Chapter 4

The space of metabolic flux distributions

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Chapter overview

- The metabolic capabilities of an organism can be related to the individual chemical reactions it can catalyze
- Elementary flux modes are minimal metabolic strategies that together span all metabolic capabilities.
- When the analysis of elementary flux modes is prohibited by computational limits, alternatives could be used, such as elementary conversion modes, flux sampling and minimal cut sets.

4.1. Modeling metabolic fluxes in cells

In the previous chapters we have seen that cells can convert substances from their environment into building blocks for cell components: their metabolism allows cells to grow, reproduce, repair themselves, and produce compounds needed to resist environmental stresses. But how does a cell manage this in detail, and does it have alternative metabolic strategies in case one does not function properly?

The overall metabolic conversion, for example from nutrients and oxygen to all necessary cell components and carbon dioxide, that a cell can use to grow and reproduce is in fact the consequence of many smaller chemical reactions working in concert. All chemical reactions that a cell can catalyze by expressing its enzymes form a very versatile 'metabolic network', which enables a cell to survive and grow, even when the availability of nutrients in its environment changes. There are various (semi-)automatized methods available that can be used to reconstruct this metabolic network from an organism's genome sequence, see Mendoza et al. for a review of the various methods [1]. In this chapter we will zoom in on this metabolic network and study the fluxes (reaction rates) through all individual reactions.

We call the combination of all reaction rates in a cell a 'metabolic flux distribution', and this flux distribution determines if and how a cell succeeds in taking up and converting the right nutrients to sustain itself. For a growing cell, we may ask: what will its flux distribution be, and how does this distribution change when its environment changes? Modeling metabolic fluxes allows us to answer specific questions, for instance about the change of a cell's metabolism after a gene is deleted: will it survive, and if so, will it take up different nutrients or produce different products? In contrast to the previous chapters, in the current and following chapters we are not satisfied with verbal descriptions, but seek predictive models that allow us to compute the state of a cell.

So how can we model metabolism in detail? Our main task is to describe and predict the uptake, conversion, and production of metabolites, as described by the metabolic fluxes. The rate at which a chemical reaction runs depends (through kinetics and thermodynamics) on metabolite concentrations and enzyme activities. Since enzymes are synthesized by the cell itself, the reaction rates are not only controlled by external nutrient supply, but also by gene expression. These dependencies make this a complicated field of study: the metabolic fluxes depend on the enzyme levels and metabolite concentrations, while the metabolite concentrations are again determined by the balance of fluxes through reactions that produce and consume the metabolites. In turn, enzyme levels are determined by gene expression, which is dependent on both external conditions and internal needs (e.g. the enzyme expression may change when different macromolecules need to be made in different phases of the cell cycle). To make matters even less transparent, most of the parameters (e.g. enzyme kinetic constants and details of enzyme regulation) are unknown.

For the moment, we therefore make some simplifying assumptions in order to obtain tractable models:

1. **Focus on small molecules** We focus on a subsystem of the cell, the metabolism of small molecules, which generates macromolecular precursors and energy carriers. All other processes (such as macromolecule synthesis) that happen "outside" our metabolic network are ignored.

2. **Ignore spatial structure** We largely ignore the spatial structure of cells: metabolite concentrations and reaction rates are assumed to be homogeneous across the cell. The exception to this rule occurs when there are cell compartments, in which case we describe the metabolites in both compartments as if they were separate compounds (e.g. cytosolic ATP vs mitochondrial ATP), which can be converted in each other through transport "reactions".

3. **Focus on fluxes as the only variables** Instead of considering metabolite concentrations, enzyme levels and metabolic fluxes together, we will only focus on metabolic fluxes. This has important consequences for the mathematical models that we will construct: many variables, and the corresponding equations, will be ignored. Additionally, fluxes cannot be computed through enzyme kinetics, so that we need to find other, non-mechanistic ways to compute the fluxes!

4. **Focus on steady-state metabolism** In a simplified picture of balanced growth (see the chapter on Balanced Growth), all metabolic processes are balanced: the rate at which material flows into the cell matches the rate at which it is converted, which again matches the production rate of macromolecule precursors. In addition, we assume that these fluxes are constant, such that the whole metabolic network is in a 'steady-state'. Taken together, we thus assume that the metabolic network can take up and produce external metabolites (e.g. extracellular metabolites and macromolecular precursors), but that all internal metabolites (inside the metabolic network) are mass-balanced, that is, for each of these metabolites, production and consumption cancel out.

5. **Describe precursor demand by a "biomass reaction"** We assume that cell growth (or: biomass production) requires a fixed set of macromolecule precursors in fixed proportions, corresponding to the average mixture of cell components that are necessary to make a cell. For metabolism, this means that the production of more macromolecule precursors only leads to more biomass production when the production of all precursors is scaled up proportionally. We formally express this by a hypothetical "biomass reaction" that consumes a mix of precursors and energy carriers in the predefined proportions. Hence, in the metabolic models we will describe the term "biomass" has a special meaning: while it usually means "the totality of compounds in a cell", here we use it for "the totality of compounds *outside* our metabolic model, which

metabolism needs to produce".

6. **Ignore dilution of small molecules** When a cell doubles its size but does not produce a certain metabolite, the concentration of this metabolite will halve. This basic principle is called 'dilution by growth', and in principle affects all compounds in the cell. During balanced growth, the production of macromolecules that are produced but not degraded should balance dilution, i.e. the number of each macromolecule should double when the cell doubles its size. This requires the rate of precursor supply to match the dilution rate, and hence the cell's growth rate. Similarly, small molecules are diluted, but since these are also degraded by consuming reactions, the rate of dilution is usually negligible compared to the production and consumption by metabolic reactions. Therefore, the models below will usually ignore the dilution of such metabolites.

7. **Constrain solutions by modeling limited resources** Since each enzyme has a maximal catalytic rate (the k_{cat} value), a reaction flux will require a certain (minimal) amount of enzyme, which takes up cellular space; since cellular space is limited, fluxes cannot increase infinitely since there is always an upper bound on a weighted sum of reaction fluxes. This constraint implies compromises between different reaction fluxes: one flux can only be increased at the expense of others.

With these assumptions, we are converging on a mathematical model: we know which variables to describe (the metabolic fluxes in steady-state metabolism), which constraints to apply (the balance of production and consumption of all internal metabolites) and what main input information we need (the metabolic network, described by a list of chemical reaction equations). Importantly, the model will be able to describe compromise: for example, with a given carbon influx and assuming mass balance, the carbon atoms can either be used to generate energy or biomass; if one function increases, the other one goes down. To obtain realistic predictions, we may introduce additional constraints, for example known flux directions or experimentally measured uptake rates. All this information will not suffice to predict metabolic fluxes precisely, but it allows us to narrow down the possible flux distributions. Importantly, all formulae in these models are linear, which makes them tractable even for very large model sizes (with thousands or even hundreds of thousands of variables).

Notably, all these assumptions depend only on the list of chemical reaction equations (the stoichiometry of the metabolic network), and nothing needs to be known about enzyme kinetics. So if the networks are already known, what do we gain from this kind of modeling? Even if a metabolic network structure is known reaction by reaction, this does not mean that we understand the network-wide behavior, i.e. which overall flux distributions are possible, and what overall flux distributions are useful for the cell. Our aim here is to make the step from structural information (about the network) to physiological insights about how the network can be used. We can learn, for example, how much biomass can be made from a certain amount of glucose, and whether an enzyme deletion is lethal because a certain precursor cannot be produced anymore.

Metabolic network structures (in the form of stoichiometric matrices) are approximately known for many microbial species, and to some extent for higher organisms. Together with the constraints outlined above, this network determines a range (or space) of possible flux distributions. In this chapter we will characterize this space of possible flux distributions according to our assumptions, and since we characterize fluxes entirely by constraints the models will be called "constraint-based models". We will get to know mathematical tools to characterize this space in a simple way: for instance, to describe all possibilities that a metabolic network provides we can use Elementary Flux Modes (EFMs).

In the next chapter, we will combine such constraint-based models with optimality principles: out of the

space of possible flux distributions, specific "optimal" flux distributions will be selected because these are supposedly "most profitable", either for the cell or for metabolic engineering purposes. Some of the flux prediction methods that we will describe refer also to concentrations; for instance, metabolite concentrations play a role in thermodynamic constraints that exclude certain flux directions, and enzyme concentrations come into play in models that associate fluxes with an enzyme demand. However, in all cases, the connection between fluxes and concentrations is very simple, and real enzyme kinetics are ignored. In later chapters, we will then see how the models change when more and more of the complex details are added about metabolite concentrations, enzyme kinetics, and thermodynamics.

