes (assuming that each flow is realised by its privileged metabolite and enzyme profiles). We obtain three types of cost functions with different arguments, but all of them describing enzyme cost (see graphics above:)

1. **Enzyme cost**
   The enzyme cost \( h(e) = h(e_1, e_2, \ldots) \) scores the enzyme levels directly. We assume a linear enzyme cost function, implying that each enzyme molecule has a fixed cost (which may nevertheless differ between different enzymes). What enzyme cost means, and in what units it is measured, may vary from model to model. It may, for example, refer to enzyme amounts (e.g. enzyme mass per cell dry weight), e.g. when predicting the enzyme demand of engineered pathways, or to the resulting growth defects in units of 1/h (for absolute growth rate changes) or unitless (for relative growth rate changes).

2. **Enzymatic metabolite cost** (or “flux-constrained enzymatic M-cost”) The set of thermodynamically feasible metabolite profiles is a convex polytope in “log-metabolite space” (i.e. in the space of logarithmic metabolite concentrations). This polytope is called M-polytope \( \mathcal{P}_m \) (or “metabolite polytope”). Its shape depends on details of the metabolic model and on the flux directions, equilibrium constants, and external metabolite concentrations (see [8]). Metabolite costs can represent various biological cost functions defined on the M-polytope. The enzymatic metabolite cost \( q^{\text{enz}}(m; v) = h(e(v, m)) \) is an overhead cost, describing the enzyme cost \( h(e) \) needed to realise a flow \( v \) at the logarithmic metabolite levels \( m = \ln c \). It refers to a given kinetic model with given external metabolite levels and with allowed ranges for the internal metabolite levels. Given a flow \( v \), the enzyme levels \( e(v, m) \) follow directly from the rate laws in the kinetic model and determine the enzymatic cost. Written as a function of logarithmic metabolite levels, the enzyme cost can serve as an indirect metabolite cost. Since it is convex [8], the optimal metabolite profile and the ensuing enzyme profiles and enzyme cost can be computed by convex optimisation (see Figure 1). By adding a direct metabolite cost \( q(m) \), we obtain the kinetic metabolite cost \( q^{\text{tot}}(m) = q(m) + q^{\text{enz}}(m) \). If the direct metabolite cost \( q(m) \) a strictly convex function, the kinetic metabolite cost will be strictly convex too, which guarantees a unique optimum for enzyme and metabolite levels.

3. **Enzymatic flux cost** (or “metabolite-optimised enzymatic F-cost”) Flux cost functions \( a(v) \) can be defined ad hoc, like the linear cost functions used in FBA, or based on kinetic models. Flux cost functions can be defined . To be biologically plausible, flux cost functions should increase with the absolute flux, i.e., \( \text{sign}(\partial a/\partial v_l) = \text{sign}(v_l) \) whenever \( v_l \neq 0 \). They may show a jump where a flux switches its sign, i.e. where \( v_l = 0 \) for some reaction \( l \). The enzymatic flux cost

\[
a^{\text{enz}}(v) = \min_m q^{\text{enz}}(m; v) = \min_m h(e(v, m))
\]

is the minimal enzyme cost at which the flow \( v \) can be realised in a given kinetic model (compare Figure 1). If we also consider a direct metabolite cost, we obtain the kinetic flux cost \( a^{\text{kin}}(v) = \min_m q^{\text{tot}}(m; v) \), i.e. the minimal sum of enzyme and metabolite costs at which \( v \) can be realised in our model.

the benefit-cost difference, the benefit/cost ratio, or the benefit at a fixed cost, or by minimising cost at a fixed benefit. By writing enzyme and metabolite cost as functions of the fluxes, we can reformulate such optimality problems in kinetic models as simple optimality problems for fluxes, leaving enzyme and metabolite levels out of the picture, but accounting for their effects.

How does this work in practice? A flux mode can be realised by various metabolic states (i.e. states with different enzyme and metabolite profiles). To score the flux mode, we pick the state with the lowest enzyme cost [16, 8] or with the smallest sum of enzyme and metabolite costs (“kinetic cost”). Their cost is then assigned to the
some useful general properties. The enzymatic flux cost requires

$$\text{flux mode itself as an “apparent flux cost”. Notably, each flux mode defines an optimal metabolite profile, but the profile is not required, but privileged by the flux profile, i.e. it is best profile among many (physically and biochemically) possible choices. The resulting apparent flux cost function can be seen as an overhead cost because it scores the indirect side-effects (or rather “side requirements”) of the fluxes. Here I will study two cases of overhead costs: the enzymatic metabolite cost, representing enzyme cost in metabolite space; and the kinetic flux cost, representing enzyme and metabolite cost in flux space.}

Let us see an example. Figure 2 shows the enzymatic flux costs in a simplified picture of central metabolism, described as a simple branch point (compare Figure 1 (a)). In the model, we assume reversible mass-action kinetics and fixed flux directions. At given fluxes $v_1$, the enzyme cost depends, effectively, on the logarithmic metabolite concentration $s = \ln c$. We obtain the formula $q(s) = \frac{a_1}{s - c_1} v_0 + \frac{a_2}{s - c_2} v_1 + \frac{a_3}{s - c_3} v_2$. The fluxes through the branch point can show different flux ratios$^4$, obtained from linear combinations of two basic flows. In one of the two, the entire flux goes to branch 1; in the other one, the entire flux goes to branch 2. All other flows can be obtained as convex combinations (i.e. by linearly interpolating between the two basic flows). Figure 2 (b) and (c) show the enzyme cost in metabolite and flux space. In metabolite space, the optimum value for a mixed flow is always between the curves for the two basic flows ($b$ blue and red). The minimal cost is achieved by one of the basic flows (in the case shown, $v_B$, blue dot). There cannot be an indifferent optimum (all black dots at the same height) unless the red and blue curves are completely horizontal and identical.

2.2 Shape of the enzymatic cost flux function

Now we know how to define enzymatic flux cost functions and how to compute them numerically (see Figure 2). But what are their mathematical properties? Even without an analytical formula to compute them, we can derive some useful general properties. The enzymatic flux cost $\alpha(v)$ is a nonlinear function on the set of possible flows.

$^4$To compare the two flows, we assume a required flux benefit of 20 ATP molecules per time unit, and we assume that reaction $v_0$ yields 2 ATP molecules (per unit of flux) and $v_2$ yields 18 ATP molecules (per unit of flux); thus, we interpolate between the modes $v_A = (10, 10, 0)$ and $v_B = (1, 0, 1)$. We then consider a range of possible concentrations $c$ in the branch point. For the three reactions, we consider simple mass action rate laws $r_0 = 10 - s; r_1 = s - 0.01; r_2 = s - 0.01$ and cost weights $h_0 = 1, h_1 = 1, and h_2 = 28$. 

Figure 2: Enzymatic flux cost in a simple branch point model. (a) Model structure. The model can be seen as a simplified model of central metabolism (see Figure 1), with reactions representing glycolysis ($v_0$), overflow ($v_1$), and respiration ($v_2$). The concentration of the branch point metabolite is called $c$, the three external concentrations are fixed. We consider a kinetic model with substrate-saturated, reversible rate laws. A linear benefit function $b(v_0, v_1, v_2)$ scores ATP production. The two basic flows $v_A$ (red) and $v_B$ (blue) are shown on the right. (b) Enzymatic metabolite cost. Cost functions for basic flows $v_A$ and $v_B$ (scaled to equal ATP production rates) are shown in red and blue. Cost functions for mixed flows $\eta v_A + (1 - \eta) v_B$, interpolating between the basic flows $v_A$ and $v_B$, are shown by black lines. For each flow, the optimum point is shown by a dot. (c) Cost in flux space. Costs (value from y-axis in (b)) are plotted against the interpolation parameter $\eta$, which varies between 0 (for flow $v_A$) to 1 (for flow $v_B$). The resulting enzymatic flux cost function is strictly concave (negatively curved) between the two basic flows.
This is not the entire flux space, but only on the set of feasible flows allowed by our model, which can further be restricted to stationary flows. This is the set of flows considered in FBA with thermodynamic constraints. The cost function is determined by rate laws, parameters, external conditions, and constraints of the kinetic model considered. A description of such models, model assumptions, and relevant mathematical notions is given in the SI. Here I focus on models with separable rate laws [20], which cover all typical reversible rate laws and ensure thermodynamic correctness – i.e. the reaction rates obtained from a given metabolite profile will always have the right signs, as required by thermodynamics. In the following sections, we sometimes consider the common modular (CM) rate law, whose graph \( v(c) \) is curved in all directions in log-metabolite space. I further assume that enzyme cost depends linearly on enzyme levels.

The set of possible flows (see Box 1) is a collection of flux polytopes, each located in a segment of flux space and realising to a particular flow pattern. Stationarity and thermodynamic constraints limit our flows to some segments in flux space, which we call “feasible”. A feasible flow pattern corresponds to a segment that contains a non-empty, convex S-polytope (signed flux polytopes, or \( \mathcal{P}_E \)). If all thermodynamically feasible flow patterns are allowed, we obtain a collection of S-polytopes, each representing a possible pattern of flux signs (e.g. determined by given chemical potentials of external compounds). Aside from fixed flux directions, we consider two more constraints: first, like in FBA, we assume stationary fluxes, which defines a subspace in flux space. Second, to avoid unrealistically large fluxes, we put bounds on individual reaction fluxes (as in classical FBA) or on linear combinations of fluxes (as in FBA with molecular crowding). The resulting feasible flows form a convex polytope in the positive segment of flux space, called the positive polytope (or P-polytope) \( \mathcal{P}_P \). To avoid complications caused by flux reversals, we will first assume that the flux directions are fixed and, whithout loss of generality, non-negative (\( v \geq 0 \)); that is, we consider the flux cost function on a single P-polytope.

To explore the possible shapes of a flux cost function, we consider two simple ways in which flows can be varied within an S-polytope (see Figure 3): we can rescale a flow, or we interpolate between flows of different shapes, but equal benefit. In the first case, we move between B-polytopes with different benefit values; in the second case, we move inside a B-polytope. In each case, the flux cost will vary. If we scale a flow, its cost will scale linearly (and the flux-specific or benefit-specific cost stays the same). If we interpolate between two flows, the flux cost is concave on the interpolation line, i.e. equal to the linearly interpolated flux costs, or even higher. In fact, many flux cost functions are strictly concave on the B-polytope, i.e. the cost of an interpolated flow is higher than the interpolated cost. Such flux cost functions are superadditive: i.e. the cost \( a(v_A + v_B) \) of a superposed flow exceeds the sum of costs \( a(v_A) + a(v_B) \). Let us consider these two basic properties, linear scaling and concavity, in more detail.

With linear enzyme cost functions \( h(e) \), the resulting flux cost \( a_{\text{enz}}(v) \) scales linearly with the flow. This is easy to see. A given flow \( v \) defines an enzymatic cost function \( q_{\text{enz}}(m) \) on the metabolite polytope. If we scale our flow \( v \) by a factor \( \eta \), the function \( q_{\text{enz}}(c) \) will retain its shape, but will be scaled by the same factor \( \eta \). The minimum point of this cost function, i.e. the privileged metabolite profile, remains unchanged. With a constant privileged metabolite profile, the privileged enzyme profile will scale proportionally to the fluxes, and given the linear enzyme cost function \( h(e) \), also the enzyme cost will scale proportionally. What about the kinetic flux cost \( a_{\text{kin}}(v) \) under flux scaling? Also the kinetic metabolite cost \( q_{\text{kin}}(m) = q(m) + q_{\text{enz}}(m) \) on the metabolite polytope remains unchanged. Therefore, a flux scaling will only change the enzymatic flux cost proportionally, but not the metabolic cost. The kinetic flux cost \( a_{\text{kin}}(v) \), which contains both terms, does not scale proportionally with the flux, but with an additional offset term: \( a_{\text{kin}}(\eta v) = \eta (a_{\text{kin}}(v) - q_{\text{opt}}) \). Here, \( q_{\text{opt}} \) is the direct metabolite cost of the privileged metabolite profile, obtained by minimising the sum of enzyme and metabolite cost.
costs in metabolite space and independent of flux scaling.

The fact that optimal enzyme levels and enzymatic cost scale proportionally with the fluxes, while the optimal concentrations remain unchanged, leads to general sum rules for gradients (or “point derivatives”). Since enzymatic flux cost scales linearly with the flow, it is a homogeneous function with degree 1: if the entire flow is scaled by a factor $\eta$, the flux cost will scale by $\eta$ as well (with exponent of 1, hence degree 1). By applying Euler’s theorem on homogeneous functions, we obtain a sum rule

$$\sum_l \frac{\partial a}{\partial \ln v_l} = \sum_l \frac{\partial a}{\partial v_l} v_l = a$$

(2)

for the point cost $a_{v_l} = \frac{\partial a}{\partial v_l} v_l = \frac{\partial a}{\partial \ln v_l} v_l$ of individual reactions. With the symbols $a_{v_l} = \frac{\partial a}{\partial v_l}$, and $a_v = \sum_l a_{v_l}$, we can write the sum rule in the compact form

$$a_v = \sum_l a_{v_l} = \sum_l a_{v_l} v_l = a,$$

(3)

stating that the enzymatic cost $a$ of a flow $v$ is given by the sum of point costs $a_{v_l}$, in each point $v$ of the S-polytope. The sum rule resembles the summation theorems of metabolic control theory, which can be derived in a similar way. Similar sum rules also exist for derivatives of the optimal metabolite or enzyme levels with respect to the fluxes (see SI section B.4). Using Eq. (2), we can show that the flux point cost $y_l = \frac{\partial h}{\partial v_l} v_l$ is given by $\frac{\partial h}{\partial v_l} v_l$ (proof in SI section E.6). For any flow $v$, the total enzymatic flux cost is the sum of enzyme point costs of all reactions, and their sum is given by the enzyme cost $h$ (assuming that $h(e)$ is linear). This may seem trivial, because the total enzyme cost was already defined as a sum of enzyme costs across all reactions. However, there is an important distinction: the terms in the sum rule do not refer to a fixed metabolite profile, but account for the optimal adjustment of metabolite levels as part of taking the derivative! The scaled flux cost $a'$ (flux cost per unit flux, or per flux benefit) is homogeneous with degree 0, and we obtain the sum rule

$$\sum_l a'_{v_l} = \sum_l a'_{v_l} v_l = 0,$$

(4)

which holds in each point $v$ of the B-polytope. Flux cost functions may be hard to compute: we need to solve an
optimality problem that depends on all details of the kinetic model and on the enzyme cost weights. However, using the sum rules (2) and (4), we can prove some general mathematical properties. A similar sum rule for combinations of flows is discussed in SI section B.4.

We saw that enzymatic flux cost functions scale linearly with the flow. What happens if we interpolate between flows of different shapes, e.g. if we gradually vary the flux ratio in the branch point in Figure 2? Now, the enzymatic flux cost may vary nonlinearly. Enzymatic flux cost functions are concave, i.e. the cost of an interpolated flow will never be lower than the corresponding interpolated cost. This is known from [9], where it has been shown by a graphical construction, and it can be easily shown if the enzymatic metabolite cost function $\tilde{q}^{\text{enz}}(m, v)$ is continuous, positive, and additive between flows (proof in section B.2). For a large class of models, the cost function is strictly concave, i.e. the cost will even be higher than the interpolated cost. Whether such a "compromise cost" arises depends on the rate laws used in the model, which determine the shape of the metabolite cost function.

To see an example of a strictly concave flux cost function, let us revisit the branch point model in Figure 2. Why is the cost profile nonlinear? The reason is that the two basic flows privilege different concentrations of the central metabolite $c$. If the flows privileged the same concentration, we could interpolate between them by simply interpolating the enzyme profiles, resulting in linear cost changes. In reality, the two flows privilege different metabolite levels, and when they are combined, they will operate with one metabolite level that is sub-optimal for each of them. This makes the flows less efficient and therefore more costly. The cost of a mixed flow is not just the combined cost of the basic flows, but is higher. Since this argument holds for any two flows on the line (taken to be "basic" flows), the cost function must be strictly concave.