4.2. The flux cone

4.2.1. Mass-balance constraints

As described in the introduction, our models will be built on the metabolic network of all chemical reactions that an organism can catalyze. We can conveniently summarize all these chemical reactions as an $(m \times n)$-dimensional stoichiometric matrix $\mathbf{N}$ where each of the m rows corresponds to a metabolite at steady state and each of the n columns to a given reaction. The entry $\mathbf{N}_{ij}$ is the coefficient of the i-th metabolite in the j-th chemical reaction equation. Then, we can gather all n reaction rates in an n-dimensional *flux vector*: $\mathbf{v} = (v_1, \ldots, v_n)^T$. This is convenient because the multiplication $\mathbf{Nv}$ now captures the net production and consumption of all m metabolites at this flux distribution, and is therefore equal to the time derivative of the metabolite concentrations: $\dot{\mathbf{c}} = \mathbf{Nv}$. Therefore, the steady-state assumption combined with the assumption that dilution of metabolites due to growth is negligible, can be mathematically captured in a set of linear equations that we call the *mass-balance constraints* on $\mathbf{v}$:

$$\dot{\mathbf{c}} = \mathbf{Nv} = \mathbf{0}. \tag{4.1}$$

Since in a typical metabolic reaction network the number of metabolites is smaller than the number of reactions ($m < n$), the equations for $\mathbf{v}$ are under-determined. This means that there are infinitely many solutions, $\mathbf{v}$, that satisfy the mass-balance constraints. The space of all such $\mathbf{v}$ is called the *null space* of $\mathbf{N}$.

In the absence of any additional constraints on $\mathbf{v}$, each v_i can take on both positive and negative values, where a negative value would mean that the reaction runs in the reverse direction. However, it will often be more intuitive to think of reaction rates as positive quantities, for example when we want to deduce necessary enzyme levels from the reaction rates by assuming that enzyme levels are directly proportional to the catalyzed reaction rate: $v_i \propto e_i$. Therefore it is often convenient to replace each reversible reaction by a forward irreversible reaction v_i^+ and a backward irreversible reaction v_i^-. Mathematically, we thus introduce non-negative variables $v_i^+, v_i^- \geq 0$ such that $v_i = v_i^+ - v_i^-$. The mass-balance constraints in these new variables become

$$\mathbf{0} = \mathbf{Nv} = \mathbf{Nv}^+ - \mathbf{Nv}^- = \begin{pmatrix} \mathbf{N} & -\mathbf{N} \end{pmatrix} \begin{pmatrix} \mathbf{v}^+ \\ \mathbf{v}^- \end{pmatrix} \tag{4.2}$$

where $\mathbf{v}^+ = (v_1^+, \ldots, v_n^+)^T$ and $\mathbf{v}^- = (v_1^-, \ldots, v_n^-)^T$, respectively. The mass-balance constraints (4.2) combined with the property that $v_i^+, v_i^- \geq 0$ can be expressed in the form

$$\mathbf{A} \begin{pmatrix} \mathbf{v}^+ \\ \mathbf{v}^- \end{pmatrix} \geq \mathbf{0} \tag{4.3}$$

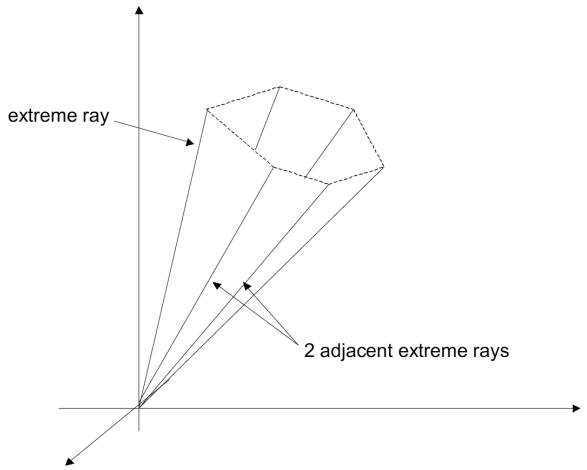


Figure 4.1: A pointed polyhedral cone,

where

$$\mathbf{A} = \begin{pmatrix} \mathbf{N} & -\mathbf{N} \\ -\mathbf{N} & \mathbf{N} \\ \mathbf{I} & \mathbf{0} \\ \mathbf{0} & \mathbf{I} \end{pmatrix}.$$

In this form the set of constraints on $(\mathbf{v}^+, \mathbf{v}^-)^T$ define a *polyhedral cone* and from the condition $\mathbf{v}_i^+, \mathbf{v}_i^- \geq 0$ we see that the cone is also *pointed*, meaning it contains no complete line and the zero vector is the only vertex (extreme point) of the cone (see Figure 4.1 for an illustration). The space of solutions that satisfies (4.3) is called the *flux cone*. It is important to remember that we only get a pointed polyhedral cone because we have chosen a representation where all reactions are irreversible; it is certainly possible to do useful analyses in the original space with reversible reactions $\mathbf{v}$. In what follows however, we will simplify notation by identifying $\mathbf{v}$ with $\mathbf{v} = (\mathbf{v}^+, \mathbf{v}^-)^T$ and use $\mathbf{N}$ in place of $(\mathbf{N}, -\mathbf{N})$ with the implicit understanding that all components of $\mathbf{v}$ are non-negative and $\mathbf{N}$ accounts for all forward and reverse reactions that exist in the network. In this notation the flux cone is defined as the space

$$\mathcal{FC} = \{\mathbf{v} \mid \mathbf{N}\mathbf{v} = \mathbf{0},\ \mathbf{v} \geq \mathbf{0}\} \tag{4.4}$$

where notation $\mathbf{v} \geq \mathbf{0}$ demands that each component of $\mathbf{v}$ is non-negative.

To provide a concrete example, we consider the simple representation of central carbon metabolism presented in Figure 4.2. In this example there are four external metabolites, G_{ex}, O, P_1, P_2 and two internal metabolites: G_{in} and P. In our model we only require mass-balance for internal metabolites, such that the steady-state constraint can be written as

$$\mathbf{N}\mathbf{v} = \begin{pmatrix} 1 & -1 & 0 & 0 & 0 \\ 0 & 2 & -1 & -1 & 1 \end{pmatrix} \begin{pmatrix} v_0 \\ \vdots \\ v_4 \end{pmatrix} = \mathbf{0}, \tag{4.5}$$

where each column thus corresponds to one of the five reactions and where the rows correspond to G_{in} and P respectively. The 1 in the first row of the first column thus corresponds to the import of one glucose molecule G_{in}. In a small example like this, it is still tractable to write out all separate steady-state equations:

$$0 = v_0 - v_1, \quad 0 = 2v_1 - v_2 - v_3 + v_4. \tag{4.6}$$

These two mass-balance constraints combine with the non-negativity conditions $v_0, v_1, v_2, v_3, v_4 \geq 0$ to define the flux cone as the space of all flux vectors, $\mathbf{v}$, that satisfy all of these constraints simultaneously.

4.2.2. Elementary flux modes

Although (4.4) already gives a mathematical description of the flux cone, we will here derive a more useful characterization of this space. One of the main problems of the description in (4.4) is that it does not give us a method to generate (or express in mathematical terms) a steady-state flux distribution, even though it makes it easy to check that any $\mathbf{v}$ is in $\mathcal{FC}$. Below, we will instead introduce an exhaustive set of generators: minimal flux distributions that can be combined to make all possible flux distributions in $\mathcal{FC}$, called *elementary flux modes* (EFMs). One can think of these EFMs as minimal building blocks that generate the flux cone, similar to how basis vectors generate a linear space, but with a particular rule for combining them as we describe below.

An important property of pointed polyhedral cones such as $\mathcal{FC}$ is that there exists a unique minimal set of n-dimensional *generators* $\{\mathbf{e}^1, \ldots, \mathbf{e}^K\}$ such that $\mathcal{FC}$ can also be represented as

$$\mathcal{FC} = \left\{ \mathbf{v} \;\middle|\; \mathbf{v} = \sum_{k=1}^{K} \lambda_k \mathbf{e}^k,\ \lambda_k \geq 0\ \forall k \right\}. \tag{4.7}$$

We must remark here that the generators $\mathbf{e}^k$ are only defined up to scalar multiplication, i.e. any $\alpha\mathbf{e}^k$ with $\alpha > 0$ could replace $\mathbf{e}^k$ in the set of generators. Each generator $\mathbf{e}^k$ represents one of the K *extreme rays* or "edges" of the pointed cone, and for metabolic reaction networks they turn out to have a particularly useful biological interpretation, which we shall see shortly. In essence, property (4.7) says that any flux vector $\mathbf{v}$ in the space $\mathcal{FC}$ can be expressed as a conical combination of generators $\{\mathbf{e}^1, \ldots, \mathbf{e}^K\}$.

To illustrate the concept of EFMs, we return to the simple representation of central carbon metabolism presented in Figure 4.2 that is associated with the stoichiometric matrix

$$\mathbf{N} = \begin{pmatrix} 1 & -1 & 0 & 0 & 0 \\ 0 & 2 & -1 & -1 & 1 \end{pmatrix} \tag{4.8}$$

and corresponding flux vector $\mathbf{v}$ with components $v_0, v_1, v_2, v_3, v_4 \geq 0$. A set of EFMs that serve as generators for the resulting flux cone is given by

$$\mathbf{e}^1 = \begin{pmatrix} 1 \\ 1 \\ 2 \\ 0 \\ 0 \end{pmatrix}, \quad \mathbf{e}^2 = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 1 \\ 1 \end{pmatrix}, \quad \mathbf{e}^3 = \begin{pmatrix} 1 \\ 1 \\ 0 \\ 2 \\ 0 \end{pmatrix}, \tag{4.9}$$

and these are depicted in Figure 4.3. In fact, because we split the reversible reactions into a forward and backward reaction, the combination of reactions v_2 and v_4 would also be an EFM, but we discard such cycles created by splitting reversible reactions. We see from our understanding of central carbon metabolism that these three EFMs represent the fundamental metabolic pathways of glycolytic fermentation ($\mathbf{e}^1$), oxidative metabolism of the fermentation product ($\mathbf{e}^2$), and oxidative metabolism of glucose ($\mathbf{e}^3$). The definition of the flux cone in terms of EFMs as in (4.7) is in this example equivalent to the statement that any flux vector

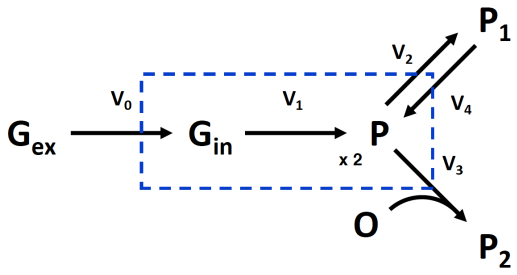


Figure 4.2: A simple representation of the metabolic reaction network for central carbon metabolism. Extracellular glucose, G_{ex}, is imported into the cell via reaction with flux v_0 and converted via intracellular glucose, G_{in}, to pyruvate, P, via the reaction with flux v_1 that has a stochiometry coefficient of two pyruvate molecules to each glucose molecule. Pyruvate can then either be converted to a fermentation product, P_1, via the reaction with flux v_2 or, in the presence of oxygen, O, converted to an oxidative phosphorylation (OXPHOS) terminal product P_2 via the reaction with flux v_3. The fermentation product P_1 can also be converted back to pyruvate via the reaction with flux v_4.

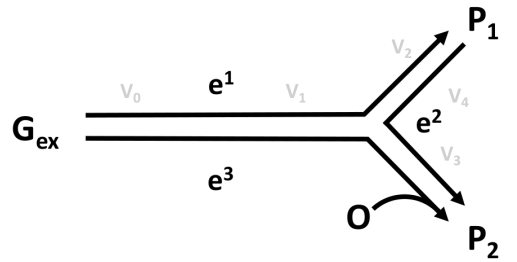


Figure 4.3: EFMs $\mathbf{e}^1, \mathbf{e}^2, \mathbf{e}^3$ overlaid on the simple metabolic reaction network in Figure 4.2 with concentrations of intracellular glucose and pyruvate assumed to be at steady state. It can be seen from our understanding of central carbon metabolism that $\mathbf{e}^1$ represents the glycolytic fermentation pathway, $\mathbf{e}^2$ the oxidative metabolism of the fermentation product, and $\mathbf{e}^3$ the oxidative metabolism of glucose.

$\mathbf{v}$ in our toy carbon metabolism network can be viewed as a (non-negative) weighted combination of these three minimal metabolic pathways. This interpretation remains true for any metabolic reaction network: *elementary flux modes represent the minimal metabolic pathways through the metabolic reaction network at steady state*.

We can make this more precise with a mathematical definition. First, let us introduce the *support* of a vector as $\text{supp}(\mathbf{v}) = \{i \mid v_i \neq 0\}$, i.e. the support of a flux vector is the set of reactions that have a non-zero rate.

Definition 1. *A vector $\mathbf{v}$ is an EFM if and only if it satisfies the following two properties:*

1. $\mathbf{v} \in \mathcal{FC}$,
2. *for all* $\mathbf{v}' \in \mathcal{FC}$, *if* $\text{supp}(\mathbf{v}') \subseteq \text{supp}(\mathbf{v})$ *then either* $\mathbf{v}' = \mathbf{0}$ *or* $\mathbf{v}' = \alpha\mathbf{v}$ *for some* $\alpha > 0$.

This means that $\mathbf{v}$ is an EFM only if there is no non-zero flux vector in the flux cone that uses only a subset of the reactions that are active in $\mathbf{v}$. This also means that if any of the flux-carrying reactions in an EFM is deleted, the flux through the resulting reactions must violate the mass-balance constraints and can therefore not occur in steady-state metabolism; the EFMs are thus minimal in the sense that they cannot be reduced further.

One may now wonder how it is possible that we have seen two definitions of EFMs. First, we introduced them as the extreme rays of the flux cone; then, we introduced them as support-minimal metabolic subnetworks. Indeed, the beautiful thing about EFMs is that these two characterizations are equivalent (see the

Mathematical details-box for a proof). These two definitions of EFMs are complementary. Understanding EFMs as minimal metabolic subnetworks enables us to interpret an EFM in terms of its biological function; an EFM can be seen as a metabolic strategy that a cell can use to obtain steady-state metabolism, and which it can combine with other strategies to reach its purpose. The extreme ray-interpretation on the other hand, allows us to write an arbitrary flux vector $\mathbf{v} \in \mathcal{FC}$ as a combination of EFMs, as is done in (4.7). This also means that we can learn something about all flux vectors $\mathbf{v}$ by learning something about all EFMs. For example, if we know that there is no EFM that produces compound Y without using reaction r, this immediately implies that there is no flux vector at all that can do this, and that reaction r is thus essential for the production of Y. In addition, as we will discuss below, the identification of EFMs as extreme rays enables the use of efficient computational algorithms to enumerate them.

Mathematical details 4.A : Characterizations of EFMs

In this box we will prove (following Gagneur and Klamt [2]) that the two characterizations of elementary flux modes that we gave in this chapter are equivalent. To prove this, we should first make precise what we mean when we define EFMs as 'extreme rays' of the flux cone. Let us start generally: a pointed polyhedral cone $\mathcal{P}$ is defined by a full rank matrix $\mathbf{A}$ such that

$$\mathcal{P} = \{\mathbf{x} \in \mathbb{R}^n : \mathbf{A}\mathbf{x} \geq \mathbf{0}\},$$

(as illustrated in Figure 4.1). Such a cone is thus defined as all points that satisfy a certain set of linear inequalities, which can be seen as a region bounded off by halfspaces. Note that the definition of the flux cone as given in (4.4) can be written in this form by taking $\mathbf{A} = [\mathbf{N}, -\mathbf{N}, \mathbf{I}_{n\times n}]^T$, where $\mathbf{I}_{n\times n}$ is the identity matrix, so $\mathcal{FC}$ is indeed a pointed polyhedral cone.
A vector, $\mathbf{r}$, is a ray of $\mathcal{P}$ if $\alpha\mathbf{r} \neq \mathbf{0}$ and for all $\alpha > 0$ we have $\mathbf{r} \in \mathcal{P}$. Each ray $\mathbf{r} \in \mathcal{P}$ has a zero set defined as $Z(\mathbf{r}) = \{i : \mathbf{A}_{i\bullet}\mathbf{r} = \mathbf{0}\}$. Thus, the zero set is the index set of inequalities that are met with equality by the ray $\mathbf{r}$. We call $\mathbf{r}$ an extreme ray when for all $\mathbf{r}' \in \mathcal{P}$ if $Z(\mathbf{r}) \subseteq Z(\mathbf{r}')$ then $\mathbf{r}' = \alpha\mathbf{r}$ for some $\alpha > 0$. In other words, a ray is called extreme if the set of inequalities that it satisfies with equality cannot be increased. With this we are ready for specifying our second definition of EFMs, after which we can prove the equivalency of the two definitions.