To study whether flux cost functions are strictly concave, we can use the notion of kinetically distinct flows. Two flows are kinetically distinct if they privilege different metabolite profiles – or, more precisely, if no metabolite profile is optimal for both of them. Proposition 3 (in SI section B.3) states: if two flows are kinetically distinct, the enzymatic flux cost is strictly concave on the line in between (proof in SI section E.4). In contrast, if two flows are not kinetically distinct, their flux costs are additive and the flux cost function between them varies linearly.

How can we tell whether two flows are kinetically distinct? For sure, they must have different shapes, because otherwise they would privilege the same optimal metabolite profile. However, flows of different shapes need not be kinetically distinct in all cases. For example, if two flows differ only in their use of isoenzymes, and if the isoenzymes have identical costs and rate laws, these flows will still privilege the same metabolite profile. Of course, this is an artificial example, but since we can construct such examples, our mathematical criteria need to be able to rule them out. In fact, with realistic rate laws, differently shaped flows will usually be kinetically distinct. This holds, e.g. whenever a model’s enzymatic metabolite cost function is strictly convex (Proposition 2 in the SI), which includes all models with common modular (CM) rate laws [19] or “convenience kinetics” (see SI section E.5, proof by Joost Hulshof).

2.3 Flux cost, enzyme levels, and metabolite levels at points of flux reversal

Until this point, our aim was to optimise the fluxes at given flux directions. Given a flow pattern, a flux benefit function $b(v)$, and a benefit value $b'$, we obtained a convex B-polytope and studied the flux cost function on this polytope. How could we optimise the flux directions themselves? By computing the cost function on all feasible B-polytopes, we obtain a comprehensive picture of flux costs in the entire flux space. We could consider

\[7\text{Here are some criteria for two flows } v_A \text{ and } v_B \text{ to be kinetically distinct. (i) The flows must not be scaled versions of each other. (ii) The flows must not have the same optimal metabolite profile; (iii) If } m_A \text{ and } m_B \text{ are their – possibly non-unique – optimal metabolite profiles, any variation of these metabolite profiles, at fixed enzyme levels, must change some of the fluxes. (iv) Any variation of } m_A \text{ and } m_B \text{ at given fluxes will change the necessary enzyme levels.}\]
all feasible flow patterns, run FCM for each of them, and pick the best flow among all solutions. This then defines the best flow pattern.

The enzymatic flux cost is concave on each B-polytope. However, what happens where polytopes touch each other, i.e. where fluxes change their directions? Within a polytope boundary, the flux cost remains well-defined. However, as we move between polytopes, the flux cost function may not be continuous, let alone differentiable or concave\(^8\) (for an example, see Figure 4). An explanation for the jump in enzyme and metabolite levels is given in SI Figure 8.

For each given flux mode, the enzymatic flux cost scales proportionally with the flow. Therefore, when approaching the point \(v = 0\) (no matter from which direction), the flux cost linearly approaches 0. Since the gradients in the different directions are different, the flux cost is not differentiable in this point. The kinetic flux cost function (which includes direct metabolite costs) is not even continuous in this point; it even converges to different values when approaching \(v = 0\) from different directions, so this function. All this shows, again, that that quadratic flux cost functions are not a good approximation of flux cost functions, and that linear flux cost functions, different for each S-polytope, are a more realistic choice.

Let me summarise what we learned so far. Two flows are kinetically distinct in a given kinetic model if their privileged metabolite profiles differ. The privileged metabolite profiles of two flows will be necessarily different if (i) the flows have different privileged metabolite profiles and if the underlying (enzymatic or kinetic) metabolite cost function (on the metabolite polytope) is strictly convex. The latter holds, for example, in models with common

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\(^8\)The enzymatic flux cost cannot be concave on the entire flux space. This is easy to see. We know that starting from the origin (at \(v = 0\)), the cost increases in all (feasible) directions. This wouldn’t be possible if the flux cost function were concave on the entire set of fluxes.
modular rate laws, and in models in which any variation \( \delta m \) of the metabolite profile has a kinetic effect on at least one of the reaction rates. In a model in which all flows on the B-polytope are kinetically distinct, there is a positive compromise cost and the flux cost function is strictly convex on the B-polytope. All this is shown in the SI. If flux reversals are allowed, we need to consider the flux cost function on all feasible S-polytopes. At the polytope boundaries, where flux reversals occur, the metabolite profile may jump from one M-polytope to another one, leading to jumps in enzymes profile and enzymatic flux cost.

### 3 Flux cost minimisation

Producing biomass (or any pathway product) at a low enzyme cost is an advantage, because it leaves resources (e.g. protein precursors or cell space) to growth-relevant proteins such as ribosomes. Then the cell can grow faster or perform extra tasks at a given growth rate. Thus, we assume that cells will choose fluxes that minimise enzyme cost at a fixed metabolic benefit. We can compute such fluxes by a nonlinear version of FBA, called Flux Cost Minimisation (FCM) (or Enzymatic Flux Cost Minimization (EFCM), in the case of enzymatic flux cost functions).

To predict such enzyme-efficient flows, FCM minimises the flux cost \( a(v) \) at a given flux benefit \( b(v) = b' \). If the flux directions are given, we can focus on a B-polytope and minimise the flux cost \( a^{\text{sf}}(v) \) on that polytope. If the flux directions are unknown, we may screen all feasible flux sign patterns, compute the best flows, and pick the best solution. Flux cost minimisation resembles FBA with flux minimisation, except for the nonlinear flux cost function. Here, we consider the enzymatic and kinetic cost functions derived from kinetic models. For more general flux cost functions, we require that flux costs are concave and that larger fluxes incur higher costs (i.e. \( a_{v_l} = a_{v_l} v_l > 0 \) whenever \( v_l \neq 0 \)).

#### 3.1 Cost-optimal metabolic flows

In FCM, we fix a benefit value (e.g. a biomass production rate) and assume that the cell’s task is to realise this flux benefit at a minimal flux cost \( a(v) \). Mathematically, we minimise \( a(v) \) on the B-polytope. How can we find the optimal flows? Within the polytope, flows cannot be linearly scaled (because this would change the benefit value, which must be fixed in the B-polytope); thus, as long as all flows on the polytope are kinetically distinct, the cost function is strictly concave and will be minimal in one of the polytope vertices (proof in section B.5).

In many cases, the vertices are elementary flux modes (EFMs). EFMs were originally defined as purely theoretical concept [21, 22] to describe minimal ways in which metabolic networks can operate. However, studies of optimality problems such as flux maximisation at a limited total enzyme level and maximisation of enzyme-specific flux suggested that enzyme-optimal flows must be elementary flux modes\(^9\) [9, 11]. Therefore, EFMs are not only a mathematical tool to characterise possible flows, but they have a biological significance by themselves. They provide a higher enzyme-efficiency, and therefore a higher biological fitness, than mixed, non-elementary flows. However, this result holds only for models in which all vertices of the flux polytope are elementary modes! Here we found that being a polytope vertex, not being an elementary mode, is a condition for enzyme optimality. In many models, the two properties coincide: as long as flows are constrained by stationarity and flux signs, and scaled to a single fixed benefit function, the resulting B-polytope will be spanned by elementary flows. In this case, all B-polytope vertices will be EFMs and our prediction matches the previous predictions. Otherwise, if there are additional active flux constraints, all polytope vertices will be EFVs, but not necessarily EFMs, and the shape of the B-polytope (and the set of corners) will explicitly depend on the prescribed flux benefit value \( b' \).

\(^9\)In the proofs, it is argued that the optimal metabolic state, if it exists, defines an optimal metabolite profile. Then, it is shown that given this metabolite profile, the cost-optimal flux profile must be elementary.
Figure 5: Vertices of the B-polytope. (a) Simple example model (metabolic branch point (top) with four elementary flux modes (EFMs; bottom); all fluxes are constrained to be positive. (b) Feasible flows in flux space (three dimensions are shown, referring to reactions 1, 2, and 3). The cone of feasible flows is spanned by the four EFMs. A given benefit value (in the example, $v_2 + v_3 = \text{const}$.) defines a plane that intersects the cone and yields a B-polytope (red rectangle), i.e. the set of feasible flows with the given benefit value. The vertices of the B-polytope are EFMs. (c) If we put an upper bound on the flux $v_2$, all flows with higher $v_2$ values are discarded; a part of the B-polytope (red rectangle) is cut off and two new, non-elementary vertices emerge (blue circles).

If additional flux constraints are used (aside from normalising the flow to a given benefit value), then each active flux constraint will cut off some of the polytope vertices, and new, non-elementary polytope vertices will emerge (see Figure 5). The new vertices will be convex combinations of EFMs. The existence of non-elementary optimal states is supported by experimental results: in the Crabtree effect, for example, yeast cells often use respiro-fermentation (a non-elementary flux mode) instead of respiration alone (an elementary flux mode).

A flow is globally optimal if it performs equally well or better than any other flow. In contrast, a flow is locally optimal if it performs better than any other similar flows. Specifically, a B-polytope vertex is locally optimal if the flux cost gradient $\nabla_{c^{\text{opt}}}(v)$, restricted to the stationary subspace, points from the vertex towards the interior of the B-polytope: in this case, any movement from the vertex into the interior of the B-polytope will increase the cost. Local optimality can be tested by applying the following criterion (“Segaula criterion”). A vertex point $v$, with privileged metabolite profile $c^{\text{opt}}$, is locally optimal if and only if $v$ is the only privileged flow of $c^{\text{opt}}$. In other words: metabolite and flux profile must exclusively privilege each other (proposition 5 in SI, proof in SI section E.7). This criterion is easy to apply: to test a flow $v$, we run ECM to compute the privileged metabolite profile $c^{\text{opt}}$ (which must be unique); then we run linear flux cost minimisation to obtain its privileged flow and check whether this yields the original flow (which must be unique, too). It also leads to a simple iterative algorithm for computing locally optimal flows, starting from an initial “seed” flow $x$ (“Segaula algorithm”, see SI section B.6).

### 3.2 Flux cost minimisation as a generalised form of minimal-flux FBA

How can we find optimal flows in practice? Given a concave flux cost function and a pattern of flux directions, the optimal flow is a vertex of the B-polytope: to find it, we may enumerate all vertices and choose the one with the smallest cost. Instead of this exhaustive search, we may directly search for local optima, either by a greedy search over polytope vertices (e.g. a simplex or amoeba algorithm) or by the iterative Segaula algorithm. If flux directions are unspecified, we may enumerate all possible flow patterns, determine the resulting B-polytopes, and apply FCM to each of them. By optimising the flow for each feasible flow pattern, we can find the globally optimal metabolic state of a kinetic model. Altogether, we apply three layers of optimisation: among the flux patterns, among the vertices of each S-polytope, and among all metabolite profiles within the M-polytope of a given vertex (see Box 1).
Enzymatic flux costs are harder to compute and to optimise than the linear cost functions used in minimal-flux FBA. This becomes problematic if costs need to be computed many times. In flux cost minimisation, we already need to inspect all vertices of the B-polytope. In flux sampling approaches, we even need to assess flows in the entire polytope (e.g. to study cell populations in which metabolic flows are randomly distributed, but with a preference for low enzymatic cost). In both cases, we need to evaluate flux costs many times, and each function evaluation involves a nonlinear optimisation problem on the M-polytope. To decrease the numerical effort, we may approximate the enzymatic flux cost by linear or nonlinear functions that are easier to evaluate (see SI section A.4). Linear approximations of our flux cost functions can also be used to define cost weights for minimal-flux FBA. Using “capacity-based” enzymatic cost scores in metabolite space [8], we would obtain the same cost weights as previously proposed. With more realistic cost scores, we obtain nonlinear flux costs. To obtain a linear approximation, we linearise the cost function around some typical reference flow. The fact that we can derive linear cost weights from a kinetic model supports the usage of linear flux costs in FBA and justifies linear flux cost minimisation as a method. The nonlinear approximation is based on multiple prototype flows, covering different parts of the flux polytope, and yields a quadratic function of the flow.

If linear flux cost minimisation is seen as an approximation of FCM, the linear flux cost weights can be systematically derived from enzymatic or kinetic flux cost functions, and thus from kinetic models. Linear flux cost minimisation can also be seen as a version of FCM with enzymatic flux costs, but fixed, predefined metabolite levels. In fact, linear flux cost minimisation is a very smart method: being linear, it is much easier to solve than FCM; and being convex, it captures a main feature of FCM, the fact that optimal flows are polytope vertices; as model parameters are changing, its solution will jump between discrete, qualitatively different flows, just like in FCM. However, it misses (i) the exact values of the cost function on these corners, (ii) the fact that the cost functions are curved, and (iii) ways to account for metabolite-dependent cost terms.

However, the biggest problem is that the flux cost weights used in linear flux cost minimisation are predefined numbers, whose relation to biochemistry and external conditions remains unclear. It has been acknowledged that flux cost weights should reflect \( k^{\text{cat}} \) values (because lower \( k^{\text{cat}} \) values, may require higher enzyme levels for compensation) and enzyme cost weights (e.g. enzyme sizes). However, a flux cost weight may depend on various other factors. We can see this by deriving flux cost weights from the enzymatic flux cost function, which depends on other rate constants (e.g. \( K_M \) values), extracellular metabolite levels, and physiological ranges on intracellular metabolite levels. Whenever these parameters change, so do the flux cost weights. For example, consider a model of a respiring cell. If the oxygen level (as a model parameter) decreases, this will affect the shape of the M-polytope, as well as the enzymatic cost \( \eta^{\text{enz}}(v) \), and will therefore alter the enzymatic flux cost function \( a(v) \). The lower driving force will lead to a higher enzyme demand. If we linearise \( a^{\text{enz}}(v) \) to define realistic flux cost weights for FBA, the flux cost weights depend on the external oxygen level and on any other model parameters. The quantitative dependence would be very hard to guess, but using FCM, we can obtain it directly from our kinetic model. Thus, our simplified linear cost functions do not only justify linear flux cost minimisation as an approximative method, but they also clarify how quantitative flux cost weights arise from quantitative cell models.

### 3.3 Flux-specific enzyme cost and cell growth rate

In a metabolic pathway or network, the enzyme investment per flux is an important quantity: it plays a central role in relating metabolic activity to growth. Cell growth can be linked to metabolism by the following argument: Under better conditions (e.g., better carbon sources, higher substrate level), the same metabolic flux can be achieved with a lower amount of enzyme. This allows the cell to increase the flux while decreasing the metabolic enzyme fraction of the proteome, so the fraction of ribosomes can be increased. The higher fluxes and ribosome levels allow cells to grow faster. Thus, cell growth depends crucially on the "proteome efficiency" of fluxes, i.e. the metabolic flux per enzyme invested (or, equivalently, on the enzyme cost of metabolic fluxes). The proteome
efficiency will vary between different metabolic strategies (e.g. respiration vs. fermentation), will depend on the external conditions (e.g. the levels of glucose and oxygen present), and will also depend on a proper allocation of enzyme resources along metabolic pathways. To predict the growth advantages provided by different metabolic flows, we need to define the external conditions, find the optimal enzyme allocation pattern, compute the enzyme investment per (biomass production) flux, and translate it into a maximal possible growth rate (possibly accounting for necessary resource rearrangements between metabolic enzymes and ribosomes).