Definition 2. *A vector $\mathbf{v}$ is called an elementary flux mode if it is an extreme ray of the flux cone $\mathcal{FC}$.*

Lemma 1. *In a metabolic network captured by stoichiometric matrix $\mathbf{N}$ in which all reactions are irreversible, the definitions of elementary flux modes as the extreme rays of the flux cone (Def. 2) and as support-minimal steady-state flux vectors (Def. 1) are equivalent.*

Proof. Let $\mathbf{v}$ be an elementary flux mode according to Definition 1. The first requirement in this definition immediately implies that $\mathbf{v}$ is a ray of the flux cone, where we can define the flux cone as all $\mathbf{x} \in \mathbb{R}^n$ such that $\mathbf{A}\mathbf{x} \geq \mathbf{0}$ with: $\mathbf{A} = [\mathbf{N}, -\mathbf{N}, \mathbf{I}_{n\times n}]^T$. To show that it is also an extreme ray, let us assume that there is another ray $\mathbf{v}'$ such that $Z(\mathbf{v}) \subseteq Z(\mathbf{v}')$. Since all rays of $\mathcal{FC}$ must satisfy the first $2m$ inequalities, this specificially means that whenever $v_i = 0$ for some $1 < i < n$, also $v'_i = 0$, i.e. $\mathrm{supp}(\mathbf{v}') \subseteq \mathrm{supp}(\mathbf{v})$, but according to Property 2 of Definition 1 we must then have $\mathbf{v}' = \alpha\mathbf{v}$. This implies that $\mathbf{v}$ is indeed an extreme ray, so it is also an EFM according to Definition 2.
For the converse, let $\mathbf{v}$ be an EFM according to Definition 2. Again, this immediately shows that $\mathbf{v} \in \mathcal{FC}$, so we should now show that it is support-minimal. For that, let $\mathbf{v}' \in \mathcal{FC}$ such that $\mathrm{supp}(\mathbf{v}') \subseteq \mathrm{supp}(\mathbf{v})$. This means that whenever $v'_i = 0$, also $v_i = 0$. Since $\mathbf{v}$ and $\mathbf{v}'$ both satisfy the first $2m$ inequalities of $\mathbf{A}\mathbf{v} \geq \mathbf{0}$ with equality, and this shows that whenever $\mathbf{v}'$ saturates one of the last n inequalities, then also $\mathbf{v}$ does this, we conclude that $Z(\mathbf{v}) \subseteq Z(\mathbf{v}')$. Using Definition 2 this implies that $\mathbf{v}' = \alpha\mathbf{v}$ for some $\alpha > 0$. This shows that $\mathbf{v}$ is indeed support-minimal, and is thus an EFM according to Definition 1. □

We note that there is currently no limit on the amount of flux that a particular EFM may carry, since $\lambda \mathbf{e}^k$ is also an EFM for any $\lambda > 0$ when $\mathbf{e}^k$ is an EFM, and consequently the absolute value of any flux vector $\mathbf{v}$ in $\mathcal{FC}$ remains unbounded. However, we will see in the next section that this is not necessarily true when additional constraints are introduced.

4.2.3. Irreversibility constraints

In principle, all reactions in a metabolic reaction network are able to run in both directions, but in many practical examples certain thermodynamic arguments can be used to justify treating a subset of reactions as *irreversible*, meaning that for all modeling purposes they can only run in one direction. This stems from their activation energy being so large for realistic levels of products and substrates that the rate of the reverse reaction is effectively negligible compared to the rate of the forward. The choice of which reactions to assume irreversible is a decision to be taken by the modeler and may affect the results of the downstream constraint-based analysis. Returning to the simple model for central carbon metabolism provided in Figure 4.2, the reactions importing glucose into the cell, converting it to pyruvate, and the production of product P_2 are assumed to be irreversible. A more detailed representation of central carbon metabolism may allow for the first two reactions to be reversible such that 'gluconeogenesis' becomes possible. This would result in the introduction of a fourth EFM associated with the conversion, via pyruvate, of the fermentation product to glucose that is then exported out of the cell.

More generally, in our mathematical description of the metabolic network (4.4), we decomposed each reaction into a forward and a reverse reaction, treating each as individual degrees of freedom, and imposing additional irreversibility constraints is thus as simple as removing a reverse reaction from the network. When this is done, the EFMs of the resulting network do not need to be re-calculated, but can be obtained by simply removing all the EFMs that use the removed directions. This nicely shows the complementarity of Definitions 1 and 2 of EFMs: although it is not easy to see that the set of extreme rays of the new flux cone will be a subset of the previous set of extreme rays, it is easy to see that each vector that is a support-minimal flux vector after the removal of some reactions, must have been a support-minimal flux vector before that. The new flux cone is therefore made up of all flux vectors that are expressed as conical combinations of the remaining EFMs, and remains non-bounded.

4.2.4. Practical uses of elementary flux modes

EFMs represent the full set of possible metabolic capacities of an organism, which can therefore make EFM analysis a useful tool for biology. To this end, application of EFM analysis to bioengineering has been proposed to guide the genetic manipulation of microorganisms to perform desirable properties such as synthesis of a bio-compound or efficient production of a recombinant protein (e.g. [3, 4]). From a more theoretical point of view, EFMs have also been used in attempts to quantify cellular robustness [5], in particular regarding robustness under genetic perturbations [6]. The relevance of elementary flux mode analysis to cellular robustness stems from the fact that there is rarely a unique conical combination of elementary flux modes for any given flux vector, which implies there are multiple combinations of minimal metabolic pathways to achieve the same desired effect. This redundancy can be interpreted as a measure for the metabolic robustness of an organism, in terms of preserving essential metabolic functionalities under loss of a gene, for example.

There have also been several ways that EFM analysis has been incorporated into analysis of multi-omics data. For example, on the basis of transcriptomic profiling of microorganisms, metabolic pathways associated with elementary flux modes have been scored according to their probability of carrying flux [7]. The principle here is that, although levels of RNA often serve as a poor proxy for flux through the reaction associated

with that particular enzyme's gene, by creating a gene set associated with an entire EFM there might be a better chance of concretely assessing whether the metabolic pathway as a whole is likely to carry flux. The study [7] suggested that the integration of EFM analysis with gene expression data enabled the identification of certain metabolic pathways activated during stress conditions, and that the organization of elementary flux mode utilization in *Saccharomyces cerevisiae* involves a disparate combination of highly specialized and multi-tasking roles. Beyond transcriptomic profiling, isotope tracing experiments in principle provide a much more direct insight into quantifying metabolic flux. To interpret isotope tracing data, an extension of the concept of an EFM was introduced in [8].

4.2.5. Computational challenges for elementary flux mode analysis

Enumerating EFMs for large networks can be computationally challenging if not impossible. In principle, EFMs can be found by removing one reaction at a time and solving the resulting mass-balance constraint problem until it is no longer possible to remove a reaction and still obtain a flux vector that satisfies the steady state conditions. However, the one-to-one identification of EFMs with extreme rays of the flux cone that we described before enables the use of algorithms that are specialized in the efficient enumeration of extreme rays of polyhedral cones, such as the double description method [9]. Various tools have been developed for elementary flux mode enumeration based on this algorithm (e.g. EFMTOOL [10] or MetaTool [11]). However, when the size of the metabolic reaction network grows, the number of EFMs scales disproportionately, leading to a combinatorial explosion that effectively makes enumeration impossible for genome-scale networks containing several thousands of reactions [12]. Currently, EFM analysis is therefore restricted to medium-scale reconstructions containing on the order of several hundreds of reactions, and results in the identification of several hundred million EFMs (e.g. enumeration based on the *Escherichia coli* core model results in approximately 272 million EFMs).

Approaches to reduce the complexity of dealing with so many EFMs even for metabolic reaction networks of modest size have also been proposed. These include invoking transcriptional regulatory constraints to eliminate most of the EFMs to be considered in downstream analysis. Imposing additional constraints based on thermodynamic conditions similarly reduces the set of EFMs considerably. A problem with these approaches is evidently that they do still depend on an initial calculation of all EFMs, and so do not solve the problem of enumeration complexity. A rigorous study of the complexity of EFM mode enumeration was performed by Acuña and colleagues [13]. They showed that the decision problem if there exists an EFM containing two specific reactions is NP-complete whilst the complexity of enumerating all EFMs remains open.

Later in this chapter we will explore some alternatives to EFM enumeration that reduce the difficulty of enumeration, cf. Section 4.4.

4.3. Additional constraints and flux polyhedra

4.3.1. Inhomogeneous linear flux constraints

We have so far been working exclusively with mass-conservation and irreversiblity constraints, which are captured entirely by the stochiometric matrix where each row is associated with a metabolite concentration at steady state. We also saw that these considerations alone result in a flux cone that is by definition unbounded, meaning that a flux vector in this space is allowed to take on any absolute value (i.e. multiplying a flux vector in the flux cone by an arbitrarily large positive number again returns a flux vector in the flux cone). However, there are physical constraints limiting the magnitude of flux vectors, especially on the values

of flux through exchange reactions that may depend on concentrations of extracellular substrates, numbers of transporter molecules in the membrane, or for which we might have direct experimental measurements. Typically, such bounds on flux values are imposed using inequality constraints of the form $v_i^{lb} \leq v_i \leq v_i^{ub}$ where v_i^{lb} and v_i^{ub} are lower and upper bounds, respectively, for the flux through the ith reaction. When reactions have been decomposed into forward and reverse directions, both upper and lower bounds are non-negative where the latter is usually zero.

In the example from Figure 4.2 one may impose an upper bound on the flux value v_0, suggesting that there is a maximal rate at which the cell or organism can import glucose from the extracellular environment. In this case the total set of constraints on the flux vector $\mathbf{v}$ take the form

$$\mathbf{N}\mathbf{v} = \mathbf{0}, \quad \mathbf{v} \geq \mathbf{0}, \quad v_0 \leq v_0^{ub} \tag{4.10}$$

where v_0^{ub} is the maximal glucose uptake rate. It is important to note that the new constraint is of a different kind than the mass-balance and irreversibility constraints: the right-hand side of the constraint is non-zero. Constraints that involve a non-zero are called *inhomogeneous constraints*. We can write these constraints in matrix form as

$$\mathbf{A}\mathbf{v} \geq \mathbf{b} \tag{4.11}$$

with

$$\mathbf{A} = \begin{pmatrix} \mathbf{N} \\ -\mathbf{N} \\ \mathbf{I} \\ \mathbf{G} \end{pmatrix}, \quad \mathbf{b} = \begin{pmatrix} \mathbf{0} \\ \mathbf{0} \\ \mathbf{0} \\ \mathbf{h} \end{pmatrix}, \tag{4.12}$$

where in this particular case

$$\mathbf{G} = \begin{pmatrix} -1 & 0 & 0 & 0 & 0 \end{pmatrix}, \quad \mathbf{h} = \begin{pmatrix} -v_0^{ub} \end{pmatrix}. \tag{4.13}$$

In general, the matrix $\mathbf{G}$ will have P rows corresponding to P linear, inhomogeneous constraints of the form

$$\sum_i w_i^p v_i \leq h_p, \quad p = 1, \ldots P \tag{4.14}$$

where each h_p corresponds to a component of the P-dimensional vector $\mathbf{h}$ and n weights w_i^p $(i = 1, \ldots, n)$ are supplied for each constraint. Many constraints can be written in this general form, for example, one might imagine modeling a bound on the total flux that a cell can catalyze, which would be captured by setting all weights equal to 1. In this form the set of constraints on $\mathbf{v}$ define a *general polyhedron* that is necessarily contained within the flux cone which was based only on the homogeneous constraints: $\mathbf{N}\mathbf{v} = \mathbf{0}$ and $\mathbf{v} \geq \mathbf{0}$. The additional inhomogeneous constraints serve to sequentially close up the cone such that various (if not all) dimensions become bounded, thus bounding the total magnitude of the flux vector $\mathbf{v}$.

In the example described above, bounding the extracellular glucose uptake rate puts an upper bound on the weights of EFMs $\mathbf{e}^1, \mathbf{e}^3$ illustrated in Figure 4.3, whose support includes the glucose transport reaction. However, the weight of the EFM associated with uptake and oxidation of the fermentation product ($\mathbf{e}^2$) can remain unbounded. In short, this restricts the values of weights $\lambda_1, \lambda_2, \lambda_3$ in the representation (4.7) to satisfy

$$\lambda_1, \lambda_2, \lambda_3 \geq 0, \quad \lambda_1 + \lambda_3 \leq v_0^{ub}.$$

Recalling that each EFM is associated with an extreme ray of the flux cone coming from mass-balance

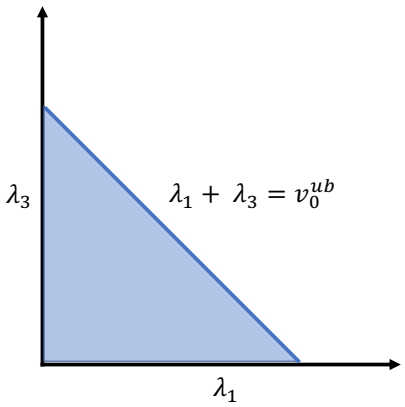


Figure 4.4: Representation of the bounded convex plane within which a flux vector satisfying the mass-balance and maximal glucose uptake constraints must lie. The possible combinations of EFM weights λ_1 and λ_3 are contained within or on the line in blue given by the equation $\lambda_1 + \lambda_3 = v_0^{ub}$.

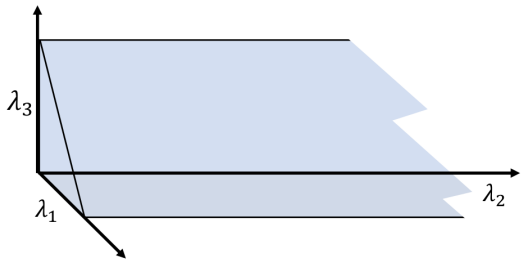


Figure 4.5: Geometry of the flux polytope (blue) containing any flux vector that satisfies the mass-balance and maximal glucose uptake rate constraints. While bounded in the directions parameterized by λ_1, λ_3, it remains unbounded in the direction parameterized by λ_2.

constraints, for this simple example it is quite straightforward to interpret the geometric consequences of the maximal glucose uptake rate. Any flux vector $\mathbf{v}$ in the resulting *flux polytope* is now constrained to lie within the bounded convex plane represented in Figure 4.4, but remains free to take on any value along the remaining extreme ray parameterized by λ_2. The maximal glucose uptake has therefore closed up the flux cone along two directions while leaving the third untouched, and the geometry of the resulting flux polytope is represented in Figure 4.5. Imposing an upper bound on the uptake rate of the fermentation product, of the form $v_4 \leq v_4^{ub}$, will serve to bound this remaining direction of the polytope such that weights of the EFMs are then restricted to the space defined by

$$\lambda_1, \lambda_2, \lambda_3 \geq 0, \quad \lambda_1 + \lambda_3 \leq v_0^{ub}, \quad \lambda_2 \leq v_4^{ub}. \tag{4.15}$$

More general constraints, for larger metabolic reaction networks will be more difficult to interpret and visualize in such simple geometric terms. Quite quickly the combinatorial complexity associated with combinations of multiple constraints and EFMs will become unmanageable. The intuitive treatment of inhomogeneous linear constraints is partially assisted using the concept of elementary flux vectors on which we will add a section in a later version of this book, but both geometrically and biologically these objects are nowhere near as easy to interpret as their EFM counterparts. We shall see that alternative computational methods for exploring flux space therefore become imperative.

As a final remark, we clarify once more that the general form of constraints (4.14) is by no means restricted to sums on the left hand side that involve just a single reaction and can of course include constraints on weighted sums of flux values for different reactions. These weighted sums are often associated with particular

biological interpretations: for example, in the metabolic reaction network from Figure 4.2 we might want to restrict our search of flux space to those flux vectors $\mathbf{v}$ that produce adenosine triphosphate (ATP) at a rate of at least v^{ATP}. Although a more elaborate model would of course include ATP as one of the metabolites, in this example we can use our biological understanding of central carbon metabolism to see that ATP is produced in reactions v_1 and v_3. A lower bound on ATP production would thus be a lower bound on a combination of v_1 and v_3 with weights determined by stoichiometry which depends on the organism under investigation. We could write such a constraint as

$$w_1 v_1 + w_3 v_3 \geq v^{ATP} \tag{4.16}$$

with appropriate weights w_1, w_3. Such a constraint forms an additional row of the matrix $\mathbf{G}$ and we leave it as an exercise for the reader to explore how this affects the geometry of the flux polytope for various values of the weights, minimal ATP production rate and maximal glucose and fermentation product uptake rates. Particular combinations of constraints will be impossible to satisfy simultaneously (i.e. when the minimal rate of ATP production is impossible to achieve under the given bounds on glucose and fermentation product uptake rates), resulting in a flux polytope that is empty. In such cases the set of constraints on $\mathbf{v}$ are called *infeasible*.