Above I assumed that cells minimise their enzyme cost while realising a fixed flux benefit. This is closely related to the assumption of microbes maximising their growth rate. In order to model fast cell growth as an objective, we can start from a metabolic model with biomass production and compute, for each flux mode, the maximal biomass production rate per enzyme investment. This quantity is called enzyme-specific biomass production rate $r$. In simplified whole-cell models [12], this quantity is a main determinant of cell growth. Using simple cost-growth conversion formulae, which translate a flux cost $a^{\text{flux}}$ into a cell growth rate $\lambda(a)$, we can represent cell growth rate as a function on the B-polytope. Under relatively mild assumptions, this function is convex (see SI section E.10), and if the flux cost function $a^{\text{flux}}$ is strictly concave, it is strictly convex. This result holds for any decreasing, convex cost-growth function $\lambda(a)$, e.g. the formulae published in [12, 10], and it means that growth rate maximisation can be formulated as the maximisation of a convex function on the convex flux polytope (implying vertex optima).

4 Switching between metabolic pathways

The shape of a flux cost function determines cells’ preferred metabolic flows. If cells minimise flux cost at a fixed flux benefit, as assumed in FCM, we can ask what metabolic strategies will emerge, especially if flux cost functions are strictly concave.

4.1 Parameter-dependent switches between metabolic flows

The optimal metabolic state in a model depends on details such as external concentrations, kinetic constants, enzyme cost weights, metabolite ranges, flux bounds or biomass production rate. Smooth changes in these
parameters will lead to smooth or abrupt changes in the optimal fluxes, metabolite levels, enzyme levels, and the resulting growth rate (see Figure 7). Using FCM, we study such changes by screening the model parameters (e.g. external metabolite levels, rate constants, etc) and computing the metabolic state. As model parameters change, the shape of the flux cost function (and even the feasibility of different S-polytopes) may change and another flow may become optimal. Since flux cost functions are concave, the state can only jump from one polytope vertex to another one. Thus, parameter changes either lead to qualitative changes or have no effect. This happens at points at which two flows become equally costly. The first case (no flux change upon parameter changes) makes flux prediction simpler; the second case (sudden flux change upon parameter changes) makes it more difficult.

Mathematically, we can distinguish three types of metabolic changes. (i) A biochemical model parameter (kinetic constant or external concentration) changes, but the B-polytope remains unchanged. Even though the flux cost function changes its shape; the flow remains the same and metabolite and enzyme levels change gradually with the parameter changes. (ii) The optimum flow jumps between polytope vertices. Such jumps are accompanied by jumps in metabolite and enzyme levels. (iii) If the changing parameters have a direct effect on the flux constraints or on the benefit constraint (e.g. a change in the predefined benefit value $b'$), the S-polytope changes its shape; polytope vertices can move, and vertices may appear or disappear. This may lead to smooth changes or to abrupt jumps between vertices.

4.2 Splitting flows in space or time can save enzyme cost

If a cell or organism needs to perform several tasks (e.g. producing different compounds), and if these tasks require different flows, these flows can either run together as a linear combination or separately, i.e. in separate
cell compartments or organs, or at different moments in time. The second strategy, “splitting the flows”, can be cheaper because the compromise cost is avoided (proof in SI section B.2). In Figure 2, we saw that mixing two flows can lead to a compromise cost because the two flows cannot operate under their respective, optimal kinetic conditions. If the flows run separately (in different cell compartments, or at different times), each of them can keep its privileged metabolite profile, and the costs are simply additive. Mixed and split flows achieve the same chemical conversions; however, they may differ in how easy they are to achieve, what metabolite and enzyme profiles they require, and, possibly, whether they are thermodynamically feasible at all!

Depending on experimental conditions, yeast grown in continuous cultures can show spontaneous metabolic oscillations involving periodic changes between respiration and fermentation, as observed in budding yeast [23, 24, 25]. Such oscillations allow cells to separate incompatible processes in “temporal compartments” in order to use them more efficiently or to reduce deleterious side effects (e.g. avoiding DNA synthesis during phases with high cellular levels of reactive oxygen species) [24]. In theory, metabolic processes may be incompatible due to thermodynamics (because running them simultaneously would require thermodynamically impossible loops) and become compatible only when they are split. However, even if processes are not strictly incompatible, splitting them may reduce enzyme cost (i.e. avoid compromise cost) because this makes them thermodynamically or kinetically more favourable. Other splitting strategies (like spatial splitting in organelles, or division of labour between cells) can provide similar benefits.

Generally, if a flux cost function is strictly concave, splitting the flows can provide an advantage. If a metabolic flow $v$ is a convex combination $v = \eta v_A + (1 - \eta) v_B$ of kinetically distinct metabolic flows $v_A$ and $v_B$, it will be cheaper to split the two flows in space (in different compartments, cells, or organs), or time (in alternating phases with relative durations $\eta$ and $[1 - \eta]$) than to run them constantly as a convex combination (“combined flow”). This affects the metabolic strategies used by cells. In theory, flows could be split further and further into combinations of elementary or non-elementary modes, e.g., MinModes [26] which run some basic “housekeeping” processes together with specific metabolic processes.

Why can splitting the flows provide an advantage? Because each flow can run at its own optimal metabolite profile without any compromise cost. There may even be cases in which a splitting of flows is the only way to make them thermodynamically feasible (e.g. by running some reactions in cell compartments with different pH values). However, this fact is already included in our previous argument: as shown in [8], poor thermodynamics leads to high enzyme cost, whereas impossible thermodynamics leads infinite enzyme cost. Whether splitting a flow will pay off depends on many factors, e.g. on how strongly the flux cost function is curved, and on extra costs for maintaining cell compartments, or achieving the temporal metabolic switches. If these extra costs are too high, splitting the flows will not be profitable.

4.3 Metabolic switches as economic phase transitions

When model parameters are smoothly changing, the optimal metabolic state (fluxes, metabolite levels, enzyme levels) may vary smoothly or sometimes abruptly. In physics, a qualitative change in a system’s state upon a smooth change in the system parameters is called a phase transition. A metabolic switch, the abrupt change from one optimal flow to another one, is an example of a zeroth-order phase transition because fluxes, metabolite levels, and enzyme levels show abrupt changes in their values, not only in their derivatives. However, they are not phase transitions (or bifurcations) in the usual dynamical sense: since they also involve an optimal choice, one might call them “economic bifurcations”. Mathematically, they resemble phase transitions in thermodynamics where the system state (and possibly phase separation) is determined by an “optimality” principle, the principle of minimal Gibbs free energy.

So far, we considered switches between simple steady states, i.e. jumps between polytope vertices. However,
cells may also split flows in space or time, and thus employ phase separation behaviour. Such switches between complex strategies correspond to another type of phase transitions in classical thermodynamics: phase separation. Instead of switching from one homogeneous phase to another one, the system (e.g. a volume filled with water) switches to a coexistence of phases (e.g. water and ice), which together minimise the Gibbs free energy. Likewise, the metabolic state of a cell may undergo a phase separation, leading to a split flow.

5 Discussion

The enzymatic flux cost \(a(v)\), or “enzyme cost in flux space”, refers to a given kinetic model and describes the minimum enzyme cost at which a flow \(v\) can be realised. The kinetic flux cost contains an extra term for metabolite cost, which has an impact on optimal metabolite and enzyme levels and also on enzyme cost. Flux cost functions may be difficult to compute and their shapes depend on details such as kinetic constants, external metabolite levels, and metabolite bounds. Here we found some simple general properties: flux costs are not additive; whenever two flows are kinetically distinct (i.e. they privilege different metabolite profiles, which is usually the case), there is an extra “compromise cost”. The compromise cost reflects the fact that the mixed flow privileges a different metabolite profile than the original basic flows. The compromise cost vanishes only if the basic flows already privilege the same metabolite profile. Therefore, enzymatic flux costs scale linearly with the metabolic flow, are concave on the S-polytope, and are strictly concave between kinetically distinct flows. Optimal flows are vertices of the flux polytope. They often represent elementary flux modes and can be computed by flux cost minimisation. Finally, strictly concave flux cost functions imply a compromise cost, which cells can avoid by splitting their flows. This provides an incentive for (spatial or temporal) compartmentalisation.

In this article, flux cost functions were used in a specific type of optimality problems: minimisation of cost at a fixed flux benefit. Since cost and benefit scale linearly with the flow, this optimisation is equivalent to minimising the cost/benefit ratio for metabolic flux modes (whose absolute scaling, by definition, remains undetermined). However, the equivalence only holds if also all active flux constraints (e.g. upper bounds on individual fluxes) are independent of such a scaling. Flux cost functions can also be used in other approaches, for example, maximising a flux benefit at a given flux cost (similar to FBA with molecular crowding). The cost/benefit ratio is an important quantity because it affects the growth rate of cells. If additional constraints are imposed (e.g. a constraint on respiration capacity, due to crowding of respiratory chain proteins on cell membranes), the absolute scaling of flows will matter in fact, and we need optimisation methods in which the absolute scaling of flows plays a role. As an even more general approach, we could treat enzyme cost, biomass production, and objectives like biomass yield by multi-criteria optimality [27].

In a cell population, different cells may show different flows. We may assume a random distribution of flows leading to different growth rates, and therefore different evolutionary advantages. Knowing the shape of a flux cost function, what would we expect about this distribution? The growth rates assigned to metabolic flows can be converted into selection coefficients (i.e. \([\text{growth rate mutant} - \text{growth rate resident}] / [\text{growth rate resident}]\) or equivalently, \(\log(\text{growth rate mutant}) - \log(\text{growth rate resident})\)). Using simple assumptions about population dynamics, we can translate the selection coefficient (as a function on the flux polytope) into a probability distribution on the same polytope. For example, treating flux cost as an “energy function”, we may assume a Boltzmann distribution on the set of possible flows. Using approximate (linear or quadratic flux cost functions, these Boltzmann distributions will be exponential, or Gaussian, respectively. Using these distributions, we may sample from them to compute average fluxes, flux variability, and flux correlations in a population ensemble. Sometimes, minimisation of enzyme cost may not play a dominant role in determining cellular states. For example, in a metabolic switch experiment, \textit{Lactococcus lactis} bacteria were found to retain apparently “useless”
protein levels even though a decrease, at the same fluxes, would have been possible [28]. An extra investment in proteins may allow cells to quickly adapt to new situations, or enzymes may have additional functions or benefits (e.g. serving as a storage of amino acids), which are neglected in the optimality problems considered here. Nevertheless, it can be helpful to know the theoretical minimal cost of a flux, and the corresponding maximal growth rate, assuming that there are no other side-objectives. Thus, the present simple approach can serve as a “baseline model” for more advanced optimality approaches that will capture preemptive expression, further side objectives for proteins or metabolites, or actual deviations from optimality.

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References

[1] J.D. Orth, I. Thiele, and B.Ø. Palsson. What is flux balance analysis? Nature Biotechnology, 28:245–248, 2010.
[2] A. Goelzer, V. Fromion, and G. Scorletti. Cell design in bacteria as a convex optimization problem. Automatica, 47:1210–1218, 2011.
[3] A. Khodayari and C.D. Maranas. A genome-scale Escherichia coli kinetic metabolic model k-ecoli457 satisfying flux data for multiple mutant strains. Nature Communications, 7(13806), 2016.
[4] Q.K. Beg, A. Vazquez, J. Ernst, M.A. de Menezes, Z. Bar-Joseph, A.-L. Barabási, and Z.N. Oltvai. Intracellular crowding defines the mode and sequence of substrate uptake by Escherichia coli and constrains its metabolic activity. PNAS, 104(31):12663–12668, 2007.
[5] H.-G. Holzhütter. The principle of flux minimization and its application to estimate stationary fluxes in metabolic networks. Eur. J. Biochem., 271(14):2905–2922, 2004.
[6] G.C. Brown. Total cell protein concentration as an evolutionary constraint on the metabolic control distribution in cells. J. theor. Biol., 153:195–203, 1991.
[7] E. Klipp and R. Heinrich. Competition for enzymes in metabolic pathways: implications for optimal distributions of enzyme concentrations and for the distribution of flux control. BioSystems, 54:1–14, 1999.
[8] E. Noor, A. Flamholz, A. Bar-Even, D. Davidi, R. Milo, and W. Liebermeister. The protein cost of metabolic fluxes: prediction from enzymatic rate laws and cost minimization. PLoS Comput Biol, 12(10):e1005167, 2016. doi:10.1371/journal.pcbi.1005167.
[9] M.T. Wortel, H. Peters, J. Hulshof, B. Teusink, and F.J. Bruggeman. Metabolic states with maximal specific rate carry flux through an elementary flux mode. FEBS Journal, 281(6):1547–1555, 2014.
[10] M.T. Wortel, E. Noor, M. Ferris, F.J. Bruggeman, and W. Liebermeister. Profiling metabolic flux modes by enzyme cost reveals variable trade-offs between growth and yield in E. coli. Preprint on BioRxiv, doi: https://doi.org/10.1101/111161, 2017.
[11] S. Müller, G. Regensburger, and R. Steuer. Enzyme allocation problems in kinetic metabolic networks: Optimal solutions are elementary flux modes. *Journal of Theoretical Biology*, 347:182–190, 2014.

[12] M. Scott, C.W. Gunderson, E.M. Mateescu, Z. Zhang, and T. Hwa. Interdependence of cell growth and gene expression: Origins and consequences. *Science*, 330:1099, 2010.

[13] E. Dekel and U. Alon. Optimality and evolutionary tuning of the expression level of a protein. *Nature*, 436:588–692, 2005.

[14] I. Shachrai, A. Zaslaver, U. Alon, and E. Dekel. Cost of unneeded proteins in *E. coli* is reduced after several generations in exponential growth. *Molecular Cell*, 38:1–10, 2010.

[15] J.G. Reich. Zur Ökonomie im Proteinhaushalt der lebenden Zelle. *Biomed. Biochim. Acta*, 42(7/8):839–848, 1983.

[16] A. Flamholz, E. Noor, A. Bar-Even, W. Liebermeister, and R. Milo. Glycolytic strategy as a tradeoff between energy yield and protein cost. *PNAS*, 110(24):10039–10044, 2013.

[17] S. Schuster and R. Heinrich. Minimization of intermediate concentrations as a suggested optimality principle for biochemical networks. *Journal of Mathematical Biology*, 29(5):425–442, 1991.

[18] N. Tepper, E. Noor, D. Amador-Noguez, H.S. Haraldsdóttir, R. Milo, J. Rabinowitz, W. Liebermeister, and T. Shlomi. Steady-state metabolite concentrations reflect a balance between maximizing enzyme efficiency and minimizing total metabolite load. *PLoS ONE*, 8(9):e75370, 2013.

[19] W. Liebermeister, J. Uhlenendorf, and E. Klipp. Modular rate laws for enzymatic reactions: thermodynamics, elasticities, and implementation. *Bioinformatics*, 26(12):1528–1534, 2010.

[20] E. Noor, A. Flamholz, W. Liebermeister, A. Bar-Even, and R. Milo. A note on the kinetics of enzyme action: a decomposition that highlights thermodynamic effects. *FEBS Letters*, 587(17):2772–2777, 2013.

[21] S. Schuster, T. Dandekar, and D. A. Fell. Detection of elementary flux modes in biochemical networks: a promising tool for pathway analysis and metabolic engineering. *Trends Biotechnol*, 17(2):53–60, 1999.

[22] S. Schuster, D. Fell, and T. Dandekar. A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. *Nature Biotech*, 18:326–332, 2000.

[23] R.R. Klevecz, J. Bolen, G. Forrest, and D.B. Murray. A genomewide oscillation in transcription gates DNA replication and cell cycle. *PNAS*, 101(5):1200–1205, 2004.

[24] B.P. Tu, A. Kudlicki, M. Rowicka, and S.L. McKnight. Logic of yeast metabolic cycle: Temporal compartmentalization of cellular processes. *Science*, 310:1152, 2005.

[25] R. Machné and D. Murray. The Yin and Yang of yeast transcription: Elements of a global feedback system between metabolism and chromatin. *PLoS One*, 7(6):e37906, 2012.