4.3.2. Thermodynamic constraints

In Chapter 3 the basic concepts of chemical thermodynamics were introduced, in particular, the Gibbs free energy of a metabolic reaction was defined in terms of the concentrations of its products and substrates. For a metabolic reaction network with stochiometric matrix $\mathbf{N}$, the vector of Gibbs free energies (one for each reaction in the network) $\mathbf{\Delta_r G'}$ can be written in matrix form as

$$\mathbf{\Delta_r G'} = \mathbf{\Delta_r G'^o} + RT \cdot \mathbf{N}^T \cdot \ln(\mathbf{c}) \tag{4.17}$$

where R is the gas constant (see section 3.3.2), T the temperature and $\mathbf{c}$ the vector of metabolite concentrations at steady state. The components of the vector $\mathbf{\Delta_r G'^o}$ are the changes in standard Gibbs free energy for each corresponding reaction. Typically, these values are not known precisely for reactions in the network, but can be estimated or approximated from experimental data using methods beyond the scope of this chapter. Similarly, although it is often difficult to accurately measure all metabolite concentrations, in principle the vector $\mathbf{c}$ can be obtained experimentally. However, in practice experimental data on $\mathbf{c}$ and $\mathbf{\Delta_r G'^o}$ are almost never available. Various methods have therefore been developed to combine estimation of $\mathbf{\Delta_r G'^o}$ (sometimes with partial measurements of $\mathbf{c}$) with advanced computational techniques that allow simultaneous optimization (see next chapter) or sampling (see below) of $\mathbf{v}$ and $\mathbf{c}$ (or equivalently: $\mathbf{\Delta_r G'}$).

The second law of thermodynamics applied to chemical reaction networks can be summarized by saying that every component of the metabolic flux vector $\mathbf{v}$ must satisfy the condition

$$\text{sign}(v_i) = -\text{sign}(\Delta_r G'_i) \tag{4.18}$$

where v_i and $\Delta_r G'_i$ are the ith components of $\mathbf{v}$ and $\mathbf{\Delta_r G'}$, respectively, and $\text{sign}(x)$ denotes the sign of a variable x, and $\text{sign}(0) = 0$. It is important to point out that this notation is different to that used previously, where we had assumed all v_i to be non-negative by decomposing each reaction into irreversible forward and backward reactions. Returning to this reversible notation simplifies the inclusion of thermodynamic constraints into constraint-based models and also their interpretation. According to the second law, a reaction can only proceed in a direction where the change in Gibbs free energy is negative. Thus, to be

consistent with mass-balance *and* the second law of thermodynamics, a flux vector $\mathbf{v}$ must simultaneously satisfy both (4.1) *and* (4.18), with $\mathbf{\Delta_r G'}$ defined in (4.17). The consequence of these additional constraints on the geometry of the space of metabolic flux distributions is to exclude quadrants incompatible with the signs of $\mathbf{\Delta_r G'}$. Equivalently, imposing the second law of thermodynamics on metabolic flux distributions removes regions of the space that are associated with combinations of thermodynamically-infeasible reaction directionalities.

The resulting space of feasible flux vectors is almost always non-convex, which means more advanced computational methods are required to explore it efficiently. The intuitive reason for this is that imposing thermodynamic constraints on top of the mass-balance constraint is usually done in terms of Boolean variables, which breaks the linearity of the problem that we had and exploited so far. Relating this to the EFMs that were discussed previously, it for example becomes clear that any EFM representing an internal cycle –not including any exchange reactions– will never be thermodynamically feasible. Thus, thermodynamic constraints also reduce the set of EFMs that are possible in a metabolic network. Interestingly, it turns out that any thermodynamically-feasible metabolic flux vector can be expressed solely in terms of thermodynamically-feasible EFMs [14], but the converse statement is not true: a linear combination of thermodynamically-feasible elementary flux mode does not necessarily satisfy the thermodynamic constraints. This shows how the workable properties of convex spaces break down as the mathematical models become more complex, in this case by accounting for thermodynamics.

4.4. Alternative methods for flux space exploration

As we described above, exploration of all possible flux distributions using EFMs can become very complex for larger models. A genome-scale model, which comprises all metabolic reactions that an organism can catalyze, typically contains thousands of reactions, which prohibits the enumeration of EFMs. At the moment, it is unclear whether, even if we would have an enormously fast computer that could compute all EFMs, the number of EFMs would not be so large that we cannot store the EFMs anywhere, nor analyze it in any meaningful way. Here we discuss several alternatives for exploring the metabolic capabilities of a cell that try to avoid the combinatorial complexity that hinders EFM analysis.

4.4.1. Elementary conversion modes

If we are interested in the metabolic capabilities of an organism, is it always necessary to know all possible flux vectors? For example, what if we want to lab-culture an organism of which we have a reconstructed metabolic network, but no idea what nutrients it needs to grow. Then we only need to know from what combinations of nutrients it can make all its cell components. Or, what if we want to model the possible cross-feeding interactions between several microbial species? Then we are mostly interested in what each of them can consume and produce, and not really in how they do that. Elementary conversion modes (ECMs), introduced in 2005 by Urbanczik and Wagner [15], capture all possible overall conversions from nutrients to products that an organism can catalyze, while ignoring which individual reactions are used for this.

ECMs focus on the net results of metabolism, i.e. on the uptake and production of compounds external to the metabolic network, such as sugars, nitrogen sources, fermentation products but also 'biomass'. To get information about these compounds we need to extend our metabolic network by including the external compounds as rows in the stoichiometry matrix; this is in general easy to do since we already had exchange reactions (reactions where an external compound was imported or exported) so we only have to find the stoichiometric coefficient in which the external compound was involved in these reactions. Let us denote the original stoichiometry matrix by $\mathbf{N}_{\text{int}}$ and the submatrix that we add by $\mathbf{N}_{\text{ext}}$; together they form $\mathbf{N}_{\text{tot}}$. We

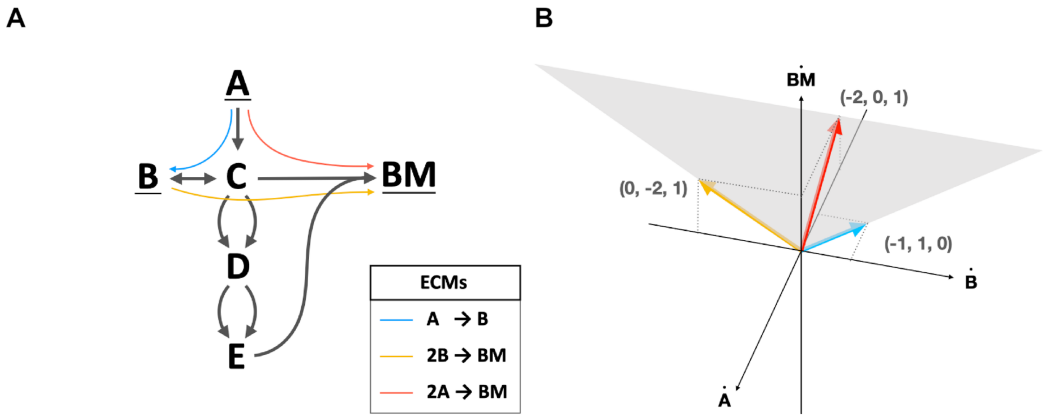


Figure 4.6: (A) Small toy network with three ECMs shown in blue, yellow and red. Note that the red mode can be decomposed as a positive combination of the blue and yellow elementary conversion modes, but that would cancel the production of B so this is not allowed. (B) The conversion cone is shown in gray, and the blue and yellow arrow correspond to the blue and yellow ECMs are the extreme rays. The red ECM needs to be added because it is on the intersection with the $\dot{B} = 0$-plane.

can then define the *conversion cone*:

$$\mathcal{C} = \{\dot{\mathbf{c}} = \mathbf{N}_{\text{ext}}\mathbf{v} \mid \mathbf{N}_{\text{int}}\mathbf{v} = \mathbf{0}, \mathbf{v} \geq \mathbf{0}\}\,. \tag{4.19}$$

If we look carefully at this definition we can see that the flux vectors $\mathbf{v}$ need to satisfy exactly the same constraints as in the flux cone (Eq. (4.4)). The only difference between flux and conversion cones is that we are either interested in the fluxes themselves, or rather in the conversions that they induce: $\dot{\mathbf{c}} = \mathbf{N}_{\text{ext}}\mathbf{v}$.

Definition 3. *The set of* ECMs *is the minimal set of conversions* $\{ecm^1, \dots ecm^K\}$ *(where* ecm^i_k *is the amount of metabolite* k *produced in the* i*th elementary conversion mode), such that*

1. *all conversions* $\dot{\mathbf{c}} \in \mathcal{C}$ *can be written as a positive sum of these elementary conversion modes:* $\dot{\mathbf{c}} = \sum_i \lambda_i ecm^i$*, with* $\lambda_i \geq 0$*,*
2. *without the production of any metabolite being canceled in that sum, i.e. for all metabolites* k *we either have for all* $\lambda_i > 0$ *that* $ecm^i_k \geq 0$ *or for all* $\lambda_i > 0$ *that* $ecm^i_k \leq 0$*.*

We will explain both parts of this definition below, but let us first remark that the definition is in fact perfectly analogous to the definition of EFMs: EFMs are the *elementary vectors* (or precisely: conformally non-decomposable vectors) of the flux cone, and ECMs of the conversion cone. The reason that the definition of ECMs has an additional requirement (2.) is just that the analogous requirement was automatically satisfied for EFMs because we assumed all reactions to be irreversible.

In Figure 4.6A we show a small metabolic network with external metabolites A, B and BM, and internal metabolites C, D and E. We can find 9 EFMs in this network: one that goes from A to B, four that produce BM starting from A and four that produce BM from B. We get four EFMs to go from A to BM because there are two ways of going from C to D and again two for converting D into E. This makes clear that having a number of modules of alternative reactions can quickly give rise to large numbers of EFMs, even though the overall conversion from nutrients to products remains the same. In contrast, we will explain that we only get three ECMs.

In Figure 4.6B we see the conversion cone in gray. Note that this cone does not live in flux space, but rather in the space of external metabolite changes, or conversions. We recognize that the cone can be spanned

by two extreme rays, which correspond to converting A into B (blue) and to using $2B$ to produce BM (yellow), so these rays correspond to elementary conversion modes following the first part of Definition 3. Now why do we have a third ECM, when the blue and yellow one already span the whole conversion cone? Indeed, the third vector in Figure 4.6B can be obtained by summing the yellow vector and two times the blue vector: $2(-1,1,0)+(0,-2,1)=(-2,0,1)$. However, note that the production of metabolite B would cancel in this sum, which is not allowed according to the second part of Definition 3. The reason that this second part of the definition is important, is that the elementary conversion modes are intended to capture all metabolic capabilities of an organism, so taking only the first two modes would not be enough: we also want to account for the possibility of making BM from A even if we decide that the elementary conversion mode from B to BM is not possible in the current environment, for example because B is not present as a nutrient in the medium.

Because many EFMs result in the same overall conversion, the exploration of metabolic capabilities can now be done in larger networks, at the cost of ignoring information about which reactions are used [16]. This way of thinking can be pushed even further: what if one is not interested in the conversions between all nutrients and products, but only between a subset of these? In that case, one would want to compute the ECMs only between the external metabolites of the most interest. This can be done with a small trick. Say that we are not interested in the production of external metabolite X. Before we start the enumeration algorithm we add a virtual reaction to the network that consumes and produces X from nothing, i.e. we add $X \rightleftarrows \emptyset$, and then we change X from an external metabolite to an internal metabolite. Consequently, it now has to satisfy the mass-balance constraint (which can always be done trivially using the added virtual reaction), and will thus never show up in the computed elementary conversions. In this way it was possible to compute all ECMs between glucose, oxygen and biomass for a real genome-scale network of *E. coli*.

4.4.2. Flux sampling

In addition to the computational complexity of EFM enumeration for large metabolic networks, these objects are not necessarily related to experimentally-derived flux measurements. This is because when a vector of experimentally-measured flux values $\mathbf{v}$ would be decomposed into EFMs, this generally does not give a unique solutions because it can be done in many ways. Flux sampling methods can be employed to solve both the computational and interpretability problems simultaneously, exploring the set of flux vectors (i.e. directly measurable in principle) by computationally sampling from the flux space. The goal of flux sampling in general terms is to produce a sequence of flux vectors that satisfy the steady state constraints until enough samples have been generated to provide an approximate representation of the entire flux space. The flux polyhedra defined by mass-balance and additional inhomogenous linear constraints are convex, and therefore uniform sampling of these flux spaces can be achieved using variants of an algorithm developed for convex analysis called the coordinate hit-and-run (CHR) algorithm [17]. Briefly, the most basic implementation of the CHR algorithm generates a Markov chain of flux vectors by starting in a random position within the flux polytope, picking a direction at random (uniform), and moving a random distance (uniform) in that direction from the current point. The resulting point is returned as a flux vector instance and the process repeats from there. It has been proven that the CHR algorithm converges to a stationary distribution of the Markov chain that is a uniform distribution in the flux space. Alternatives to uniform sampling (i.e. alternative distributions across the flux polytope) can also be achieved using variants of the CHR algorithm.

As highlighted previously in Section 4.3.2, mass-balance and inhomogeneous linear constraints alone often do not contain enough information to sufficiently reduce the space of biologically-feasible flux vectors. For example, thermodynamic constraints on flux vectors are important for ruling out a large proportion of the sampled flux vectors as infeasible, but this may disproportionately dominate the resulting sampling distribu-

tions. Unfortunately, for mathematical reasons too deep to go into here, simply removing these infeasible flux distributions post-sampling will not result in a uniform distribution over the thermodynamically-feasible portion of flux space. In fact, this relevant subset of flux space cannot be defined explicitly, and is usually neither convex nor connected meaning that no Markov chain methods exist for sampling. As an alternative, a recent method [18] has been developed to combine thermodynamic constraints, physiological observations and estimated thermodynamic parameters, with mass-balance and inhomogeneous linear constraints to provide a probabilistic thermodynamic analysis of metabolic reaction networks. Advances such as these will almost certainly aid a more complete characterization of flux space as data and methods become available.

4.4.3. Minimal cut sets

A minimal cut set (MCS) is a set of reactions that, when disabled, disables a set of modes, which in turn can represent a biological function, such as the secretion of a side product. This enables the prediction of gene deletion targets, given that the genes coding for the involved reactions are known. A cut set is minimal if the removal of one or more reactions from the set leads to at least one of the targeted modes not being disabled.

In order to avoid also disabling desired functionalities, such as product secretion and growth, the concept of constrained minimal cut sets (cMCSs) has been developed. cMCSs enable targeting a set of modes while at the same time making sure that some elements of another set of modes will remain active.

Motivation for (constrained) Minimal Cut Sets The concept of MCSs was introduced by Klamt and Gilles in 2004 [19] and subsequently generalized and improved [20, 21, 22]. As briefly outlined above, the idea is to define a set of EFMs which should be disabled, for example because they generate an unwanted side product or because they don't generate the product of interest with a sufficiently high yield. Since EFMs are minimal, removing a single reaction will disable it. A cut set is a set of reactions of which at least one is active in each of the EFMs in the targeted group. Thus, disabling the reactions contained in the cut set will disable all of the targeted EFMs, and each cut set therefore represents the prediction of a set of gene deletions. Since it would be pointless to remove reactions which only target EFMs that were already targeted by other reactions, cut sets are required to be minimal. This means that removing a single reaction from the cut set would lead to one or more of the targeted EFMs to survive the intervention and also that adding a single reaction to the cut set would have no additional effect on the set of target EFMs.

The pitfall when using MCSs is that while they guarantee the elimination of the targeted EFMs, all other EFMs may be affected as well. This means that modes with desired phenotypes, such as high growth and/or high product yield, may become impossible. Therefore, cMCSs were developed [23]. In this extension of the concept of MCSs it is now possible to additionally define a set of EFMs which are desired, i.e. which can not be disabled by the cMCSs. This is usually implemented by the requirement that at least a specified minimum number of EFMs of the desired set need to remain active. Summarizing, cMCSs are sets of reactions which guarantee that (i) the full set of target EFMs is disabled and (ii) a certain minimum of desired EFMs has to remain unaffected. The drawback, with both MCSs and cMCSs, is that the target (and desired) EFMs need to be defined. This is generally achieved by defining cut-offs in terms of product yield and growth, which is, however, ultimately arbitrary.

Calculation of (constrained) Minimal Cut Sets Since minimal cut sets in a metabolic network are EFMs in a dual network [24], methods used for calculating EFMs can be used to calculate MCSs. Among other approaches [25] one based on binary integer programming has been developed [26, 27]. While it requires that the EFMs are calculated before it can be applied, the advantage is that the algorithm is very intuitive. After having calculated the modes, each is represented as a binary vector which is zero for reactions with zero flux

and one otherwise. The EFMs are then divided into either targeted or desired. A binary vector, corresponding to the cMCSs being calculated is introduced. It will have a one if the corresponding reaction remains active and zero if the reaction is disabled. The first requirement is that cMCS needs to disable all target modes and thus the vector must have zero elements such that each target EFM must have at least one corresponding non-zero element. The second requirement is that at least a defined minimum of desired modes must remain active. This is achieved by introducing a second binary vector. This vector has an element for each EFM and is calculated so that it has a zero when the mode is disabled by the cMCS and one otherwise. By adding the constraint that the number of ones in this vector must at least equal the previously defined minimum, the second requirement is met. Maximizing the vector corresponding to the cMCS yields the first solution. The next solution can be found by adding constraints to make sure that the current one is excluded.