[26] S. Hoffmann, A. Hoppe, and H.-G. Holzhütter. Composition of metabolic flux distributions by functionally interpretable minimal flux modes (inModes). *Genome Informatics*, 17(1):195–207, 2006.

[27] R. Schuetz, N. Zamboni, M. Zampieri, M. Heinemann, and U. Sauer. Multidimensional optimality of microbial metabolism. *Science*, 336(6081):601–604, 2012.

[28] A. Goel, T.H. Eckhardt, P. Puri, A. de Jong, F. Branco dos Santos, M. Giera, F. Fusetti, W.M. de Vos, J. Kok, B. Poolman, D. Molenaar, O.P. Kuipers, and B. Teusink. Protein costs do not explain evolution of metabolic strategies and regulation of ribosomal content: does protein investment explain an anaerobic bacterial Crabtree effect? *Molecular Microbiology*, 97(1):77–92, 2015.
A Metabolic flows and flux cost functions

To describe metabolic flows and flux cost functions, I use the following terminology.

A.1 Metabolic models and metabolic flows

Metabolic models We consider kinetic models with reversible rate laws of the form \( v_l = e_l \cdot r(c) \). The catalytic rate \( r(c) \) can be separated into capacity, reversibility, saturation, and regulation factors [8]. To score a metabolic state, we consider three fitness objectives: \( b(v) \) (linear flux benefit function), \( q(c) \) (concave metabolite benefit function), and \( h(e) \) (linear enzyme cost function). Typically, flux benefits describe the production of valuable compounds or biomass, and the enzyme cost describes growth disadvantages from higher protein levels. From the ratio \([\text{enzyme cost}]/[\text{flux benefit}]\), estimates of the cell growth rates can be derived (see section E.10).

Metabolic flows, scaled flows, and flux modes Stationary flux distributions \( v \), satisfying the stationarity condition \( N v = 0 \), are also called metabolic flows. If we disregard the absolute scaling of \( v \), we obtain a flux mode, which can also be represented by a scaled flow. For example, with a reaction flux \( v_r \) (e.g. biomass production) chosen as our “target flux”, we can define the scaled flow \( v/v_r \) and the overall flux cost \( a'(v) = a(v)/v_r \), i.e. the flux cost per target flux. Likewise, we can scale all flows to the same (linear) flux benefit. If a flow \( v \) is multiplied by some factor \( \eta \), its scaled cost remains unchanged. An elementary flux mode is a flux mode with a minimal set of active reactions: if one more reaction flux were restricted to be zero, the remaining set of active reactions would not support any stationary flux distribution anymore.

Flow patterns and conformal flows The sign vector of a flow \( v \), a vector with elements 1,0, and -1, described the set of active reaction and flux directions. Generally, such vectors are called flow patterns. A flow or a flux mode conforms to a flow pattern if all active fluxes match the prescribed signs (i.e. zero fluxes are allowed even where signs 1 or -1 are prescribed). A flow \( v \) has (or “strictly conforms to”) a flow pattern if the flux signs exactly match the prescribed signs (in this case, a prescribed sign of 0 means that the flux must vanish). Two flows are called conformal if they conform with a common flow pattern, i.e. if all their shared active reactions show the same flux directions. Two flows are strictly conformal if they have exactly the same flux pattern. All these definitions also hold, mutatis mutandis, for flux modes instead of metabolic flows.

Feasible flow patterns Among the possible flow patterns in a metabolic model, only some are physically and physiologically feasible. To be kinetically realisable (with reversible rate laws), a flow must be thermo-physiologically feasible, i.e. it must be possible to realise the fluxes, satisfying the thermodynamic constraint on flux directions and with a choice of metabolite levels that respect the physiological concentration ranges defined in the model. If a flow is thermo-physiologically feasible, then its flow pattern is called thermo-physiologically feasible as well. Briefly, in our terminology, a feasible flux sign pattern is a flow pattern that allows for fluxes that conform to this flow pattern, are stationary, thermodynamically feasible, and realise the predefined flux benefit.

A.2 Flux polytopes and metabolite polytopes

Flux polytopes Each flow pattern corresponds to a segment in flux space, i.e. an orthant or a lower-dimensional surface of an orthant. If a segment is cut by the subspace of stationary fluxes, the resulting set is called an S-polytope (“signed flux polytope”) \( P_S \). If the flow pattern forbids negative fluxes, the corresponding S-polytope is called a positive flux polytope \( P_P = \{ v | N v = 0; v \geq 0 \} \). If two flows are conformal, they will belong to the same S-polytope. All flows in the interior of an S-polytope are strictly conformal.
**Benefit-scaled polytope** By restricting an S-polytope to flows with a fixed benefit $b(v) = b'$, we obtain a B-polytope ("benefit-restricted flux polytope"), which is convex and lower-dimensional than the S-polytope\(^{10}\). Feasible flux sign patterns correspond to segments of flux space that contain non-empty B-polytopes, for the kinetic model, concentration ranges, and flux benefit function in question.

**Metabolite polytope** Flow patterns do not only define an S-polytope for fluxes, but also a corresponding metabolite polytope $\mathcal{P}_M$ for thermo-physiologically feasible metabolite profiles: it is the set of (natural) log-concentration vectors $m = \ln c$ that are thermodynamically feasible for the given flux directions and bounds on individual concentrations. In a given metabolic model, each flow pattern defines an S-polytope and an associated M-polytope.

**Reorienting the reaction directions** The flux signs in models are a matter of convention. They depend on how the reactions are formulated, i.e. which compounds are treated as substrates or as products. Due to this arbitrariness, an S-polytope can always be converted into a P-polytope by reorienting the reactions. Whenever we consider flows with a predefined flow pattern, we can assume (without loss of generality) that the fluxes are non-negative. All statements about enzyme or metabolite levels (in particular, the convexity proof for enzyme cost functions on the M-polytope and the concavity proof for flux cost functions on the P-polytope) remains valid after reorienting the fluxes.

**Mixed flows** With two flows $v_A$ and $v_B$ and an interpolation parameter $0 < \eta < 1$, we can form the mixed flow $v_C = \eta v_A + [1 - \eta] v_B$; in this combination, the flows $v_A$ and $v_B$ are called the basic flows. Combinations of three or more flows are defined accordingly. If several flows belong to one S-polytope, their mixed flows will all belong to the same S-polytope. If flows belong to one B-polytope, their mixed flows will belong to the same B-polytope.

### A.3 Flux cost function and compromise cost

**Definition A.1 Flux cost functions** We consider a kinetic model with metabolite cost $q(m)$ and enzyme cost $h(e)$ and define

\[
\begin{align*}
q^{\text{enz}}(m; v) &= h(e(m; v)) & \text{(Enzymatic M-cost)} \\
q^{\text{kin}}(m; v) &= h(e(m; v)) + q(m) & \text{(Kinetic M-cost)} \\
a^{\text{enz}}(v) &= \min_m q^{\text{enz}}(m; v) & \text{(Enzymatic F-cost)} \\
a^{\text{kin}}(v) &= \min_m q^{\text{kin}}(m; v) & \text{(Kinetic F-cost)},
\end{align*}
\]

where the log-metabolite profile $m = \ln c$ contains the logarithmic metabolite levels and the enzyme demand $e(m; v)$ is the enzyme profile that is needed to realise the flow $v$ with the log-metabolite profile $m$.

**Definition A.2 Concave flux cost functions** A flux cost function on a B-polytope is concave if it satisfies, for all $v_A$ and $v_B$ from the B-polytope and for all $\eta \in [0, 1]$,

\[
\forall \eta \in [0, 1]: \quad a([1 - \eta]v_A + \eta v_B) \geq [1 - \eta]a(v_A) + \eta a(v_B). \tag{6}
\]

Eq. (6) states that the enzymatic flux cost $a(v_C)$ of the interpolated flow $v_C = [1 - \eta]v_A + \eta v_B$ (where $0 < \eta < 1$) is equal or larger than the (additive) combined cost $q^{\text{interp}} = [1 - \eta]a(v_A) + \eta a(v_B)$. If a flux cost function satisfies Eq. (6) with $>$ instead of $\geq$ signs, it is called strictly concave.

**Remarks**

\(^{10}\)One may also apply several benefit constraints (i.e. a set of equalities $B v = b'$), but we do not consider such cases here.
1. **Scaled flux polytopes** In the definition above, the “benefit function” defining the B-polytope need not be biologically motivated. It can be any linear function used to establish a unique scaling of flows.

2. **Compromise cost** A concave flux cost function can be split into an additive combined cost plus a non-negative compromise cost. If a flux cost function is strictly concave, the compromise cost will be positive.

3. **Cost of mixed and split flows** A combination of flows \( v_A \) and \( v_B \) on one S-polytope can be realised in two ways: as a mixed flow (a weighted sum of the two flows), or as a split flow. The enzymatic flux cost is a concave function, so the cost of a mixed flow \( v = \sum_{\alpha} \eta_{\alpha} v^{(\alpha)} \) (with coefficients \( 0 \leq \eta_{\alpha} \leq 1 \)) will always be at least as high as the combined cost \( a^{\text{lin}}(v) = \sum_{\alpha} \eta_{\alpha} a(v^{(\alpha)}) \). In many cases, it will be strictly concave, and there will often be an additional positive compromise cost. However, the combined cost can be achieved by splitting the mixed flows, and running the basic flows separately in different cell compartments or at different times (with relative durations \( \eta \) and \( 1 - \eta \)). In this case there is no compromise cost, and costs of the flows are additive.

### A.4 Linear and nonlinear approximations of enzymatic flux cost functions

**Linear approximation of the enzymatic flux costs** To obtain a linear flux cost function with realistic cost weights, we linearise the enzymatic flux cost around a reference flow \( v^{\text{ref}} \). The approximation works best near the reference flow (or, more precisely, for flows \( v \) that privilege similar metabolite profiles as the reference flow itself). Therefore, \( v^{\text{ref}} \) should be “typical”, resembling the flows for which \( a(v) \) will be computed. The cost gradient in a point \( v \) is given by

\[
\frac{\partial a(v)}{\partial v_l} = \frac{h_{\alpha_l} e^{\text{opt}}_l(v)}{v_l},
\]

where \( e^{\text{opt}}_l(v) \) is the optimal enzyme profile realising our flow \( v \) (see SI E.8 and E.6). Equation (7) holds for kinetic flux cost functions as well (i.e. cost functions that include a direct metabolite cost). By linearising the cost function around the reference state \( v^{\text{ref}} \), we obtain the linear approximation

\[
a(v) = \sum_l \frac{\partial a}{\partial v_l} v_l \approx \sum_l \left( \frac{\partial a}{\partial v_l} \right)_{v^{\text{ref}}} v_l = \sum_l a_{v_l} v_l
\]

with flux weights \( a_{v_l} = \frac{h_{\alpha_l} e^{\text{opt}}_l(v^{\text{ref}})}{v_l^{\text{opt}}} = \frac{h_{\alpha_l}}{v_l} \). There is no offset term, and this makes in fact sense: otherwise, our approximated cost function (unlike the original cost function) would not be linearly scalable. The formula (8) holds only for P-polytopes (i.e. where non-negative fluxes are required). For other S-polytopes, with negative fluxes, we may reorient the reactions and obtain the same formula, but with different prefactors (obtained from different reference flows). The signs of these prefactors match the flux signs. If we assume the same (absolute) prefactors for all S-polytopes, we obtain the formula \( q^{\text{lin}}(v) = \sum_l a_{v_l} |v_l| \), the formula assumed by linear flux cost minimisation. The linear approximation (8) leads to linear flux cost weights for linear flux cost minimisation or FBA with molecular crowding. Not surprisingly, the linearised flux cost function is exact for the reference flow and scaled versions of it. For other flows, the approximation error depends on how much the specific rates \( (v_l/e_l) \), for optimised enzyme levels) differ between \( v \) and the reference flow \( v^{\text{ref}} \).

**Nonlinear approximation of flux cost functions, based on prototype flows** For a nonlinear approximation, we join information from several prototype flows \( v^{(\alpha)} \). To approximate the cost \( a(v) \), we first approximate our flow \( v \) by a convex combination \( v \approx \sum_{\alpha} \eta_{\alpha} v^{(\alpha)} \) of the prototype flows (with positive weights \( \eta_{\alpha} \) satisfying \( \sum_{\alpha} \eta_{\alpha} = 1 \)). Then we use these weights \( \eta_{\alpha} \) to define a weighted average of the inverse specific rates \( 1/r_l^{(\alpha)} = \sum_{\alpha} \eta_{\alpha}/r_l^{(\alpha)} \). We
obtain the approximated flux cost (see appendix E.9)

\[
a^{\text{non}}(v) \approx \sum_{l} \frac{h_{ci}}{r'_{l}} v_{l} = \sum_{\alpha} \frac{\eta_{\alpha}}{r'_{\alpha l}} h_{ci} v_{l}.
\]  

(9)

This flux cost function is nonlinear in \(v\) because the prefactors \(1/r'_{l}\) are weighted averages of the prototype flows’ \(1/r_{l}\), with weights depending on \(v\). As shown in section E.9, this cost function is quadratic. If we use a single prototype, the curvature vanishes and we reobtain the linear approximation. To improve the quadratic approximation (9), it is more important to use prototype points close to \(v\), the flow of interest, than many prototype points elsewhere. In fact, considering additional prototype points far from \(v\) may worsen the approximation.

B  Mathematical properties of flux cost function

In this section, I first discuss the conditions under which enzymatic M-cost functions have a unique minimum point. Then, I show that enzymatic flux cost functions are concave on the S-polytope and strictly concave when interpolating between kinetically distinct flows.

B.1  Shape of the enzymatic metabolite cost function

Conditions for strictly convex metabolite cost functions  
Under certain conditions, we can prove that the enzymatic M-cost will be strictly convex on the metabolite polytope. In a given point of the M-polytope, the enzymatic metabolite cost function will be positively curved in some directions and constant in others. These “cost-neutral” directions are due to the structure of the model and the rate laws used. To see this, we consider the Hessian matrix of the enzymatic M-cost function, which I call the cost curvature matrix \(H\). If \(H\) is positive definite (i.e. regular), the enzymatic metabolite cost is strictly convex on the M-polytope, and the ECM problems has a unique solution. By contrast, if \(H\) has vanishing eigenvalues (i.e. it is singular), the enzyme cost function is non-strictly convex, and the ECM problem has a subspace of solutions. To see whether a cost curvature matrix is regular, we need to determine the cost-neutral subspace of the model.

Shape of the enzymatic metabolite cost function  
A given flow \(v\) defines a feasible M-polytope, and each metabolite profile \(m\) in this M-polytope defines a feasible metabolic state. Optimal profiles are determined by the shape of the enzymatic M-cost function on the polytope. There are two cases: a cost function can be strictly convex (with a regular cost curvature matrix on the entire M-polytope), with a single optimal metabolite profile; or there exist directions of constant cost in metabolite space (i.e. the cost curvature matrix will have a non-empty nullspace), and there is a whole set of optimal metabolite profiles along these directions. Whether such “cost-neutral” directions exist depends on the model’s network structure and type of rate laws. Thus, to see whether the ECM problem has a unique solution, we need to check for “cost-neutral” metabolite variations \(\delta m\) that leave enzymatic metabolite cost unchanged, no matter in what metabolic state they are applied.

Cost-neutral variations and the nullspace of the cost curvature matrix matrix  
Let us look at the enzymatic metabolite cost function \(q^{\text{enz}}(m)\) and its curvature matrix matrix \(H\) more closely. If \(q^{\text{enz}}(m)\) stems from separable rate laws, \(H\) will have the following properties (proof in section E.2):

1. \(H\) is positive semidefinite (proof in SI E.4)
2. Given a flow \(v\), the cost curvature matrix of the enzymatic metabolite cost in a point \(m\) can be split into contributions from the single reactions \(H(m; v) = \sum_{l} H^{(l)}(m; v)\). Each of these cost curvature matrix is positive semidefinite (because the enzymatic metabolite cost for each reaction is convex), and the nullspace
of $\mathbf{H}$ is the intersection of all nullspaces of the matrices $\mathbf{H}^{(l)}$. If this intersection is empty, $\mathbf{H}$ will be regular. This happens, for instance, in models with CM rate laws (proof see section E.5).

3. The nullspace $\ker(\mathbf{H})$ is structurally determined, i.e. determined by rate laws and network structure only, and is independent of $\mathbf{m}$ and $\mathbf{v}$ (here, a fixed flow pattern of $\mathbf{v}$, with strictly defined zero values, is assumed).

4. Let $\mathbf{v}_A$ and $\mathbf{v}_B$ be two flux modes in the same S-polytope, and $\mathbf{m}_A$ and $\mathbf{m}_B$ be the respective optimal metabolite profiles. Then the cost curvature matrices in the points $(\mathbf{v}_A, \mathbf{m}_A)$ and $(\mathbf{v}_B, \mathbf{m}_B)$ have identical nullspaces.

5. In models with reversibility-based rate laws [8], $\ker(\mathbf{H})$ is the nullspace of the transposed stoichiometric matrix $\mathbf{N}^\top$; in models with saturation-based rate laws, $\ker(\mathbf{H})$ is the nullspace of the molecularity matrix $(\mathbf{M}^S_{\mathbf{M}^P})$; in models with CM rate laws, $\ker(\mathbf{H})$ is empty, i.e., $\mathbf{H}$ is regular (proof in section E.5).

**Conditions for a singular cost curvature matrix** Under what conditions will the cost curvature matrix be singular, resulting in non-unique optimal metabolite profiles?

1. If a metabolite $i$ does not affect any of the reaction rates. Then any variation vector $\delta \mathbf{m} = (0, 0, \ldots, \delta m_i, 0, 0)^\top$, with an entry at position $i$ is a nullvector of $\mathbf{H}$.

2. If the rate laws do not directly depend on metabolite levels, but only on thermodynamic forces. The thermodynamic driving forces are given by $\Theta_l = -RT \sum n_{il} \ln c_i$. If the rate laws depend on these forces only (for holds, e.g. for the “reversibility-based” rate laws in [8]), then any variation $\delta \mathbf{m}$ that leave the driving forces unchanged is a nullvector of $\mathbf{H}$. Such vectors $\delta \mathbf{m}$ are given by the nullvectors of $\mathbf{N}^\top_{\text{tot}}$ (i.e. conserved moiety vectors).

3. If the rate laws do not directly depend on metabolite levels, but only on mass-action products. The mass-action products are given by $\prod c_i^{m_i^S}$ or $\prod c_i^{m_i^P}$, with molecularity in the matrices $\mathbf{M}^S$ and $\mathbf{M}^P$ (e.g. the “saturation-based” rate laws in [8]). Variations $\delta \mathbf{m}$ that do not affect the mass-action products are nullvectors of $\mathbf{H}$. Such vectors $\delta \mathbf{m}$ are given by the nullvectors of $(\mathbf{M}^S_{\mathbf{M}^P})$.

4. If there exist variation vectors $\delta \mathbf{m}$ that do not affect any of the reaction rates. A reaction $l$ defines, by its enzyme cost, a cost curvature matrix $\mathbf{H}^{(l)}$ with a nullspace $\ker(\mathbf{H}^{(l)})$. The nullspace of $\mathbf{H}$ is the intersection of all these nullspaces. A variation that is cost-neutral for each reaction is therefore a nullvector of $\mathbf{H}$. In contrast, if any possible variation $\delta \mathbf{m}$ affects at least one reaction, then $\mathbf{H}$ is regular.

Thus, nullvectors of the cost curvature matrix may arise structurally, from rate laws and network structure alone. In fact, for models with separable rate laws (and non-constant thermodynamic and kinetic efficiency factors), the entire nullspace of the cost curvature matrix arises in this way. This is stated by the following lemma (proof in SI section E.3).

**Lemma 1 (The nullspace of the enzymatic metabolite cost curvature matrix is given by the cost-neutral subspace)** In models with separable rate laws (and non-constant thermodynamic and kinetic efficiency factors), all neutral metabolite variations $\delta \mathbf{m}$ are nullvectors of the cost curvature matrix $\mathbf{H}$ and, conversely, every nullvector of $\mathbf{H}$ is a neutral metabolite variation. This holds in any point $\mathbf{m}$ of the M-polytope. A nullvector of $\mathbf{H}$ in a point $\mathbf{m}$ is also a nullvector of $\mathbf{H}$ in any other point.

Thus, given a kinetic model (network structure, rate laws, and model parameters) and a flow pattern, the nullspace of the cost curvature matrix is structurally determined and is independent of the metabolite profile $\mathbf{m}$ and the flow $\mathbf{v}$.
Remark According to Lemma 1, if a model has an empty cost-neutral subspace, the enzymatic metabolite cost is strictly convex on the M-polytope and the ECM problem has a unique solution. This holds, in particular, in all models with common modular rate laws, because their cost-neutral subspace is empty (proof in SI section E.5).

Two metabolite profiles $m_A$ and $m_B$ are called cost-equivalent if their difference vector $m_A - m_B$ is cost neutral, i.e. in the cost-neutral subspace. To obtain a practical test, we introduce the notion of rate equivalence. Rate equivalence is defined as follows. With unit enzyme concentrations $e_l = 1$, the metabolite profile $m_A$ will yield the reaction rates $\nu(m, 1)$. We now ask whether changing from $m_A$ to $m_B$, at fixed enzyme levels, will change these reaction rates. If there is no change, $m_A$ and $m_B$ are rate-equivalent. Rate-equivalent metabolite profiles are also cost-equivalent.

B.2 The enzymatic flux cost is a concave function on the S-polytope

Lemma 2 Concave flux cost functions (proof in SI section E.1) Consider a cost function $f(m; v)$ that depends on metabolite profiles $m$ (in the M-polytope), is linear in the fluxes $v$ (in the S-polytope), $f(m; \alpha v_A + \beta v_B) = \alpha f(m; v_A) + \beta f(m; v_B)$, and is bounded from below. Let $g(m)$ be a cost function on the M-polytope that is also bounded from below. Then the flux cost function $a(v) = \min_{m \in P_S} f(m; v) + g(m)$ is concave on the $S$-polytope $P_S$.

From Lemma 2, we obtain

Proposition 1 (Enzymatic flux cost is a concave function) The enzymatic flux cost $a^{enz}(v) = \min_m q^{enz}(m; v)$ and the kinetic flux cost $a^{kin}(v) = \min_m q^{enz}(m; v) + g(m)$ are concave functions on the S-polytope.

Remarks

1. Linear enzyme cost function In Lemma 2, the enzymatic metabolite cost function $q^{enz}(m; v)$, must be linear in the fluxes; this requires that the enzyme cost function $h(e)$ is also linear. If $h(e)$ has a more general form $f(g(m, v))$, where $f(\cdot)$ is increasing and convex and $g(m, v)$ is linear in the fluxes, the enzymatic flux cost function may not be concave.

2. Non-stationary fluxes Proposition 1 holds not only for stationary, but also for non-stationary flux distributions. In this case, we only require that the flux distributions $v_A$ and $v_B$ and all their interpolations must be thermodynamically feasible for the equilibrium constants and external metabolite concentrations in our model. Let us consider one thermodynamically feasible flux sign pattern. If non-stationary flux distributions are allowed, then any flux distribution in the positive orthant $O_P = \{v | v \geq 0\}$ can be a solution. Since the flux cost is concave on this orthant, it is also concave on all B-polytopes within this orthant.

B.3 The enzymatic flux cost is strictly concave between kinetically distinct flows

The enzymatic flux cost is not only concave, but in many models strictly concave on the B-polytope. This means: the costs of flows are not simply additive; instead, mixed flows contain an additional, positive compromise cost. The most general criterion for strict concavity is that all flows on the B-polytope must be kinetically distinct.

Definition B.1 Kinetically distinct metabolic flows Consider two strictly conformal metabolic flows $v_A$ and $v_B$ (i.e. flows with the same active reactions and flux directions). The flows are kinetically distinct for a given kinetic model if they privilege necessarily different metabolite profiles, i.e. if there is no metabolite profile that is cost-optimal for $v_A$ and $v_B$. 

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Remark Why does the word “necessarily” appear in this definition? If each flow privileges a single metabolite profile only (i.e. if the enzymatic M-cost function is strictly convex.), then flows with different privileged profiles are kinetically distinct. However, a flow may also privilege several metabolite profiles (if the solution to the ECM problem is not unique). In this case, the criterion requires that no single profile may be privileged by the two flows.

Proposition 2 Consider a kinetic model and a flow pattern. If the enzymatic M-cost is strictly convex (on the M-polytope), then all fluxes on the B-polytope are kinetically distinct.

With this definition, we can now state: an enzymatic flux cost function will be strictly concave on the B-polytope if (and only if) all flows in the B-polytope are kinetically distinct (proof in SI E.4). To prove this statement, we consider metabolic models with given rate laws and flux directions and analyse the resulting ECM problem. We assume that the external metabolites have fixed concentrations and the internal metabolites have variable concentrations to be optimised.

Proposition 3 (Conditions for strictly concave flux cost functions) Let $\alpha(v)$ be an enzymatic flux cost function on a P-polytope. If $v_A$ and $v_B$ are kinetically distinct flows in the interior of that polytope, then $\alpha(v)$ is strictly concave on the line between $v_A$ and $v_B$ (proof in section E.4).

Remarks

1. The condition also holds for combinations of multiple flows $v_A, v_B, v_C, ...$ Instead of a line, we consider the simplex spanned by the flows (i.e. their convex hull).

2. Strict concavity also holds for kinetic flux cost functions (i.e. flux cost functions that contain direct metabolite costs) if the following conditions are satisfied: the kinetic metabolite cost $q(m)$ (i.e. enzymatic plus direct metabolite cost) must be convex and its cost curvature matrix must have the same nullspace everywhere in the M-polytope. In this case, adding it to the enzymatic metabolite cost may change the cost curvature matrices, but these matrices, in the entire M-polytope, will still have the same nullspaces. Therefore the concavity proof in SI E.4 still applies.

Corollary 1 If all flows in a B-polytope are kinetically distinct, the enzymatic flux cost $\alpha(v)$ is strictly concave on the interior of this B-polytope.

By combining Proposition 2 and Corollary , we obtain the following proposition.

Proposition 4 We consider a kinetic model and prescribe a feasible flow pattern. If the enzymatic M-cost is strictly convex on the M-polytope, the enzymatic flux cost $\alpha(v)$ is strictly concave on the interior of the B-polytope.

Typically, different metabolic flows will be kinetically distinct. However, let us see some flows that are not kinetically distinct and for which the flux cost function varies linearly on the line between the two flows.

1. Flows that differ only by scaling The enzymatic flux cost scales linearly with the flow. Therefore, if two flows $v_A$ and $v_B$ are identical except for their absolute scaling, the flux cost is not strictly concave on the interpolation line.

2. Flows that employ nominally different (but practically equivalent) chemical reactions The enzyme molecules catalysing a reaction R may be arbitrarily assigned to two pools (E1, “blue” enzyme molecules) and
(E2, "red" enzyme molecules), and our reaction may be formally represented by two reactions with identical properties, R1 (catalysed by blue enzyme) and R2 (catalysed by red enzyme). Since the subdivision is a pure matter of description and has no effect on the system dynamics, it cannot change the flux cost. Thus, reactions R1 and R2, as well as any convex combination, have the same cost; the cost function is constant between R1 and R2 and therefore not strictly concave.

In these examples, the flows \( v_A \) and \( v_B \), privilege the same metabolite profile. Therefore, the interpolated flows can be optimally realised by a linear interpolation of the enzyme levels; for linear cost functions \( h(e) \), this implies a linear change in enzyme cost. Thus, for strict concavity to hold, our basic flows must not privilege the same metabolite profile. However, if the optimal metabolite profiles are not uniquely determined, i.e. if a flow can privilege several metabolite profiles, will strict concavity still hold?

1. **Flows that privilege kinetically equivalent metabolite profiles.** In some models, different metabolite profiles \( m_A \) and \( m_B \) may yield the same reaction rates at the same enzyme levels, and the same reaction rates may occur for all metabolite profiles on the line between \( m_A \) and \( m_B \). As an example, consider two reactions \( A \leftrightarrow B + C \leftrightarrow D \) with reversible mass-action kinetics. If \([B]\) is scaled by a factor 10 and, at the same time, \([C]\) is scaled by a factor 0.1, the reaction rates remain unchanged. In the metabolite polytope (on logarithmic scale), we can linearly interpolate between two metabolite profiles. This is a case in which rates (and therefore cost) remain constant on a subspace in log-metabolite space, indicated by a nullvector of the cost curvature matrix of \( e^{enz}(m; v) \).

2. **Metabolites that do not affect any of the reaction rates** As a second case, we may consider models in which some reaction rates are independent of all metabolite levels. This holds for "capacity-based rate laws" \([8]\), describing enzymes that are completely substrate-saturated and forward-driven. In models containing such rate laws, even small enzyme perturbations can make a steady state impossible. In such models, it is also likely that some metabolites have no actual impact on reaction rates. A similar case occurs in models in which certain metabolites formally appear, but do not participate in any reactions.

In these two examples, the privileged metabolite profiles of our flows are not unique. However, there are still flows that can privilege the same metabolite profiles. In this case, when interpolating between two flows, one could obtain different metabolite profiles, and still realise the flux changes by linear changes in enzyme levels (i.e. at varying metabolite levels, but constant specific rates!). Therefore, whenever two flows can privilege the same metabolite profiles, we do not regard them as kinetically distinct.

**B.4 A sum rule for the enzymatic costs of mixed flows**

The sum rules Eqs (2) and (4) for absolute and scaled enzymatic flux cost functions can be obtained from Euler’s theorem on homogeneous functions. Similarly, we obtain sum rules for the derivatives of the optimal metabolite or enzyme levels with respect to the fluxes:

\[
\sum_l \frac{\partial c_i^{opt}}{\partial \ln v_l} = \sum_l \frac{\partial c_i^{opt}}{\partial v_l} v_l = 0,
\]

\[
\sum_l \frac{\partial e_i^{opt}}{\partial \ln v_l} = \sum_l \frac{\partial e_i^{opt}}{\partial v_l} v_l = e_i^{opt}.
\]

At first glance, Eq. (2) seems to state something obvious: that the total enzyme cost of a flow consists of the sum of enzyme costs \( a(v) = \sum_i h(e_i) \). However, the two statements differ in one point: the derivatives \( \frac{\partial h(e_i)}{\partial v_l} \) in the sum rule are not derivatives \( \frac{\partial h(e_i)}{\partial v_l} \) assuming fixed metabolite levels. Instead, they imply an optimal
metabolic adjustment whenever fluxes are changing. This reflects an important difference between our enzymatic flux cost function and simple flux costs as in FBA with molecular crowding, which assume a proportionality between enzyme levels and stationary fluxes, as if metabolite levels were constant and independent of the choice of enzyme levels and fluxes. Our sum rule acknowledges that fluxes and enzyme levels are globally coupled through metabolite levels, whereas simple flux costs are assumed to be additive between reactions\(^{11}\). We can formulate a similar sum rule for flows that are represented by convex combinations \(v = \sum \eta_\alpha v^{(\alpha)}\) of prototype flows \(v^{(\alpha)}\): now the cost of \(v\) is homogenous (with degree 1) with respect to the coefficients \(\eta_\alpha\), where the basis may be complete or even overcomplete, and we obtain the sum rule:

\[
a = \sum_\alpha \frac{\partial a}{\partial \eta_\alpha} \eta_\alpha \quad (11)
\]

for the coefficients \(\eta_\alpha\). The derivatives are given by \(\frac{\partial a}{\partial \eta_\alpha} = \sum_\iota \frac{\partial a}{\partial v^{(\iota)}} \frac{\partial v^{(\iota)}}{\partial \eta_\alpha} = \frac{\partial a}{\partial v} \cdot v^{(\alpha)}\). Again, there is an analogous sum rule for scaled flux costs.

B.5 The optimum points of concave flux cost functions are polytope vertices

If all flows in a B-polytope are kinetically distinct, the optimal (enzyme-cost-minimising) flow must be a polytope vertex. If several optimal flows exist, one of them must be a polytope vertex. We can see this as follows.

A **concave flux cost function has a minimum point in a polytope vertex** The enzymatic flux cost assumes its minimum value on a B-polytope in one of the vertices. However, the same minimal value may also occur in other, non-vertex points, but no lower values. We can see this as follows. Any polytope point \(v\) can be seen as a convex combination \(\sum_\alpha \eta_\alpha v^{(\alpha)}\) of the vertices. If the enzymatic flux cost is concave on the B-polytope, the cost \(a(v)\) must be equal or higher than the combined cost \(\sum_\alpha \eta_\alpha a(v^{(\alpha)})\). This combined cost, in turn, must be equal or higher than the lowest cost \(\min_\alpha a(v^{(\alpha)})\) among all vertices. Thus, if an interior point \(v\) assumes the minimum cost value on the B-polytope, there will always be a vertex point with the same minimum value.

**Concave flux cost functions have minimum points only on polytope vertices** If the enzymatic flux cost function is strictly concave on the B-polytope, its optima can only be vertex points. With strict concavity, the cost of an interpolated flow \(v = \sum_\alpha \eta_\alpha v^{(\alpha)}\) (with \(\eta_\alpha \in [0, 1], \sum_\alpha \eta_\alpha = 1\)) will be higher than the linearly combined cost. Therefore, whenever a non-vertex point has a cost \(a\), there will always be a vertex point with an even lower cost.

B.6 Locally optimal flows

**Definition B.2 Locally optimal flow.** A flow is called **locally optimal** if it has a lower flux cost than any other flow in a small region around \(v\) within the B-polytope.

**Proposition 5 (\textit{"Segaula criterion"})** A polytope vertex \(v\), with privileged metabolite profile \(m\), represents an optimal flow if if \(v\) is the only privileged flow of \(m\) (proof in section E.7).

**Remark** The proposition leads to an algorithm for constructing a locally optimal state (\textit{"Segaula algorithm"}): (i) Start from a given flow mode (e.g. obtained by running linear flux cost minimisation. (ii) Use ECM to compute the privileged metabolite profile for this flow. (iii) Compute the apparent catalytic rates in the resulting state, use them to define a linear flux cost minimisation problem, and solve it. (iv) Iterate steps (ii) and (iii) until convergence. Note that the flows computed by linear flux cost minimisation are likely to be sparse (in models\(^{11}\)).
Figure 8: During a flux reversal, some metabolite and enzyme levels change abruptly. (a) Schematic explanation. Curves show enzymatic metabolite cost (y-axis) in log-metabolite space (x-axis), for one selected metabolite. The dark blue curve represents the enzymatic cost from one reaction (whose direction is changing), and the light blue curve shows the enzymatic cost in all other reactions. Their sum (red curve) determines the optimal metabolite level (dot, x-coordinate) and enzyme cost (dot, y-coordinate). At a large forward flux (top), small metabolite levels are thermodynamically impossible (shaded region). In the conditions shown further down, the flux changes from positive to negative values. The cost of the flux-changing reaction is changing, while all other costs remain almost constant (close to a flux reversal, this is a plausible assumption). At the point of flux reversal, the flux-reverting reaction has zero flux and incurs no cost, and the optimal metabolite level is above the threshold value. In the state with an (infinitesimally small) negative flux, the metabolite level must be below the threshold value. This requires a jump in metabolite levels, and therefore enzyme levels and enzyme cost. (b) Simulation results from the branch point model.

without flux constraints: EFMs) and that therefore, some metabolite levels may be ill-determined. This can be fixed by using a regularisation term during ECM.

C Flux costs around points of flux reversal

For flows without predefined flux directions, i.e. flows in a single S-polytope, the flux cost function is concave. However, what is the shape of this cost function in the entire flux space, if any feasible flow patterns are allowed? By merging all feasible S-polytopes, we obtain a big (and typically non-convex) polytope of possible flux distributions with any feasible flux signs. The flux cost function is concave within each of the S-polytopes. However, what happens at the boundaries, where S-polytopes touch, and where fluxes change their directions? Will the function remain concave, smooth, or at least continuous?

Flux reversals can be caused in two ways: by a change in external metabolite levels (which are model parameters), or by changes in internal concentrations (which result from enzyme adaptations to any parameter changes in the model). To study a flux cost function at the point of flux reversal, we revisit our branch point model from Figure 4. In this model (with three active reactions and a stationarity constraint for the branch point metabolite), there
Figure 9: Metabolic changes during flux reversals. The drawings show enzymatic cost functions on the metabolite polytope (schematic, model not shown). Physiological and thermodynamic constraints are shown by dashed lines, enzymatic M-cost by colours from bright (low) to dark (high). Compare Figure 8 for a one-dimensional example. (a) A flux change without flux reversal. The metabolite polytope remains unchanged, but the shape of the cost function changes due to the changing desired flux, and the optimum point moves. (b) Flux reversal: a forward, zero, or backward flux in one reaction leads to different M-polytopes. At the point of flux reversal (centre), the optimum point is in the lower sub-polytope. Before the reversal (left), this optimum can already be realised. After the reversal, at a slightly negative flux, it becomes inaccessible and another point, near the boundary of the new M-polytope, becomes optimal. At larger negative fluxes, the optimum moves further away from the boundary (not shown).

There are $2^3 = 8$ possible flow patterns, corresponding to the eight orthants in flux space. The plane of stationary fluxes intersects six of these orthants, while any other two flow patterns can be discarded (these are patterns that would lead to accumulation or depletion of the branch point metabolite). Thermodynamically, the flux directions are determined by chemical potential differences: e.g. a positive flux $v_1$ in Figure 4 requires that $\mu_X > \mu_c$. In the example, we assume that $\mu_X > \mu_Y > \mu_Z$, corresponding to a fixed choice of external metabolite levels. There are two possible choices of $\mu_c$ that lead to stationary flows: either $\mu_c$ must be lower than $\mu_x$ and higher than $\mu_y$; then all fluxes run in forward direction; or it must be lower than $\mu_x$ and higher than $\mu_y$; then the flux $v_2$ is reversed. With this choice of external chemical potentials, only two orthants in flux space remain feasible, and the chemical potential $\mu_c$ determines which of them will be used (blue or red). Thus, given a choice of external metabolite levels, the enzymatic flux cost is a function on the two coloured triangles. With a predefined objective (e.g. flux $v_3$), we obtain the B-polytope (thick black line in Figure 8). Within each part of the line (blue or red), the cost function is concave. However, the flux cost function shows a jump at the polytope boundary. Figures 8 and 9 show what happens at the point of flux reversal. Between two S-polytopes, the flux cost function is not continuous, except for some very unlikely cases (requiring fine-tuned model parameters). Also metabolite levels, enzyme levels, and enzyme cost are discontinuous, so the enzymatic flux cost has discontinuities between S-polytopes. As the flow moves smoothly between the polytopes, the optimal metabolite and enzyme levels show a jump in their values rather than in their derivatives, i.e. a zeroth-order phase transition.

Generally, in order for fluxes to change their directions, the thermodynamic driving force and thus the metabolite levels must change. In flux space, the metabolic flow will move to another segment and will cross the boundary between two S-polytopes. At the same time, in metabolite space, the feasible metabolite polytope flips along the boundary associated with the reversed reaction (see Figure 9) and the metabolite profile jumps to the new polytope.
D Variants of flux cost minimisation

D.1 Optimisation of enzyme activities at given desired fluxes and fixed enzyme concentrations

In our search for optimal flows in FCM assume that each flow is realised by its own optimal enzyme profile. However, cells may sometimes not be able to adapt their enzyme levels, for example on very short time scales. Anticipating fast, unpredictable metabolic changes, the cell may provide sufficient, constant enzyme levels for various possible (or likely) situations, and then inhibit enzymes, whenever needed, by phosphorylation. In [8], we showed that this problem – finding an optimal enzyme profile that will suffice to realise a finite set of flux distributions – can be formulated as a convex optimality problem. Here we consider a slightly different scenario: we assume that the cell has already chosen an enzyme profile, not matter how, and needs to realise a flow with a maximal flux benefit (e.g. a maximal biomass production, or a maximal substance conversion), where the enzymes may be inhibited. What is the optimal flow, the optimal metabolite profile, and the optimal profile of enzyme activities? To model such a scenario, we consider a optimality problem in which the equality

\[ v_l = e_l r_l \]  

is replaced by an inequality \( v_l \leq e_l r_l \) (because the cell may inhibit some enzymes by phosphorylation), the enzyme profile \( e \) is predefined, and we search for a flow (and a corresponding log-metabolite profile \( m \)) that maximises a benefit value \( b'_v \cdot v \). Instead of maximising the benefit numerically, we employ a trick: we compare flows \( v \) providing a fixed nominal benefit \( b' = b'_v \cdot v \) (which allows us to consider optimality problems on the B-polytope); then, for each flow, we assume that the benefit can be scaled by a factor \( \sigma \), and we determine the maximal value \( \sigma_{\text{opt}}(v) \) at which the enzyme constraint Eq. (12) can be satisfied. A high value of \( \sigma_{\text{opt}} \) means that the nominal benefit \( b' \) can be achieved at lower enzyme levels, or that a time-integrated benefit \( \int b' \, dt \) can be reached in a shorter period of time. To determine \( \sigma_{\text{opt}} \) for each flow \( v \), we consider a variable \( \sigma \) to be maximised under the constraint

\[ \forall l: \sigma v_l \leq e_l r_l(m). \]  

Here the fluxes \( v_l \) and enzyme levels \( e_l \) are given, \( r_l(m) \) denotes the catalytic rates, and the log-metabolite levels \( m_i = \ln c_i \) are the variables to be optimised. For a flow \( v \), the optimality problem reads

\[ \text{Maximise } \sigma \text{ with respect to } \sigma \text{ and } m \in \mathcal{P}_M \text{ subject to } \forall l: \sigma \leq \frac{e_l r_l(m)}{v_l}. \]  

The inequality constraints can be reformulated by defining \( \sigma_{\max}(m) = \min_l \frac{e_l r_l(m)}{v_l} \), the maximal possible value of \( \sigma \) that satisfies all the inequalities. We obtain

\[ \text{Maximise } \sigma_{\max}(m) = \min_l \frac{e_l r_l(m)}{v_l} \text{ with respect to } m \in \mathcal{P}_M. \]  

Equivalently, we can minimise the inverse value:

\[ \text{Minimise } \frac{1}{\sigma_{\max}(m)} = \max_l \frac{v_l}{e_l r_l(m)} \text{ with respect to } m \in \mathcal{P}_M. \]
Since all terms on the right are convex in \( m \), their maximum is convex, too\(^{12}\). Thus, for each flux \( v \), the maximal possible value of \( \sigma \), called \( \sigma^{opt}(v) \), is obtained by solving a convex optimality problem for \( 1/\sigma_{\text{max}}(m) \) on the metabolite polytope. The function \( \sigma^{opt}(v) \) is monotonically decreasing\(^{13}\) in all fluxes \( v_l \). Given \( \sigma^{opt}(v) \), we can define \( a(v) = 1/\sigma^{opt} \), the cost/benefit ratio of flux \( v \) at fixed enzyme levels\(^{11}\). In contrast to our usual flux cost function, this function need not be concave in flux space. For example, if two flux distributions \( v_A \) and \( v_B \) hit the enzyme constraints Eq. (12) in different reactions, then by using a convex combination of the two flows, one could relax both constraints and increase the flux at the given enzyme levels. As a rule of thumb, this means: if enzyme levels are already given, then cells will rather use the available enzyme than using sparse flux distributions.

### D.2 Models with non-enzymatic reactions

So far, we assumed that all reactions in our kinetic model are enzyme-catalysed. This makes each reaction flux costly, but also directly controllable. In reality, there are non-enzymatic reactions, which changes the cost of metabolic flows and may even render certain flows impossible. Non-enzymatic reactions include, for example, (i) the damaging of molecules by free radicals; (ii) molecules leaving the cell by passive diffusion; (iii) the dilution of substances in growing cells (which formally resemble a linear degradation reaction). In ECM, non-enzymatic reactions will impose specific constraints on metabolite levels. For example, an irreversible reaction with mass-action rate law and given flux defines a feasible plane within the M-polytope. (ii) A reversible reaction in quasi-equilibrium will fix the ratio between its substrate and product levels. (iii) In models with dilution fluxes \( \lambda c_i \), a given flux distribution \( v \) determines all dilution fluxes and, thereby, all metabolite levels! Such constraints can restrict the M-polytope to lower-dimensional sub-polytopes or even to an empty set (i.e. the flow will not be realisable). For flux cost minimisation, this means that some of the flows, even thermodynamically feasible ones, may have to be omitted because they cannot be realised kinetically. Geometrically, parts of the flux polytope are excluded for kinetic reasons, and the remaining set of lows may be non-convex. Instead, different flows in the same S-polytope may correspond to different sections of an M-polytope; this means that the functions \( q(v,m) \), obtained from different flows, reside on different sets in metabolite space and are not comparable anymore. In addition, the flux cost function itself will change its shape, and our concavity proof does not hold anymore.

### E Proofs

#### E.1 Proof of Lemma 2

To prove Lemma 2, we consider a kinetic flux cost \( a(v) = \min_{m \in P_S} f(m;v) + g(m) \), where \( f(m;v) \) and \( g(m) \) are bounded from below, and where \( f(m;v) \) is linear in \( v \):

\[
\forall m \in P_S : f(m;\eta v_A + \mu v_B) = \eta f(m;v_A) + \mu f(m;v_B).
\]

(18)

To prove that \( a(v) \) is concave, we need to show that

\[
a(\eta v_A + \mu v_B) \geq \eta a(v_A) + \mu a(v_B)
\]

(19)

\(^{12}\)This optimality problem resembles the standard ECM problem

\[
\text{Minimise } q^{\text{max}}(m;v) = \sum_{l} \frac{h_l v_l}{r_l(m)} \text{ with respect to } m \in P_M.
\]

Here, since each sum term is convex on \( P_M \), these sum (i.e., the enzymatic metabolite cost) is also convex.

\(^{13}\)Here is a proof. Whenever a flux \( v_l \) in (17) increases, the corresponding sum term increases as well. The maximum can only increase, and \( \sigma \) will either decrease or remain the same. On the contrary, if a flux \( v_l \) decreases, then \( \sigma \) must increase or remain the same.
for all $\eta \in [0, 1]$ and $\mu = 1 - \eta$. We compute

$$
a(\eta v_A + \mu v_B) = \min_{m \in P_\Theta} f(m; \eta v_A + \mu v_B) + g(m)
= \min_{m \in P_\Theta} [\eta f(m; v_A) + \eta g(m) + \mu f(m; v_B) + \mu g(m)]
\geq \min_{m \in P_\Theta} [\eta f(m; v_A) + \eta g(m)] + \min_{m \in P_\Theta} [\mu f(m; v_B) + \mu g(m)]
= \eta \min_{m \in P_\Theta} [f(m; v_A) + g(m)] + \mu \min_{m \in P_\Theta} [f(m; v_B) + g(m)]
= \eta a(v_A) + \mu a(v_B). \quad (20)
$$

To obtain the inequality in the third line, we used the fact that by taking the minimum value of a sum of functions, one cannot obtain a lower value than by summing the minimum values of the original functions.

### E.2 Metabolite variations and their effect on enzymatic metabolite cost

When metabolite levels are changing (and the enzyme levels are fixed), this will usually change the reaction rates. However, there are exceptions. To see which variations of the metabolite profile can affect the reaction rates, and therefore the enzymatic cost at a given flux, we first introduce some terminology. We generally assume a given flow pattern, and flux and metabolite profiles in the corresponding feasible polytopes.

**Definition (Thermodynamically neutral metabolite variations)** The thermodynamic force of reaction $l$, given by $\Theta_l = -\Delta_G / RT = -\sum_{i=1}^n n_{il} s_i$, is a linear function on the metabolite polytope. A metabolite variation $\delta m$ is called thermodynamically neutral if adding $\delta m$ to any metabolite profile $m$ leaves all thermodynamic forces $\Theta_l$ unchanged. The set of thermodynamically neutral metabolite variations $\delta m$ is called thermodynamically neutral subspace. It is given by the nullspace of $N_{\text{tot}}^\top$ (where $N_{\text{tot}}$ is the stoichiometric matrix referring to all – internal and external – metabolites).

**Definition (Mass-action neutral metabolite variations)** In some rate laws, the reaction rate depends on metabolite levels only through mass-action terms $S_i = \prod_i c_i^{m_{ii}^{\text{tot}}}$ (for substrates) and $P_i = \prod_i c_i^{m_{ii}^{\text{tot}}}$ (for products). A metabolite variation $\delta m$ is called mass-action neutral if adding $\delta m$ to any metabolite profile $m$ leaves all these terms in a model unchanged. The set of mass-action neutral metabolite variations is called mass-action neutral subspace. It is given by the nullspace of $M_{\text{tot}}^\top$.

**Definition (Kinetically neutral metabolite variations)** A metabolite variation $\delta m$ is called kinetically neutral if adding $\delta m$ to any metabolite profile $m$ leaves the rates of all reactions unchanged. The set of kinetically neutral metabolite variations is called kinetically neutral subspace.

**Definition (Cost-neutral metabolite variations)** A metabolite variation $\delta m$ is called cost-neutral if adding $\delta m$ to any metabolite profile $m$, at a fixed flow $\nu$, leaves the enzymatic metabolite cost $q^{\text{enz}}(m)$ unchanged. The set of cost-neutral metabolite variations is called cost-neutral subspace.

All four criteria can be defined for entire models or single reactions. For example, a metabolite variation $\delta m$ is thermodynamically neutral for reaction $l$ if it can be added to any metabolite profile $m$ in the metabolite polytope without changing the thermodynamic force of reaction $l$. The four criteria help us describe the nullspace of the cost curvature matrix $H$ for different types of rate laws:

1. “Reversibility-based” rate laws depend on metabolite concentrations solely through thermodynamic forces [8]. In models with such rate laws, all thermodynamically neutral metabolite variations are both kinetically neutral and cost-neutral. In such models, any nullvector of $N^\top$ will also be a nullvector of $H$.

2. “Saturation-based” rate laws depend on metabolite concentrations solely through the principal concentration terms [8]. In models with such rate laws, all mass-action neutral metabolite variations are also kinetically neutral.
neutral and cost-neutral. In such models, any nullvector of \( \frac{M}{M^*} \) will also be a nullvector of \( H \).

3. Let \( S \) be the cost-neutral subspace of a model, i.e., the set of all cost-neutral metabolite variations. For any difference vectors \( \mathbf{m}_A - \mathbf{m}_B \), we can infer the implication \( \mathbf{m}_A - \mathbf{m}_B \in S \Rightarrow \mathbf{q}^\text{enz}(\mathbf{m}_A) = \mathbf{q}^\text{enz}(\mathbf{m}_B) \). This means: any kinetically neutral variation \( \delta \mathbf{m} \) is also cost-neutral and is therefore a nullvector of \( H \), i.e., \( H \delta \mathbf{m} = 0 \). Thus, for reversibility-based rate laws, all thermodynamically neutral variations are cost-neutral (and are therefore nullvectors of the cost curvature matrix), and for saturation-based rate laws, all mass-action neutral variations are cost-neutral.

### E.3 Proof of Lemma 1

The cost curvature matrix of the cost function \( \mathbf{q}^\text{enz}(\mathbf{m}) \) depends on the desired flow \( \mathbf{v} \) and on the metabolite profile \( \mathbf{m} \). Lemma 1 states that the nullspace of this matrix does not depend on the specific choices of \( \mathbf{v} \) and \( \mathbf{m} \), but only on the flux polytope and the metabolite polytope from which they are taken; it is structurally determined by rate laws, network structure, and flow pattern (specifying the active reactions and flux directions). To prove this, we first note that all separable rate laws can be written as functions of the metabolite log-concentrations \( \mathbf{m}_i = \ln c_i \) in the form

\[
r(\mathbf{m}) = k_{\text{cat}} \frac{1 - e^{-RT(\ln k_{eq} + \mathbf{n} \cdot \mathbf{m})}}{\sum_t a_t \mathbf{g}_t \cdot \mathbf{m}},
\]

where the vector \( \mathbf{n} \) contains the stoichiometric coefficients and the vectors \( \mathbf{g}_t \) follow from the rate law denominator (see [8]). We first show two additional lemmas:

**Lemma 3** The function

\[
f(y) = \frac{e^{\alpha y}}{1 - e^y}
\]

with real-valued coefficient \( \alpha \) is positively curved for all \( y < 0 \). To prove this lemma, we consider the second derivative

\[
\frac{\partial^2 f}{\partial y^2} = \frac{a^2 e^{\alpha y}}{1 - e^y} + e^{\alpha y} \left( \frac{e^y}{(1 - e^y)^2} + \frac{2 e^2 y}{(1 - e^y)^3} \right) + \frac{2 a e^{\alpha y}}{(1 - e^y)^2},
\]

which is positive for all \( y < 0 \).

**Lemma 4 (Curvature of inverse rate law functions)** The inverse of a separable rate law \( r(\mathbf{m}) \), as a function on the metabolite polytope, is positively curved in the subspace spanned by \( \mathbf{n} \) and the vectors \( \mathbf{g}_t \), and it has zero curvature in any direction orthogonal to that subspace. Here is the proof. After omitting the constant prefactor \( k_{\text{cat}} \), the inverse of the rate law Eq. (21) can be written as

\[
\frac{1}{r(\mathbf{m})} = \sum_t \frac{e^{\alpha_t + \mathbf{g}_t \cdot \mathbf{m}}}{1 - e^{\beta_t + \mathbf{n}' \cdot \mathbf{m}}},
\]

where the vector \( \mathbf{n}' \) is parallel to our original vector \( \mathbf{n} \) and \( \beta + \mathbf{n}' \cdot \mathbf{m} \) yields a negative number (because any point \( \mathbf{m} \) in the metabolite polytope must yield positive thermodynamic forces). If a metabolite variation \( \delta \mathbf{m} \) is orthogonal on \( \mathbf{n} \) and on all \( \mathbf{g}_t \), then \( 1/r(\mathbf{m} + \delta \mathbf{m}) = 1/r(\mathbf{m}) \), and the function Eq. (24) has a vanishing curvature in that direction. If a variation \( \delta \mathbf{m} \) lies in the space spanned by \( \mathbf{n} \) and all \( \mathbf{g}_t \), then at least one of the sum terms must depend on \( \delta \mathbf{m} \). Each sum term, as a function of \( \mathbf{m} \), is identical to Eq. (22) except for an affine transformation, and must therefore be positively curved with respect to a scaling of \( \delta \mathbf{m} \).
**Proof of Lemma 1** The enzymatic metabolic cost function is given by \( q(m) = \sum_l \frac{h_l m_l}{r_l(m)} \), and its cost curvature matrix is given by \( H(m) = \sum_l h_l v_l \frac{\partial^2}{\partial m^2} \frac{1}{r_l(m)} \). Since \( \frac{\partial^2}{\partial m^2} \frac{1}{r_l(m)} \) is convex (see [8]), all sum terms are positive semidefinite, and the nullspace of \( H \) is given by the intersection of the nullspaces of all matrices \( \frac{\partial^2}{\partial m^2} \frac{1}{r_l(m)} \). To show that the nullspace of \( H \) does not depend on the specific choices of \( m \) and \( v \), we just need to show that the nullspace of \( \frac{\partial^2}{\partial m^2} \frac{1}{r_l(m)} \), for single reactions \( l \), does not depend on \( m \). This can be proven by applying Lemma 4.

**E.4 Proof of Proposition 3**

Proposition 3 states that the enzymatic flux cost on the interpolation line between two conformal, kinetically distinct flows is strictly concave. For the proof, we consider two flows \( v_A \) and \( v_B \) on the same P-polytope. By solving the ECM problems for these two flows, we obtain optimal metabolite vectors \( m_A \) and \( m_B \), as well as the cost curvature matrices \( H_A \) and \( H_B \) in these points. Since our flows \( v_A \) and \( v_B \) are kinetically distinct by assumption, the profiles \( m_A \) and \( m_B \) must be different \( (m_A \neq m_B) \) and cost-distinct \( (H_A (m_A - m_B) \neq 0 \) and \( H_B (m_A - m_B) \neq 0) \). The latter condition holds because otherwise, each of the two, \( m_A \) and \( m_B \), would be optimal for both of the flows. In the following proof, we consider a convex combination \( v_C \) of \( v_A \) and \( v_B \) (with interpolation parameter \( 0 < \eta < 1 \)) and its optimal metabolite vector \( m_C \).

1. We first show that the cost \( q^{enz}(m; v) \) at a given flow \( v \) can be Taylor-expanded with respect to \( m \) in each point of the metabolite polytope. Here is the proof: our cost function is a composition of exponentials and rational functions (with no singularity points inside the metabolite polytope) and is therefore holomorphic in every variable \( m_i \). By Hartogs’ theorem, it must also be holomorphic as a function of several variables. Therefore, it can be approximated by a convergent power series in each interior point of the metabolite polytope.

2. To show that the flux cost function \( a(v) \) is strictly concave, it is sufficient to show this for similar flows \( v_A \approx v_B \). Since the cost function \( q^{enz}(m; v) \) is continuous in \( m \) and \( v \), the optimal metabolite profiles \( m_A \), \( m_B \), and \( m_C \) will be similar, too. To simplify the notation, we write \( q_A^{enz}(m) = q^{enz}(m; v_A) \) and \( q_B^{enz}(m) = q^{enz}(m; v_B) \). Now we approximate the cost by a quadratic expansion (see point 1)

\[
q_A^{enz}(m) = q_A^{enz}(m_A) + \frac{1}{2} (m - m_A)^\top H_A (m - m_A) \\
q_B^{enz}(m) = q_B^{enz}(m_B) + \frac{1}{2} (m - m_B)^\top H_B (m - m_B). 
\]

(25)

For each given metabolite profile \( m \), the two functions are additive between \( v_A \) and \( v_B \). Therefore, the enzymatic metabolite cost for the interpolated flow \( v_C \) reads

\[
q_C^{enz}(m) = [1 - \eta] q_A^{enz}(m) + \eta q_B^{enz}(m) \\
= [1 - \eta] q_A^{enz}(m_A) + \frac{1}{2} (m - m_A)^\top H_A (m - m_A) + \eta q_B^{enz}(m_B) + \frac{1}{2} (m - m_B)^\top H_B (m - m_B) \\
= [1 - \eta] q_A^{enz}(m_A) + \eta q_B^{enz}(m_B) \\
= \frac{1}{2} [1 - \eta] (m - m_A)^\top H_A (m - m_A) + \eta (m - m_B)^\top H_B (m - m_B)] \\
\Delta q \]

(26)

The first term describes the linearly interpolated costs of \( v_A \) and \( v_B \). The second term, called compromise cost \( \Delta q \), is non-negative. This already shows that the flux cost function must be concave. In the next step, we show that \( \Delta q \) is actually positive, i.e. the flux cost function is strictly concave.

3. To find the optimal metabolite profile \( m_C \) for our combined flow \( v_C \), we minimise th e compromise cost \( \Delta q \)
with respect to \( \mathbf{m} \). First, we rewrite \( \Delta q \) as

\[
\Delta q = [1 - \eta] \left[ \mathbf{m}^\top \mathbf{H}_A \mathbf{m} - 2 \mathbf{m}^\top \mathbf{H}_A \mathbf{m}_A + \mathbf{m}_A \right]^\top \mathbf{H}_A \mathbf{m}_A + \eta \left[ \mathbf{m}^\top \mathbf{H}_B \mathbf{m} - 2 \mathbf{m}^\top \mathbf{H}_B \mathbf{m}_B + \mathbf{m}_B \right].
\]  

(27)

Omitting some constant terms, we obtain

\[
\Delta q' = [1 - \eta] \left[ \mathbf{m}^\top \mathbf{H}_A \mathbf{m} - 2 \mathbf{m}^\top \mathbf{H}_A \mathbf{m}_A + \eta \left[ \mathbf{m}^\top \mathbf{H}_B \mathbf{m} - 2 \mathbf{m}^\top \mathbf{H}_B \mathbf{m}_B \right] \right] = \mathbf{m}^\top \left[ [1 - \eta] \mathbf{H}_A + \eta \mathbf{H}_B \right] \mathbf{m} - 2 \mathbf{m}^\top \left[ [1 - \eta] \mathbf{H}_A \mathbf{m}_A + \eta \mathbf{H}_B \mathbf{m}_B \right].
\]  

(28)

Minimising \( \Delta q' \) with respect to \( \mathbf{m} \), we obtain the optimal metabolite profile \( \mathbf{m}_C \):

\[
\mathbf{m}_C = \mathbf{H}_C^{-1} \left[ [1 - \eta] \mathbf{H}_A \mathbf{m}_A + \eta \mathbf{H}_B \mathbf{m}_B \right].
\]  

(29)

4. For later use, we now show that \( \mathbf{m}_C \neq \mathbf{m}_A \) and \( \mathbf{m}_C \neq \mathbf{m}_B \) (and still assume that \( 0 < \eta < 1 \)). We prove \( \mathbf{m}_C \neq \mathbf{m}_A \) by contradiction: we first assume that \( \mathbf{m}_C = \mathbf{m}_A \), and obtain

\[
\mathbf{m}_A = \mathbf{m}_C = \mathbf{H}_C^{-1} \left[ [1 - \eta] \mathbf{H}_A \mathbf{m}_A + \eta \mathbf{H}_B \mathbf{m}_B \right] \Rightarrow \mathbf{H}_C \mathbf{m}_A = [1 - \eta] \mathbf{H}_A \mathbf{m}_A + \eta \mathbf{H}_B \mathbf{m}_B \Rightarrow \mathbf{H}_B (\mathbf{m}_A - \mathbf{m}_B) = 0.
\]  

(30)

This would imply that either \( \mathbf{m}_A = \mathbf{m}_B \), or that \( \mathbf{m}_A - \mathbf{m}_B \) is a nullvector of \( \mathbf{H}_B \) and therefore cost-neutral. This contradicts our initial assumptions. The proof for \( \mathbf{m}_C = \mathbf{m}_B \) is analogous.

5. We now start again from the expression for \( \Delta q \) in Eq. (26), insert the optimal metabolite profile \( \mathbf{m}_C \), and obtain

\[
\Delta q = [1 - \eta] \left( \mathbf{m}_C - \mathbf{m}_A \right)^\top \mathbf{H}_A \left( \mathbf{m}_C - \mathbf{m}_A \right) + \eta \left( \mathbf{m}_C - \mathbf{m}_B \right)^\top \mathbf{H}_B \left( \mathbf{m}_C - \mathbf{m}_B \right).
\]  

(31)

To complete our proof, we need to show that this expression is strictly positive. It cannot be negative, because the matrix products are symmetric and the prefactors \( [1 - \eta] \) and \( \eta \) are non-negative. Thus, we only need to show that it cannot vanish. We already saw that \( \left( \mathbf{m}_C - \mathbf{m}_A \right) \) and \( \left( \mathbf{m}_C - \mathbf{m}_B \right) \) cannot vanish. Thus, in order for \( \Delta q \) to vanish, \( \left( \mathbf{m}_C - \mathbf{m}_A \right) \) would have to be in the nullspace of \( \mathbf{H}_A \), and \( \mathbf{m}_C - \mathbf{m}_B \) would have to be in the nullspace of \( \mathbf{H}_B \). According to Lemma 1, the two nullspaces are structurally determined and therefore identical. Thus, for \( \Delta q \) to vanish, the difference \( \mathbf{m}_A - \mathbf{m}_B \) must be in this nullspace, which contradicts our assumptions.

### E.5 The enzymatic metabolite cost obtained from common modular rate laws is strictly convex

In models with common modular (CM) rate laws, the enzymatic metabolite cost function has a strictly positive cost curvature matrix and is therefore strictly convex. In the following proof, found by Joost Hulshof (VU Amsterdam),
this is first shown for one reaction and then for an entire network. We consider an CM rate law
\[
v = E \frac{k_+ \prod_{i \in \text{sub}} (\frac{c_i}{k_i})^{m_{i}^{\text{sub}}} (1 - e^{-\Theta(x)})}{\prod_{i \in \text{sub}} (1 + \frac{c_i}{k_i})^{m_{i}^{\text{sub}}} + \prod_{i \in \text{prod}} (1 + \frac{c_i}{k_i})^{m_{i}^{\text{prod}}} - 1},
\]
where \(m_{i}^{\text{sub}}\) and \(m_{i}^{\text{prod}}\) denote the molecularities (i.e. positive stoichiometric coefficients) of substrates and products. We focus on one reaction and consider all internal metabolites that appear in the rate law. The enzyme cost, as a function of the log-concentrations of these metabolite only, reads
\[
y = h_e v \frac{\prod_{i \in \text{sub}} (1 + \frac{c_i}{k_i})^{m_{i}^{\text{sub}}} + \prod_{i \in \text{prod}} (1 + \frac{c_i}{k_i})^{m_{i}^{\text{prod}}} - 1}{k_+ \prod_{i \in \text{sub}} (\frac{c_i}{k_i})^{m_{i}^{\text{sub}}} (1 - e^{-\Theta(x)})}.
\]
The numerator is a polynomial of the form
\[
1 + \prod_{i} (\frac{c_i}{k_i})^{n_i} + ..., \tag{34}
\]
where all following terms are powers of metabolite concentrations with positive prefactors. Following the proof by Joost Hulshof, we now expand the denominator. The thermodynamic term alone would yield \(\frac{1}{1 - e^{-\Theta(x)}} = 1 + e^{-\Theta(x)} + e^{-2\Theta(x)} + ...\) (for positive driving forces \(\Theta\), this series converges). Multiplying this by \(k_+ \prod_{i \in \text{sub}} (\frac{c_i}{k_i})^{-m_{i}^{\text{sub}}}\), we obtain
\[
k_+ (\prod_{i \in \text{sub}} (\frac{c_i}{k_i})^{-m_{i}^{\text{sub}}})(1 + e^{-\Theta(x)} + e^{-2\Theta(x)} + ...)
= k_+ \prod_{i \in \text{sub}} (\frac{c_i}{k_i})^{-m_{i}^{\text{sub}}} + k_+ \prod_{i \in \text{sub}} (\frac{c_i}{k_i})^{-m_{i}^{\text{sub}}} e^{-\Theta(x)} + ...
= k_+ \prod_{i \in \text{sub}} (\frac{c_i}{k_i})^{-m_{i}^{\text{sub}}} + \prod_{i \in \text{prod}} (\frac{c_i}{k_i})^{m_{i}^{\text{prod}}} + ...
\]
where all remaining terms are powers of metabolite concentrations with positive cofactors. Multiplying this with the numerator and writing it in terms of log-concentrations, we obtain a series of terms, each describing a function that is positively curved in all log-concentrations. Thus, the cost curvature matrix for this reaction is positive definite with respect to the log-concentrations of all metabolites participating in the reaction. If we consider a kinetic model with CM rate laws, the cost curvature matrix will be a sum of cost curvature matrices for the single reactions. It will therefore be positive definite in all metabolites that appear in the enzymatic rate laws.

**E.6 Gradient of the enzyme-based flux cost Eq. (7) and identity between flux and enzyme point cost**

To compute the gradient \(\partial a / \partial v\) of an enzyme-based flux cost function, we consider a reference flow \(v^{\text{ref}}\) with optimal metabolite profile \(m^{\text{ref}} = m^{\text{opt}}(v^{\text{ref}})\). A small variation \(\delta v\) will change the optimal metabolite profile by \(\delta m\). The new enzyme-based flux cost can be approximated to first order:
\[
a(v^{\text{ref}} + \delta v) = q^{\text{enz}}(m^{\text{ref}} + \delta m; v^{\text{ref}} + \delta v)
\approx q^{\text{enz}}(m^{\text{ref}}; v^{\text{ref}}) + \frac{\partial q^{\text{enz}}(m^{\text{ref}}; v)}{\partial v} |_{v = v^{\text{ref}}} \delta v + \frac{\partial q^{\text{enz}}(m; v^{\text{ref}})}{\partial m} \delta m. \tag{36}
\]
The prefactor of \( \delta v \) in this formula yields the flux cost gradient \( \frac{\partial a}{\partial \nu_l} \):

\[
\frac{\partial a(\nu)}{\partial \nu_l} = \frac{\partial q_{enz}(m_{opt}(\nu); \nu)}{\partial \nu_l} = \frac{\partial}{\partial \nu_l} \frac{h_{e_l} \nu_l}{r_l(m_{opt}(\nu))} = \frac{h_{e_l}}{r_l(m_{opt}(\nu))},
\]

where \( m_{opt}(\nu) \) is the optimal metabolite profile for \( \nu \). We can therefore linearly expand the flux cost function as

\[
a(\nu + \delta \nu) \approx q_{enz}(m_{opt}(\nu); \nu + \delta \nu) = a(\nu) + q_{enz}(m_{opt}(\nu); \delta \nu)
\]

and obtain the flux point cost

\[
\frac{\partial a(\nu)}{\partial \nu_l} \nu_l = \frac{h_{e_l}}{r_l} \nu_l = \frac{h_{e_l}}{r_l} e_l = \frac{\partial h}{\partial e_l} e_l.
\]

The flux point cost is therefore identical to the enzyme point cost.

### E.7 Criterion for locally optimal flows (proposition 5)

Let \( \nu^{(1)} \) a polytope vertex to be checked from being locally optimal. Let \( \nu^{(2)}, \nu^{(3)}, \ldots \) be all other polytope vertices. For \( \nu^{(1)} \) to be locally optimal, any infinitesimal movement \( \delta \nu \) towards the interior of the B-polytope must increase the cost, i.e., \( a(\nu^{(1)} + \delta \nu) > a(\nu^{(1)}) \). According to SI section E.6, we can write the left side as \( a(\nu^{(1)}) + q(m; \delta \nu) \) and obtain the condition \( q(m; \delta \nu) > 0 \). Any vector \( \delta \nu \) can be written as a convex combination \( \varepsilon \sum_{k \neq 1} \eta_k (\nu^{(k)} - \nu^{(1)}) \) with an infinitesimal prefactor \( \varepsilon \), and since \( q \) is linear with respect to \( \nu \), we obtain the condition \( \varepsilon \sum_{k \neq 1} \eta_k q(m; \nu^{(k)} - \nu^{(1)}) > 0 \). To show that this holds true, we just need show that \( q(m; \nu^{(k)} - \nu^{(1)}) > 0 \) for any other vertex \( \nu^{(k)} \). Again, by linearity, this is equivalent to \( q(m; \nu^{(k)}) > q(m; \nu^{(1)}) \), which is true by assumption (\( \nu^{(1)} \) being the only privileged flow of \( m \)).

### E.8 Linear approximations of the enzyme-based flux cost function

The linearised flux cost function Eq. (8) is obtained by a linear expansion

\[
a(\nu) \approx a(\nu^{ref}) + \sum_l \frac{\partial a}{\partial \nu_l}|_{\nu^{ref}} (\nu_l - \nu^{ref}_l) = a(\nu^{ref}) + \sum_l \frac{\partial a}{\partial \nu_l}|_{\nu^{ref}} \nu_l - \sum_l \frac{\partial a}{\partial \nu_l}|_{\nu^{ref}} \nu^{ref}_l = \sum_l \frac{\partial a}{\partial \nu_l}|_{\nu^{ref}} \nu_l.
\]

The sum rule Eq. (2) was used to simplify the sum expression. This proof also holds for effective metabolite cost functions \( q(m) + q_{enz}(m; \nu) \) and the resulting kinetic flux cost functions. The gradient \( \frac{\partial q(m)}{\partial \nu_l} \) of the metabolite cost vanishes, so the formula for \( \frac{\partial a}{\partial \nu_l} \) remains unchanged. However, the optimal \( m \) vector, at which the gradient is evaluated, will be different.

### E.9 Nonlinear approximation of enzyme-based flux cost, computed from prototype flows

Enzymatic flux cost functions can be linearly or nonlinearly approximated. For a linear approximation, we choose a reference flow \( \nu^{ref} \) (in which all reactions must be active) and determine its privileged metabolite levels \( e^{ref}_l \). The enzyme-specific reaction rates in this state are called \( r^{ref}_l = r_l(e^{ref}) \). For our reference flow \( \nu^{ref} \), this yields the flux-specific enzyme costs

\[
\left( \frac{q_l}{\nu_l} \right)_{\nu^{ref}} = h_{e_l} \frac{e^{ref}_l}{\nu^{ref}_l} = \frac{h_{e_l}}{r^{ref}_l}.
\]
To approximate the flux cost function around $v_{\text{ref}}$, we treat these flux-specific costs as constant numbers and set

$$a(v) = \sum_{l} \frac{q_l}{v_{l}} v_l \approx \sum_{l} \left( \frac{q_l}{v_{l}} \right)_{\text{ref}} v_l = \sum_{l} \frac{h_{l}}{r_{l}^{'\text{ref}}} v_l,$$

(42)

where we used the sum rule Eq. (2) as well as Eq. (40). We obtain a linear flux cost function with cost weights defined by the specific rates $r_l$ obtained from our reference flow. For a nonlinear approximation, we apply the same procedure, but with several prototype flows $v(\alpha)$ instead of a single reference flow. For each prototype flow $v(\alpha)$ we determine the optimal metabolite profile and the resulting specific rates $r_l^{(\alpha)}$. To obtain a flux cost $a(v)$, we approximate the flow $v$ by a convex combination $v \approx \sum_{\alpha} \eta_{\alpha} v(\alpha)$ of the prototype flows. Then we use the weights $\eta_{\alpha}$ from this expansion to define a weighted mean of the prototype fluxes. The calculation works even if our prototype flows contain vanishing reaction fluxes, provided that the specific rates $r_l^{(\alpha)}$ do not vanish. If a reaction is exactly in thermodynamic equilibrium, or if it is completely inhibited, then $r_l^{(\alpha)}$ will vanish, and this reaction cannot be active, even with an arbitrarily high enzyme investment. Flows that contain such reactions cannot be used as prototype flows.

E.10 The cell growth rate as a convex function on the B-polytope

**Proposition 6** We assume that the enzyme cost $a(v)$, at a given biomass production can be converted into a cell growth rate $\lambda$ by a decreasing, convex cost-growth function $\lambda(a)$. The resulting growth rate $\lambda(a(v))$ is a convex function on the B-polytope. If the enzymatic flux cost $a(v)$ is strictly concave on the B-polytope, the growth rate $\lambda(a(v))$ is strictly convex on the B-polytope.

**Proof** Consider a strictly concave enzymatic flux cost $a(v)$ on the B-polytope and a monotonically decreasing, convex cost-growth function $\lambda(a)$. We consider two flows $v_A$ and $v_B$. Since $a(v)$ is strictly concave, we obtain

$$a(\eta v_A + [1 - \eta] v_B) > \eta a(v_A) + [1 - \eta] a(v_B),$$

(45)

where $0 < \eta < 1$. Since $\lambda(a)$ is monotonically decreasing, we obtain

$$\lambda(\eta v_A + [1 - \eta] v_B)) < \lambda(\eta a(v_A) + [1 - \eta] a(v_B))$$

(46)

and since $\lambda(a)$ is convex,

$$\lambda(\eta v_A + [1 - \eta] v_B)) < \eta \lambda(a(v_A)) + [1 - \eta] \lambda(a(v_B)).$$

(47)

---

14It may sometimes be preferable to approximate $v$ by a non-convex combination with positive weights $\eta_{\alpha}$ that do not sum to 1. In this case, the weighted mean $1/r_l^{'}$ is computed using the scaled weights $\eta_{\alpha}^{'} = \eta_{\alpha}/(\sum_{\alpha} \eta_{\alpha})$.

15The approximated flux cost function Eq. (44) is quadratic. To see this, we collect the prototype vectors $v_A, v_B, v_C, \ldots$ in a matrix and set $v = (v_A v_B v_C, \ldots)^{\top} \eta$. We can determine $\eta = (v_A v_B v_C, \ldots)^{\top} v$ by using the pseudoinverse of this matrix. Therefore, the vector $\eta$ is linear in $v$, and inserting $\eta$ into the expansion $a \approx \sum_{\alpha} \eta_{\alpha} h_{l} v_l = \sum_{l} \left( \frac{h_{l}}{r_{l}^{'\text{ref}}} \right) v_l$ yields a quadratic function in $v$. 

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Thus $\lambda(a(v))$ is strictly convex. Likewise, if $a(v)$ is non-strictly concave, then $\lambda(a(v))$ will be non-strictly convex (same proof, with inequality $\geq$ instead of $>$).

Typical cost-growth functions (as proposed in the literature [12, 10] are hyperbolically shaped and decreasing, and therefore convex. To derive such a growth formula $\lambda(r_{BM})$, we consider the biomass concentration $c_{BM}$ (given, e.g. in carbon moles / cell volume) and a biomass production rate $v_{BM}$. For a simple growth formula, we assume that the metabolic enzymes in the cell have a fixed total concentration $c_{ME}$. In this case, the cell growth rate is given by $\lambda = v_{BM} / c_{BM} = \frac{c_{BM}}{c_{ME}} = r_{BM} \rho_{ME}$ with the enzyme-specific biomass production rate $r_{BM} = 1/a$ (where the enzymatic cost $a$ refers to fluxes with unit biomass production) and the enzyme fraction (metabolic enzyme concentration / biomass concentration) $\rho_{ME})$. The growth rate is proportional to $r_{BM}$, and therefore inversely proportional to the enzymatic flux cost $a$. To obtain a second, more realistic growth formulae, we consider the fact that the enzyme fraction in the biomass is growth-rate dependent. This can be explained by a compromise between investments in metabolic enzymes and ribosomes. Following the resource allocation model of Scott et al. [12], one obtains a saturable (Michaelis-Menten-like) formula of the form $\lambda = \lambda_{max} \frac{r_{BM}}{r_{BM} + k}$ with a maximal growth rate $\lambda_{max}$ and a constant Monod constant $k$. The resulting cost-growth function has the hyperbolic form $\lambda(a) = \lambda_{max} \frac{k'}{k' + a}$ (see [10]).