4.5. Concluding remarks

In this chapter we studied how the individual reactions that an organism can catalyze together give rise to the overall conversion of nutrients into cell components and secretion products. For that, we studied the cell's metabolism under a number of simplifying assumptions, most notably, we model metabolism in steady-state. Given this steady-state constraint, we explained how all feasible flux distributions form a space of a specific type: a pointed polyhedral cone. By exploring this 'flux cone' we can chart the metabolic capabilities of an organism.

We have seen that an exhaustive charting of these metabolic capabilities is the computation of all *elementary flux modes*: minimal subnetworks that can individually give rise to steady-state flux distributions, and that may be interpreted as minimal metabolic strategies. An especially important use of EFM analysis can be found in the prediction of the effect of gene knockouts: when all EFMs that produce compound Y use reaction r, then the organism cannot make this compound when the gene is knocked out that codes for the enzyme that catalyzes r. And conversely, sometimes gene knockouts can be found such that the cell cannot grow anymore without producing a certain compound of interest. Clearly, these analyses can be very useful for the design of organisms in bio-industry.

On the other hand, we also saw that for large models the computation of all EFMs becomes impossible. There are simply too many of these minimal subnetworks. We presented several alternatives. One could use *elementary conversion modes* if one still desires an exhaustive list of the metabolic capabilities of the cell. The ECMs are easier to enumerate because one can choose to focus only on all possible conversions between (a subset of) the nutrients and products, instead of requiring all information about which reactions are used to get these conversions. For the design of gene knockouts specifically, *minimal cut sets* may be used. Finally, we discussed that the flux cone can be sampled randomly to characterize the flux cone, if this characterization does not need to be exhaustive.

In many cases we have additional information that determines that part of the flux cone is infeasible. For example, some metabolic fluxes may have been measured so that these reaction rates can be fixed to their observed value. In other cases, one may want to use thermodynamic properties to prohibit reactions from occurring that would violate the second law of thermodynamics. These additional constraints can be imposed on top of the mass-balance constraint to further bound the space of feasible flux distributions; each correctly-imposed constraint narrows down the space of feasible fluxes, and thus increases our knowledge of the metabolic state of the cell.

All explorations of the space of feasible flux distributions show one unavoidable conclusion: the metabolic network is incredibly flexible. Even when several constraints are imposed, a genome-scale metabolic model

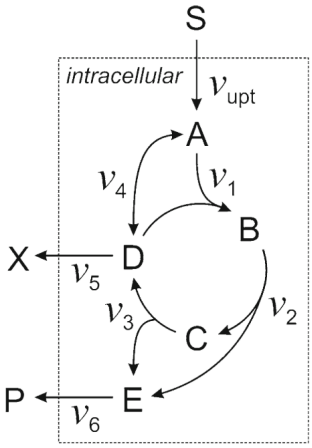


Figure 4.7: *Spirallus insilicus* network, adapted from [28]

will allow for an almost incomprehensible number of modes in which the metabolic network can function. Consequently, to predict the metabolic state of a cell in more detail we need to make an additional assumption. In the following chapter, we will study what predictions we can make when we assume that the metabolic state is optimized to perform a certain function.

Recommended readings

Elementary flux modes A nice paper that gives an understandable introduction of elementary flux mode analysis and its applications: Jürgen Zanghellini, David E. Ruckerbauer, Michael Hanscho, Christian Jungreuthmayer (2013). Elementary flux modes in a nutshell: Properties, calculation and applications. Biotechnology Journal 8 (9), 1009. doi: doi.org/10.1002/biot.201200269

Elementary Flux Vectors were introduced as an analog of Elementary Flux Modes in the case that the flux mode is further bound by at least one inhomogeneous constraint. A nice review of these EFVs is can be found in: Steffen Klamt, Georg Regensburger, Matthias P Gerstl, Christian Jungreuthmayer, Stefan Schuster, Radhakrishnan Mahadevan, Jürgen Zanghellini, and Stefan Müller (2017). From elementary flux modes to elementary flux vectors: Metabolic pathway analysis with arbitrary linear flux constraints. PLoS Computational Biology, 13(4):e1005409, doi: doi.org/10.1371/journal.pcbi.1005409.

Problems

Problem 4.1 A small metabolic network (2) *Spirallus insilicus*, a completely fictional organism [29], is characterized by the the metabolic network depicted in Figure 4.7 X, S and P represent the biomass, one substrate and one product, while metabolites A to E denote intracellular metabolites. One directional arrows indicate irreversible reactions (all but v_4)

1. How many intracellular metabolites, intracellular reactions and transport reactions are involved in the model?
2. Obtain the stoichiometric matrix ($\mathbf{N}$) and the vector of fluxes. How many elements are in the product $\mathbf{Nv}$ and what do they represent?
3. Is the matrix $\mathbf{N}$ of full rank? How many fluxes should be specified to have a unique solution?
4. Transform the set of constraints so that they define a pointed cone. Determine the number of variables

(fluxes) and constraints.

Problem 4.2 A small metabolic network (2) Consider the following small metabolic network:

$$\begin{aligned} \mathrm{S}_e &\xrightarrow{\mathrm{v}_0} \mathrm{S}_c \\ \mathrm{S}_c &\xrightarrow{\mathrm{v}_1} \mathrm{P}_c \\ \mathrm{P}_c &\xrightarrow{\mathrm{v}_2} \mathrm{C}_c \\ \mathrm{P}_c &\xrightarrow{\mathrm{v}_3} \mathrm{D}_c \\ \mathrm{P}_c + 2\,\mathrm{C}_c &\xrightarrow{\mathrm{v}_4} \mathrm{X} \end{aligned}$$

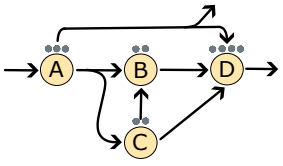
Metabolites with a c subscript are located in the cytosol (intracellular) while e stands for extracellular and X represent biomass. All fluxes are positive.

1. Represent the model as a reaction network (a sketch with metabolites and reactions)
2. Obtain the stoichiometric matrix ($\mathbf{N}$) and list the variables of the metabolic model ($\mathbf{v}$)
3. Show that there is no solution to the mass balance equation $\mathbf{Nv} = \mathbf{0}$ producing metabolite D. Identify why this is so and modify the model so the production of D is allowed ($v_3 > 0$)

Problem 4.3 Elementary Flux Modes (1) Assume reaction v_4 is irreversible from A to D in *Spirallus insilicus* (Problem 4.1). Calculate all the Elementary Flux Modes.

1. By hand.
2. Using a software of your choice (e.g. https://pypi.org/project/efmtool/)

Problem 4.4 Elementary Flux Modes (2) Consider the following metabolic network



$$\mathbf{N} = \begin{pmatrix} 1 & -2 & 0 & 0 & 0 & 0 & -2 \\ 0 & 1 & -2 & 1 & 0 & 0 & 0 \\ 0 & 2 & 0 & -1 & -2 & 0 & 0 \\ 0 & 0 & 1 & 0 & 1 & -1 & 1 \end{pmatrix}$$

Please note that some stoichiometric coefficients in $\mathbf{N}$ are different from 1 (not shown in the graphics).

1. In the network drawing, grey dots denote carbon atoms. Check that carbon atoms are conserved in all reactions. What's the carbon content of the byproduct (not shown) of the reaction from A to D?
2. All metabolites are treated as internal, that is, they need to be mass-balanced. Find all EFMs (by pure reasoning or by using a software). Determine all EFMs in which all fluxes are in forward direction, i.e. along the conventional directions indicated by arrows.
3. Which of the EFMs are thermodynamically realizable? Explain why.

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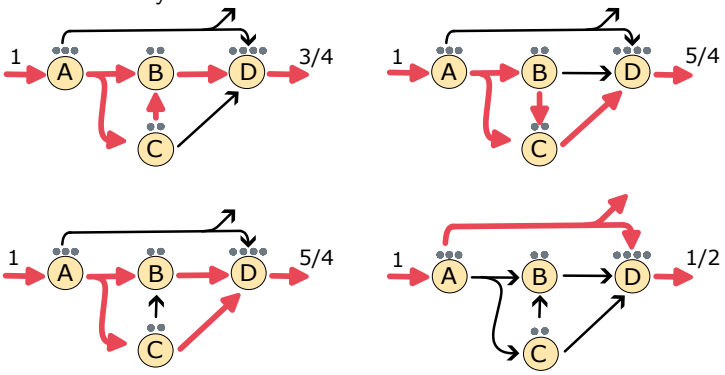
Solutions to problems

Problem 4.4 (Elementary Flux Modes (2))

EFMs containing forward fluxes only:



EFMs containing forward and backward fluxes: