Chapter 5
Towards the Modular Decomposition of the Metabolic Network

Anne Goelzer and Vincent Fromion

Abstract Modular systems emerged in biology through natural selection and evolution, even at the scale of the cell with the cellular processes performing elementary and specialized tasks. However, the existence of modules is questionable when the regulatory networks of the cell are superimposed, in particular for the metabolic network. In this chapter, a theoretical framework that allows the breakdown of the steady-state metabolic network of bacteria into elementary modules is introduced. The modular decomposition confers good systemic and control properties, such as the decoupling of the steady-state regime with respect to the co-metabolites or co-factors, to the entire system. The biological configurations and their impact on the module properties are discussed in detail. In particular, the presence of irreversible enzymes was found to be critical in the module definition. Moreover, the proposed framework can be used to qualitatively predict the dynamics of the module components and to analyse biological datasets.

Keywords Modular · Metabolic network · Steady-state · Metabolite · RNA · DNA · Enzyme · Bacillus subtilis · Genetic · Regulatory network · Pathway · Genetic control · End product control structure (EPCS)

5.1 Introduction

Modularity emerged at all scale in living organisms, from organs in mammals to cellular processes in bacteria such as DNA replication. These sub-systems, empirically identified through their functions, perform elementary specialized tasks, that

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are coordinated to achieve the growth and the survival of the organism. Despite the existence of these specialized sub-systems, the existence of modules is questionable when the regulatory networks of the cell are superimposed, and in particular for the metabolic network. The metabolic network is a central cellular process whose main function is to produce energy and the main building blocks for biomass synthesis like amino acids or nucleotides. It is composed of a large set of highly connected chemical reactions (more than 2,000 reactions for the bacterium *Escherichia coli* [11]) catalysed by enzymes. The questions that we adressed in this chapter is: can we identify modules and, more generally any structure in the metabolic network when the metabolic regulatory network is considered? Can we establish intrinsic and structural properties associated to this organisation? These questions are ambitious and require, as a preliminary step, to have the regulatory network of the metabolic pathways of an organism, enough complete and exhaustive to tackle these questions. To this purpose, we focused on the metabolic network of the "simplest" organism, the bacterium. However, since the metabolic pathways are highly conserved in higher organisms, the results obtained in this chapter are also interesting.

In previous works [17], we inferred the genetic and metabolic regulatory network for the model bacterium *Bacillus subtilis* using information in the literature and databases. From the analysis of this network, we pointed out, in agreement with the results of [21], the key role of metabolites in the genetic control of metabolic networks. Moreover, we identified (a) two main control structures of metabolic pathways and (b) the standard biological configurations that are found in the metabolic network. Most existing studies focus on the behaviour of metabolic pathways (or signalling pathways) and consider a specific metabolic configuration [3, 28, 34, 36]. Because of the strong non-linearity of the dynamical model that is used to describe the system, these authors mainly focused on identifying the stability conditions for a simplified model. Moreover, their results are rarely discussed from a biological point of view. Some work has dealt with more realistic metabolic configurations [1, 2], but these models do not integrate genetic regulation.

In contrast to these studies, our approach analyses the existence and uniqueness of a structural steady-state regime for any metabolic pathway, regardless of its configuration and its genetic and enzymatic regulatory mechanisms. We identified two types of well-defined elementary modules that have specific mathematical properties. This module definition can then be used to study the decomposition of a complete metabolic network into modules.

This chapter is organised as follows. Section 5.2 briefly introduces the main results of our work [17] and details the identification of two control structures in the metabolic network, which are considered elementary modules. Sections 5.3 and 5.4 discuss the existence and uniqueness of a steady state in the two elementary modules and in a large diversity of biological configurations. Section 5.5 examines the connection and the coordination between modules. Section 5.6 focuses on the decomposition of the metabolic network of *B. subtilis* into modules.

5.2 Two Main Control Structures in Metabolic Pathways

The analysis of the *B. subtilis* metabolic network (see Fig. 5.1 (top) and [17]) led to the identification of two distinct control structures in metabolic pathways. In the first control structure, which we named end-product control structure (EPCS), the last metabolite of the metabolic pathway is the key factor because it inhibits the activity of the first enzyme and its synthesis through a genetic regulator. The second structure, which is called initial-product control structure (IPCS), involves the first metabolite of the pathway. Increasing concentrations of the first metabolite induces the synthesis of the enzymes in the pathway through a genetic regulator. Based on previous results [17], we defined two levels of control in metabolic pathways: local regulation and global regulation [see Fig. 5.1 (bottom)]. The local regulation of a metabolic pathway corresponds to any type of genetic regulation (transcriptional, translational, and post-translational) that involves the concentration of an intermediate metabolite in the controlled pathway. The global regulation of a metabolic pathway is defined as all non-local regulations. For practical purposes, the local regulation ensures the induction or repression of enzymes of the pathway according to the concentration of an intermediate metabolite of the pathway. The global regulation, however, can change or bypass the local regulation.

The choice of these structures as elementary sub-systems, even if it seems simple at first, is based on their intrinsic mathematical properties, which will be presented in the next sections. From an input/output perspective, these control structures correspond to sub-systems, or **modules**. In addition, these allow the breakdown of the metabolic network into sub-systems, which usually correspond to the empirical organisations of the metabolic network that are defined by biologists.

5.3 The End-Product Control Structure

In this section, the theoretical properties that are related to the end-product control structure are analysed. In addition, the consequences of these properties will be assessed from a biological point of view. Compared to previous studies, this work systematically studies the impact of different biological configurations of metabolic pathways, which are deduced from the work by [17]. These configurations include changes in the reversibility/irreversibility of metabolic pathways, the presence of cofactors, and isoenzymes and the organisation of the genes in an operon.

The EPCS system is shown in Fig. 5.2. As shown, the system is a linear metabolic pathway that is composed of n metabolites $(X_1, \ldots, X_n)$ and $n-1$ enzymes $(\mathbb{E}_1, \ldots, \mathbb{E}_{n-1})$ and is controlled by the concentration of the end-product, which represses the synthesis of the first enzyme $\mathbb{E}_1$ (genetic level) and inhibits the activity of $\mathbb{E}_1$ (metabolic level).

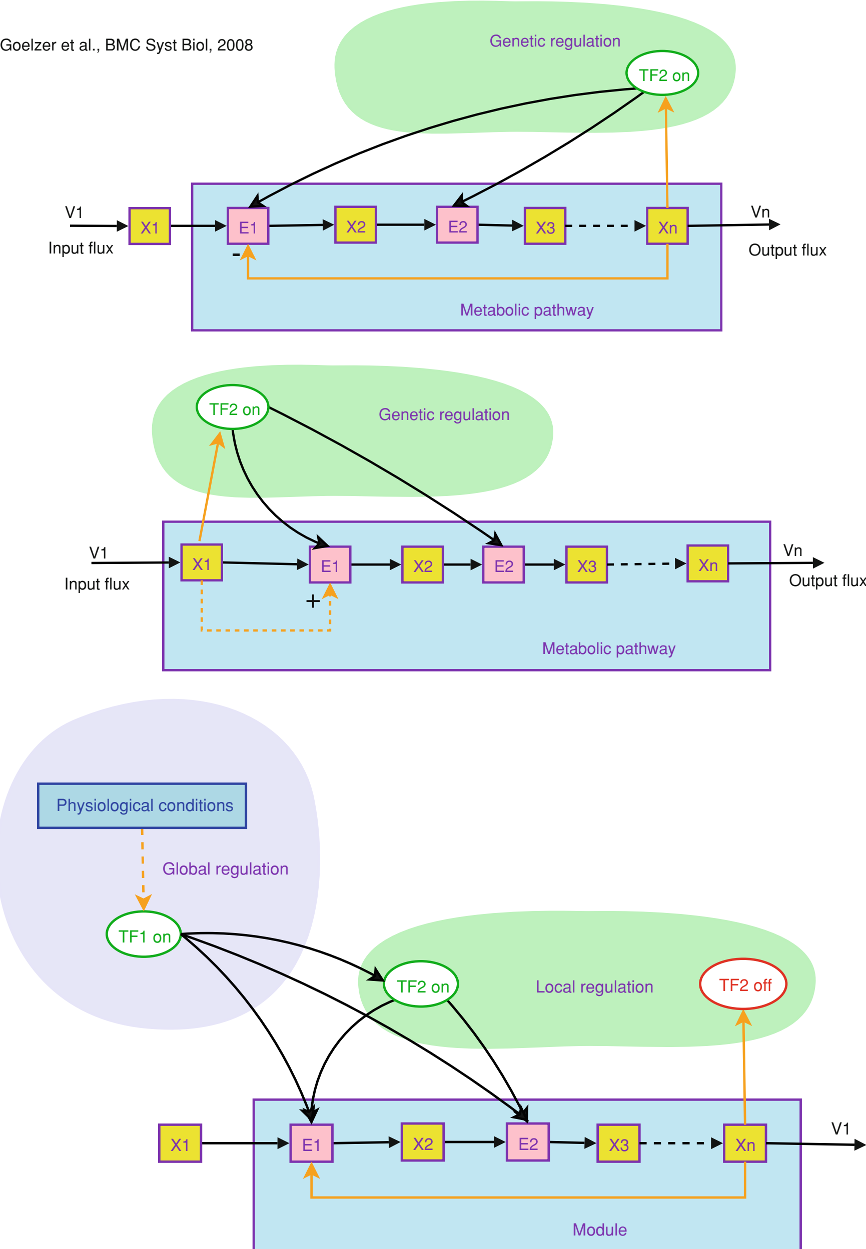


Fig. 5.1 *Top* two control structures in the metabolic network: one controlled by the last metabolite (end-product), one controlled by the first metabolite (initial-product). Enzymes (resp. metabolites) are in *pink* (resp. *yellow*). The transcription factor (TF) is the ellipsoid, and the *orange arrows* refer to the regulation by metabolites on the enzyme activity and on the TF activity. *Bottom* two control levels of a metabolic pathway

Fig. 5.2 A metabolic pathway controlled by the end-product

Enzymatic and genetic control

Model of metabolic level: Following the standard representation of enzymatic reaction of [38], the dynamics of the metabolite concentrations can be described by the following set of ordinary differential equations:

$$\begin{cases} \dot{x}_1(t) = \nu_0(t) - E_1(t) f_1(x_1(t), x_2(t), x_n(t)) \\ \dot{x}_2(t) = E_1(t) f_1(x_1(t), x_2(t), x_n(t)) - E_2(t) f_2(x_2(t), x_3(t)) \\ \vdots \quad \vdots \quad \vdots \quad \vdots \\ \dot{x}_n(t) = E_{n-1}(t) f_{n-1}(x_{n-1}(t), x_n(t)) - \nu_n(t) \end{cases} \tag{5.1}$$

where $\nu_n(t) \triangleq E_n(t) f_n(x_n(t))$ and the characteristics of the enzyme activity f_i are such that:

(a) **Reversible enzymes**:

- for intermediate enzymes, $\mathbb{E}_i$ for $i \in \{2, \ldots, n-1\}$: f_i is continuous, increasing in x_i and decreasing in x_{i+1} such that $f_i(0, 0) = 0$, $f_i(x_i, 0) > 0$ for all $x_i > 0$, and $f_i(0, x_{i+1}) < 0$ for all $x_{i+1} > 0$. Moreover, there exist $M_i > 0$, $M'_i \geq 0$[1] such that $f_i(x_i, x_{i+1}) \in (-M'_i, M_i)$ for all $x_i > 0$ and $x_{i+1} \geq 0$. We assume that for $x_i > 0$, there always exists $x_{i+1} > 0$ such that $f_i(x_i, x_{i+1}) = 0$.
- for the first enzyme, $\mathbb{E}_1$: $f_1(0, 0, x_n) = 0$ for all $x_n \geq 0$, $f_1(x_1, 0, x_n) > 0$ for all $x_1 > 0$ and $x_n \geq 0$, and $f_1(0, x_2, x_n) < 0$ for all $x_2 > 0$ and $x_n \geq 0$. Moreover, there exist $M_1 > 0$, $M'_1 \geq 0$ such that $f_1(x_1, x_2, x_n) \in (-M'_1, M_1)$, for all $x_1 > 0$, $x_2 \geq 0$ and $x_n \geq 0$. Moreover, we also assume that for all $x_1 > 0$ and $x_n \geq 0$, there exists $x_2 > 0$ such that $f_1(x_1, x_2, x_n) = 0$. In addition, $f_1(x_1, x_2, x_n)$ is continuous and increasing (resp. decreasing) in x_1 (resp. x_2). Moreover, if $f_1(x_1, x_2, 0) < 0$ for all $x_1 > 0$ and $x_2 > 0$, then f_1 is increasing in x_n; similarly, if $f_1(x_1, x_2, 0) > 0$ for all $x_1 > 0$ and $x_2 > 0$, then f_1 is decreasing in x_n. Moreover, for all $x_1 > 0$ and $x_2 > 0$, $\lim_{x_n \to +\infty} f_1(x_1, x_2, x_n) = 0$.
- for the last enzyme, $\mathbb{E}_n$: $\mathbb{E}_n$ represents the set of chemical reactions that consume x_n and summarises the link between the flux that is produced by the metabolic pathway and the final concentration. The characteristics of f_n mainly depend on other modules. In addition, f_n is continuous and increasing in x_n such that $f_n(0) = 0$.

[1] All constants introduced in this chapter are assumed to be finite.

(b) **Irreversible enzymes**:

- for the intermediate enzymes, $\mathbb{E}_i$ for $i \in \{2, \ldots, n-1\}$: f_i is continuous and increasing in x_i such that $f_i(0) = 0$. Moreover, there exists $M_i > 0$ such that $f_i(x_i) \in (0, M_i)$ for all $x_i > 0$ and $\lim_{x_i \to +\infty} f_i(x_i) = M_i$.
- for the first enzyme, $\mathbb{E}_1$: $f_1(0, x_n) = 0$ for all $x_n \geq 0$. There exists $M_1 > 0$ such that $f_1(x_1, x_n) \in (0, M_1)$ for all $x_1 > 0$ and $x_n \geq 0$. Moreover, f_1 is continuous, increasing in x_1 and decreasing in x_n such that for all $x_1 > 0$, $\lim\limits_{x_n \to +\infty} f_1(x_1, x_n) = 0$.

Model of the control at the genetic level: Enzyme synthesis occurs in two steps: the gene is first transcribed by the RNA polymerase to produce the RNA messenger (mRNA), which is then translated by the ribosomes to produce the protein. By noting m, Y_L and R_L as the concentrations of mRNAs, free RNA polymerases and free ribosomes, respectively, a simplified dynamic model of the synthesis of an enzyme $\mathbb{E}$ can be written:

$$\begin{cases} \dot{m}(t) = k_m Y_L(t - \tau_m) - k_d m(t) \\ \dot{E}(t) = k_e m(t) R_L(t - \tau_e) - \mu E(t) \end{cases} \tag{5.2}$$

where

(a) k_m, k_d and k_e are the affinity of the promoter for the RNA polymerase, the degradation of mRNA and the affinity of the ribosome for the mRNA, respectively;
(b) μ is the growth rate of the bacterium in exponential growth (μ can then be calculated such that $\dot{N}(t) = \mu N(t)$, where $N(t)$ is the concentration of the bacterial population);
(c) τ_m is the transcriptional delay, which corresponds to the time required for mRNA availability for ribosomes; and
(d) τ_e is the translational delay, which corresponds to the time required for translation of the mRNA.

Moreover, if the synthesis of the mRNA or the enzyme is inhibited by a factor, such as a metabolite, the previous equations also depend on the factor. For example, if the synthesis of the mRNA is inhibited by the metabolite X, which has a concentration of x, then the first equation is now

$$\dot{m}(t) = k_m f_I(x(t)) Y_L(t - \tau_m) - k_d m(t)$$

where $f_I(x(t))$ is continuous, positive and decreasing in x.
If the concentration of the metabolite x has a constant steady state regime $\bar{x}$, then

$$\bar{m} = \frac{k_m}{k_d} f_I(\bar{x}) \bar{Y}_L, \; \bar{E} = \frac{k_e k_m}{\mu k_d} f_I(\bar{x}) \bar{Y}_L \bar{R}_L.$$

The steady state does not depend on the delays τ_m and τ_e. Since we are only interested by the study of the steady state, then for the sake of readability and by abuse of notations, the differential system of enzyme synthesis will be rewritten as

$$\dot{E}(t) = g(x(t)) - \mu E(t) \quad \text{or} \quad \dot{E}(t) = h(t) - \mu E(t) \tag{5.3}$$

where the delays have been removed. The left equation is used if the enzyme synthesis is regulated by a metabolite with concentration x. The right equation corresponds to unregulated enzymes. The functions g and h are continuous, positive, monotonous with respect to their arguments and, because maximal values structurally exist for $\bar{Y}_L$ and R_L, bounded.

Remark 1 The half life of mRNA ($log(2)k_d^{-1}$) is approximately 2–3 min [10] and the growth rate μ, which depends on the medium composition, corresponds to a generation time ($\log(2)\mu^{-1}$) that is between 30 min and several hours [29]. Therefore, the mRNA equation is usually assumed to be at steady state with respect to the enzyme equation. Moreover, the value of the translational delay τ_e is less than 1 min [5] and is therefore often neglected compared to μ.

For the metabolic pathway shown in Fig. 5.2, where $\mathbb{E}_1$ is inhibited by x_n, we have

$$\begin{cases} \dot{E}_1(t) = g(x_n(t)) - \mu E_1(t) \\ \dot{E}_i(t) = h_i(t) - \mu E_i(t) \quad \text{for} \quad i \in \{2, \ldots, n\} \end{cases} \tag{5.4}$$

where

(a) The function g is continuous, positive and decreasing in x_n. Moreover, we assume that $g(0) = P_{\max}$, where $P_{\max} > 0$ and $\lim_{x \to +\infty} g(x) = 0$.
(b) The functions h_i are assumed to be constant.

Remark 2 In the rest of the chapter, we will assume that a steady-state regime of the unregulated enzymes always exists and is determined by the steady-state regimes of other cellular components. The associated differential equations will therefore be removed from the system for the sake of readability.

The final description of the metabolic pathway shown in Fig. 5.2 is the following:

$$\begin{cases} \dot{x}_1(t) = \nu_0(t) - E_1(t) f_1(x_1(t), x_2(t), x_n(t)) \\ \dot{x}_2(t) = E_1(t) f_1(x_1(t), x_2(t), x_n(t)) - E_2(t) f_2(x_2(t), x_3(t)) \\ \vdots \qquad \vdots \qquad \vdots \qquad \qquad \vdots \\ \dot{x}_n(t) = E_{n-1}(t) f_{n-1}(x_{n-1}(t), x_n(t)) - E_n(t) f_n(x_n(t)) \\ \dot{E}_1(t) = g(x_n(t)) - \mu E_1(t) \end{cases} \tag{5.5}$$

If the genes coding for enzymes $(\mathbb{E}_1, \ldots, \mathbb{E}_{n-1})$ are organised in an operon, then there exists $\alpha_i > 0$ such that

$$E_i(t) = \alpha_i E_1(t) \tag{5.6}$$

for $i \in \{2, \ldots, n-1\}$.

5.3.1 Characteristics of the Steady-State Regime

In this section, we introduce the main theoretical properties of the elementary modules on the end-product control structure. To this purpose, we consider the main biological configurations identified from our model [17] and describe the associated steady-state regime. These elementary configurations are realistic enough to describe by combination the entire set of configurations of metabolic pathways of *Bacillus subtilis*.

5.3.1.1 The Enzymes are Irreversible and the Genes are in the Same Operon

All enzymes are irreversible and the genes coding for $(\mathbb{E}_1, \ldots, \mathbb{E}_{n-1})$ belong to the same operon, which means that these are transcribed in the same mRNA. Thus, the concentrations of the enzymes are assumed to be proportional [see Eq. (5.6)].
The system (5.5) can be written as:

$$\begin{cases} \dot{x}_1 = \nu_0 - E_1 f_1(x_1, x_n) \\ \dot{x}_2 = E_1(f_1(x_1, x_n) - \alpha_2 f_2(x_2)) \\ \vdots \;=\; \quad \vdots \qquad\qquad \vdots \\ \dot{x}_n = \alpha_{n-1} E_1 f_{n-1}(x_{n-1}) - E_n f_n(x_n) \\ \dot{E}_1 = g(x_n) - \mu E_1 \end{cases} \tag{5.7}$$

and the steady-state regime is characterised by this result.

Proposition 1 *For all $\mu > 0$, $E_n > 0$ and $\bar{x}_1 > 0$ there exists a unique steady-state regime $(\bar{E}_1, \ldots, \bar{E}_{n-1})$ and $(\bar{x}_2, \ldots, \bar{x}_n)$ for the system (5.7) given by*

$$\begin{cases} \bar{E}_1 = \dfrac{g(\bar{x}_n)}{\mu} \\ f_1(\bar{x}_1, \bar{x}_n) g(\bar{x}_n) = \mu E_n f_n(\bar{x}_n) \\ \nu_0 = E_n f_n(\bar{x}_n) \end{cases} \tag{5.8}$$

and for $i \in \{2, \ldots, n-1\}$,

$$\bar{E}_i = \alpha_i \frac{g(\bar{x}_n)}{\mu} \quad \textit{and} \quad \bar{x}_i = f_i^{-1}\left(\frac{\mu E_n f_n(\bar{x}_n)}{\alpha_i g(\bar{x}_n)}\right) \tag{5.9}$$

if and only if for $i \in \{2, \ldots, n-1\}$

$$\alpha_i M_i > \frac{\mu E_n f_n(\bar{x}_n)}{g(\bar{x}_n)}. \tag{5.10}$$

The proof of this proposition is a particular case of the proof of Proposition 3, which is shown in page 10. Proposition 1 indicates that the system (5.7) has a unique steady-state regime if and only if all of the enzymes that belong to the metabolic pathway do not saturate (the condition (5.10) holds true). Moreover, x_n and thus implicitly f_1 and g have key roles in the definition of the steady state. The monotonicity of f_1 and g with respect to x_n allows to deduce the unicity of x_n. Surprisingly, the characteristics denoted by f_i and the concentrations of the intermediate enzymes have no impact on the definition of the steady state $\bar{E}_1$, $\bar{x}_n$ and the output flux $E_n f_n(\bar{x}_n)$ if none of the intermediate enzymes saturate. Consequently, the sensitivity of the steady-state regime to a constant perturbation in the concentration of enzyme E_n (or to a flux demand ν_n) only depends on the genetic characteristics g and the characteristics f_1 of the first enzyme. The prediction of the steady-state behaviour of the metabolic pathway can therefore be dramatically simplified, even if it is composed of a large number of intermediate reactions.

Remark 3 The condition (5.10) can be written as

$$\frac{\mu E_n f_n(\bar{x}_n)}{g(\bar{x}_n)} < \alpha_i M_i \iff f_1(\bar{x}_1, \bar{x}_n) < \alpha_i M_i.$$

Therefore, if $M_1 < \alpha_i M_i$ for all $i \in \{2, \ldots, n-1\}$, then condition (5.10) is satisfied.

5.3.1.2 Behaviour of the Components of the Metabolic Pathway

The variation of the flux demand with respect to the variation of the concentration of $\mathbb{E}_n$ will now be discussed. Based on the definition of the steady-state regime,

$$\frac{f_1(\bar{x}_1, \bar{x}_n) g(\bar{x}_n)}{\mu f_n(\bar{x}_n)} \stackrel{\Delta}{=} E_n.$$

Therefore, $\bar{x}_n$ is decreasing when E_n is increasing. In addition, the final flux demand $\bar{\nu}_n \stackrel{\Delta}{=} E_n f_n(\bar{x}_n)$ is by definition equal to $f_1(\bar{x}_1, \bar{x}_n) g(\bar{x}_n) = \bar{\nu}_n$. Because the left side of equation is a decreasing function of $\bar{x}_n$, then, when E_n is increasing, $\bar{\nu}_n$ is also increasing (as long as none of the enzymes saturate). Consequently, the metabolic pathway has a maximal flux capability, which is given by the following corollary.

Corollary 1 *Let the assumptions of Proposition 1 be satisfied. Then the flux demand has the following upper bound at steady state*

$$\frac{g(0)}{\mu} f_1(\bar{x}_1, 0). \tag{5.11}$$

The outer flux is then bounded and the superior value only depends on the characteristics f_1 and g of the first enzyme; this is only true if none of the intermediate enzymes saturate.
The impact of variations in (a) the flux demand and (b) the concentration x_1 on the intermediate metabolite concentrations will now be discussed.

Corollary 2 *Let the assumptions of Proposition 1 be satisfied. Then,* (a) *for all* $i \in \{2, \ldots, n-1\}$, $\bar{x}_i = \bar{x}_i(E_n)$ *is increasing in* E_n *and* $\bar{x}_n = \bar{x}_n(E_n)$ *is decreasing in* E_n *and,* (b) *for all* $i \in \{2, \ldots, n\}$, $\bar{x}_i(\bar{x}_1)$ *and* $\bar{\nu}_n(\bar{x}_1)$ *are increasing in* $\bar{x}_1$.

The intermediate metabolite concentrations are increasing functions of the flux demand and of x_1, whereas the end-product is a decreasing (resp. increasing) function of the flux demand (resp. x_1).

Remark 4 $\bar{x}_n$ can be written as a function of $\bar{x}_1$: $\bar{x}_n \stackrel{\Delta}{=} H(\bar{x}_1)$. Therefore, at steady state, the input and output flux and the concentration of the first metabolite $\bar{x}_1$ are linked by the monotonously increasing relationship $\nu_0 = E_n f_n(H(\bar{x}_1))$. We then obtain an input/output description that corresponds to a fictitious enzyme, which links ν_0 to $\bar{x}_1$ and integrates all of the module properties through the functions H and f_n.

Remark 5 A metabolic flux corresponds to a material flow through an enzyme such that $\nu = E f_E(x)$. A metabolic flux is thus an intensive quantity, whereas the metabolite concentration is an extensive quantity. This fact explains why, in most mechanisms of gene regulation, only the concentration of a metabolite is used (and not the flux). As in Ohm's law ($U = RI$), in which the current I is measured through the measurement of the voltage U for the resistance R, the cell senses the flux ν through the measurement of the concentration x and a specific mechanism, such as an enzyme or a genetic regulator.

5.3.1.3 Consequences of Enzyme Saturation

Several factors can result in enzyme saturation; these include an inadequate concentration of the enzyme or its limitation by a cofactor. The effect of enzyme saturation will now be discussed.

Corollary 3 *Let the assumptions of* Proposition 1 *be satisfied and let us define*

$$\psi_{i^*,sat} = \min_{i \in \{2,\ldots,n-1\}} \alpha_i M_i$$

and i^*, the value of i for which the minimum is reached (i^* can also correspond to a set of possible values). If $\psi_{i^*,sat}$ is such that $\psi_{i^*,sat} < f_1(\bar{x}_1, 0)$, then there exists E_n^* and $\bar{x}_n^*$ such that

$$\frac{\mu E_n^* f_n(\bar{x}_n^*)}{g(\bar{x}_n^*)} = \psi_{i^*,sat}$$

and

$$\lim_{\substack{< \\ E_n \to E_n^*}} \bar{x}_{i^*} = +\infty.$$

In addition, for $E_n \geq E_n^*$, the regime of the metabolic pathway is saturated.

The output flux is fixed through the saturation of the enzyme $\psi_{i^*,sat}$ and by the characteristics g of $\mathbb{E}_1$. Moreover, the concentration of the metabolite $\bar{x}_{i^*}$, which is the substrate of enzyme i^*, goes theoretically to infinity when E_n goes to E_n^*. Obviously, thermodynamical laws prevent the metabolite concentration to go to infinity. Very high concentrations of metabolites lead to reverse the direction of the chemical reaction, i.e. the irreversible enzyme becomes reversible (see Sect. 5.3.1.6).

5.3.1.4 Biological Interpretation

The biosynthesis pathways of amino acids are generally regulated by the end product. The enzyme $\mathbb{E}_n$ and the output flux v_n correspond to the tRNA synthase and the flux of charged-tRNA that is consumed by the ribosomes for the production of proteins at steady-state, respectively. Thus, an increase in the ribosomal demand usually results in an increase in the concentration of tRNA synthase (E_n) due to a genetic regulation that induces a decrease in the concentration of the amino acid x_n. A decrease in x_n leads to the readjustment of the entire pathway (enzyme and metabolites) to provide the requested flux (assuming that the intermediate enzymes do not saturate). In other words, for fixed $\bar{x}_1$, the concentration of the amino acid x_n must decrease to increase the capacity of the synthesis pathway and thus satisfy the flux demand within the limit defined by the characteristics of the first enzyme (Corollary 1).

5.3.1.5 The Genes are Independent

In the following analysis, the genes belonging to the metabolic pathway are not in the same operon. We assume that a steady state for the intermediate enzyme exists and is given by $(E_i)_{i \in \{2,\dots,n\}} > 0$.

Proposition 2 *For all $\mu > 0$, $\bar{x}_1 > 0$ and $E_i > 0$ for $i \in \{2, \dots, n\}$, there exists an unique steady-state regime $\bar{E}_1$ and $(\bar{x}_2, \dots, \bar{x}_n)$ for (5.7) such that*

$$\begin{cases} \bar{E}_1 = \dfrac{g(\bar{x}_n)}{\mu} \\ f_1(\bar{x}_1, \bar{x}_n)g(\bar{x}_n) = \mu E_n f_n(\bar{x}_n) \\ \nu_0 = E_n f_n(\bar{x}_n) \end{cases} \tag{5.12}$$

and for all $i = \{2, \ldots, n-1\}$, $\bar{x}_i = f_i^{-1}\left(\dfrac{E_n f_n(\bar{x}_n)}{E_i}\right)$ *if and only if* $E_n f_n(\bar{x}_n) < E_i M_i$.

Compared to Proposition 1, only the condition of saturation changes. The link between the flux demand and the concentrations of the first and last metabolite that are obtained in Proposition 1 is unchanged as long as none of the intermediate enzymes saturate. All of the previous results of Sect. 5.3.1.1 can be easily extended.

5.3.1.6 All Enzymes are Reversible

We now assume that all of the enzymes in the metabolic pathway (including the first enzyme) are reversible. This configuration dramatically changes the properties obtained in Proposition 1. In contrast, the results in Proposition 1 can be partially recovered through the presence of a single irreversible enzyme.

Proposition 3 *If the genes coding for* $(\mathbb{E}_1, \ldots, \mathbb{E}_{n-1})$ *belong to the same operon (see Eq. (5.6)) and if the enzymes* $\mathbb{E}_i$ *for all* $i \in \{1, \ldots, n-1\}$ *are reversible, then, for all* $\mu > 0$, $E_n > 0$ *et* $\bar{x}_1 > 0$, *there exists a unique steady-state regime for the system (5.5),* $(\bar{E}_1, \ldots, \bar{E}_{n-1})$ *and* $(\bar{x}_2, \ldots, \bar{x}_n)$ *such that*

$$\begin{cases} \bar{x}_n = H_n(H_2(\ldots(H_{n-1}(\bar{x}_n, \bar{x}_1), \bar{x}_1)\ldots, \bar{x}_1), \bar{x}_1) \\ \bar{x}_i = H_i(H_{i+1}(\ldots(H_{n-1}(\bar{x}_n, \bar{x}_1), \bar{x}_1)\ldots, \bar{x}_1), \bar{x}_1)\ \textit{for}\ i \in \{2, \ldots, n-1\} \\ E_1 = \dfrac{g(\bar{x}_n)}{\mu} \\ E_i = \alpha_i \dfrac{g(\bar{x}_n)}{\mu}\ \textit{for}\ i \in \{2, \ldots, n-1\} \\ \nu_0 = E_n f_n(\bar{x}_n), \end{cases} \tag{5.13}$$

where, for all $i \in \{2, \ldots, n-1\}$, *the functions* H_i *are increasing with respect to their arguments and the function* H_n *is decreasing (resp. increasing) with respect to its first (resp. second) argument.*

Proof The proof is inductive.

Step 1: Let us first prove that there exists $x_2^* > 0$ such that, for all $\bar{x}_2 \in [0, x_2^*]$, there exists a unique $\bar{x}_n \geq 0$ such that

$$\frac{g(\bar{x}_n)}{\mu} f_1(\bar{x}_1, \bar{x}_2, \bar{x}_n) = E_n f_n(\bar{x}_n). \tag{5.14}$$

The monotonicity of the functions of the left and the right side of the equation with respect to $\bar{x}_n$ means that, for all $\bar{x}_1 > 0$, there exists $x_2^* > 0$ such that $f_1(\bar{x}_1, \bar{x}_2, 0) > 0$

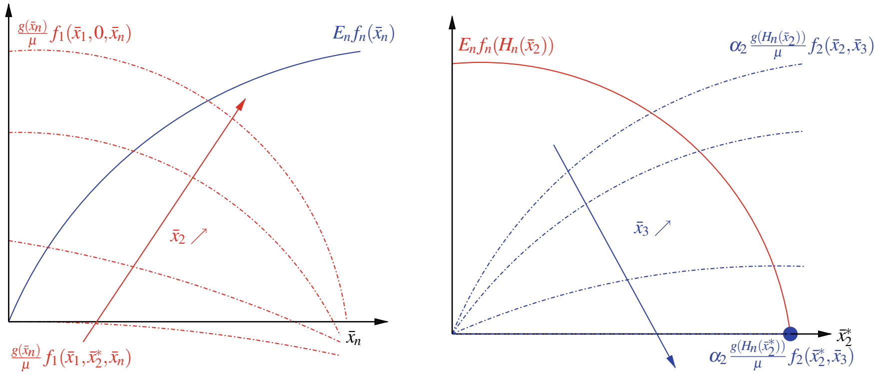


Fig. 5.3 Intersection of *curves* $E_n f_n(\bar{x}_n)$ and $f_1(\bar{x}_1, \bar{x}_2, \bar{x}_n)$ for all $\bar{x}_2 \in [0, x_2^*]$ (*left*) and of *curves* $E_n f_n(H_n(\bar{x}_2))$ and $\alpha_2 \dfrac{g(H_n(\bar{x}_2))}{\mu} f_2(\bar{x}_2, \bar{x}_3)$ (*right*)

for all $\bar{x}_2 \in [0, x_2^*)$ with $f_1(\bar{x}_1, x_2^*, 0) = 0$. Then, for all $\bar{x}_2 \in [0, x_2^*)$, the left side of Eq. (5.14) is a decreasing function of $\bar{x}_n$, is positive for $\bar{x}_n = 0$ and tends to 0 when $\bar{x}_n$ goes to infinity. In addition, the right side of the Eq. (5.14) is an increasing function of $\bar{x}_n$ and is equal to 0 when $\bar{x}_n = 0$. Thus, for all $\bar{x}_2 \in [0, x_2^*)$, the two curves with respect to $\bar{x}_n$ necessarily have a unique intersection point. In addition, for $\bar{x}_2 = x_2^*$, $\bar{x}_n = 0$ is the only solution to the Eq. (5.14) (see Fig. 5.3 left), which concludes the proof of Step 1.

Thus, the function $\bar{x}_n \stackrel{\Delta}{=} H_n(\bar{x}_2, \bar{x}_1)$ is continuous and decreasing in $\bar{x}_2$ and can be defined for $\bar{x}_2 \in [0, x_2^*]$ such that $H_n(0, \bar{x}_1) > 0$ and $H_n(x_2^*, \bar{x}_1) = 0$. For the sake of readability, we omitted the dependence of the equations on $\bar{x}_1$ in the rest of the proof.

Step 2: The rest of the proof is by induction. If the steady-state regime exists, then $\bar{x}_2$ and $\bar{x}_3$ are linked by

$$\alpha_2 \frac{g(H_n(\bar{x}_2))}{\mu} f_2(\bar{x}_2, \bar{x}_3) = E_n f_n(H_n(\bar{x}_2)), \tag{5.15}$$

where $\bar{x}_n$ has been substituted by its expression. As in the first step, we can prove that there exists[2] $\bar{x}_3^* > 0$ such that, for all $\bar{x}_3 \in [0, x_3^*]$, there exists $x_2 \in [0, x_2^*]$ such that Eq. (5.15) is true. Thus, the function $\bar{x}_2 \stackrel{\Delta}{=} H_2(\bar{x}_3)$ can be defined, which is well defined, continuous, increasing in $\bar{x}_3$ for all $\bar{x}_3 \in [0, x_3^*]$, and such that $H_2(0) > 0$ and $H_2(x_3^*) = x_2^*$ (See Fig. 5.3 right).

Step 3: Step 2 is repeated for all $i \in \{3, \ldots, n-1\}$. By definition, $\bar{x}_i$ has to be the solution of the following equation:

[2] In fact, x_3^* is such that $f_2(x_2^*, x_3^*) = 0$, which guarantees that $f_2(x_2^*, \bar{x}_3) > 0$ for all $[0, x_3^*)$.

$$\alpha_i \frac{g(H_n(H_2(...(H_{i-1}(\bar{x}_i)))))}{\mu} f_i(\bar{x}_i, \bar{x}_{i+1}) = E_n f_n(H_n(H_2(\ldots(H_{i-1}(\bar{x}_i))))). \tag{5.16}$$

Then, as in the previous step, it is easy to prove the existence of the function H_i such that $x_i \triangleq H_i(x_{i+1})$ is well defined, continuous, increasing in $\bar{x}_{i+1}$ for all $\bar{x}_{i+1} \in [0, x^*_{i+1}]$, and such that $H_i(0) > 0$ and $H_i(x^*_{i+1}) = x^*_i$.

Step 4: Through the combination of the results of the previous steps, we can deduce that $\bar{x}_n$ exists if the following equation has a solution:

$$\bar{x}_n = H_n(H_2(\ldots(H_{n-1}(\bar{x}_n)))). \tag{5.17}$$

By definition, H_{n-1} is defined on $[0, x^*_n]$ such that $H_{n-1}(0) > 0$ and $H_{n-1}(x^*_n) = x^*_{n-1}$. Let us note that $H_n(H_2(\ldots(H_{n-1}(0))) > 0$ and $H_n(H_2(\ldots(H_{n-1}(x^*_n)))$ $= H_n(x^*_2) = 0$, and because the right side (resp. left side) of Eq. (5.17) is a decreasing (resp. increasing) function in $\bar{x}_n$, we can deduce that there exists a unique $\bar{x}_n \in [0, x^*_n)$, solution to Eq. (5.17), which concludes the proof.

Remarkably, when all enzymes are reversible, the steady-state regime of the metabolic pathway always exists. We will not develop the results of this structure because the systematic analysis of metabolic pathways indicates the presence of at least one irreversible enzyme per module [17, 20]. In most cases, the irreversible step corresponds to the first or second enzyme. The presence of an irreversible enzyme means that the results of Proposition 1 hold:

Corollary 4 *Let the assumptions of* Proposition 3 *be satisfied. If enzyme* $\mathbb{E}_1$ *is irreversible, then, for all* $\mu > 0$, $E_n > 0$ *and* $\bar{x}_1 > 0$, *there exists a unique steady-state regime* $(\bar{E}_1, \ldots, \bar{E}_{n-1})$ *and* $(\bar{x}_2, \ldots, \bar{x}_n)$ *for the system* (5.5) *such that*

$$\begin{cases} \bar{E}_1 = \dfrac{g(\bar{x}_n)}{\mu} \\ f_1(\bar{x}_1, \bar{x}_n) g(\bar{x}_n) = \mu E_n f_n(\bar{x}_n) \\ \nu_0 = E_n f_n(\bar{x}_n) \end{cases} \tag{5.18}$$

if and only if $(\bar{x}_2, \ldots, \bar{x}_{n-1})$ *exists such that, for* $i \in \{2, \ldots, n-1\}$, $\alpha_i \dfrac{g(\bar{x}_n)}{\mu} f_i(\bar{x}_i, \bar{x}_{i+1}) = E_n f_n(\bar{x}_n)$.

As in Proposition 1, the steady-state regime is only defined by f_1, g and f_n as long as none of the enzymes saturate. However, the condition of saturation explicitly depends on the steady-state regime and is then less useful.

5.3.2 *Integration of the Main Biological Configurations*

We will now discuss the impact of different biological configurations in more detail. These configurations involve changes due to the presence of isoenzymes, co-factors and co-metabolites. The mathematical results are presented in the most general case of the end-product control structure, in which only the first enzyme is irreversible and the genes are not organised in a single operon.

5.3.2.1 Impact of Co-Metabolites and Co-Factors

External factors (co-metabolites and co-factors) usually modulate the rate of enzymatic reactions. Co-metabolites, such as ATP/ADP, NAD/NADH or glutamine/glutamate, are also substrates of the enzymes and are transformed into products. Co-factors, such as ions (e.g. Mg^{2+}, Zn^{2+}) or vitamins, are generally bounded to the enzyme and are therefore considered to be an enzyme component. Both of these types of factors can be easily included in our analysis through the introduction of a new argument in the reaction rate f_i of the enzyme. Assuming that the ith reaction requires a co-metabolite, which is labelled as X_P with concentration p, then the rate of reaction for an irreversible enzyme (resp. reversible) is given by $f_i(x_i, p)$ (resp. reversible: $f_i(x_i, x_{i+1}, p)$) such that $f_i(x_i, 0) = 0$ (resp. reversible: $f_i(x_i, x_{i+1}, 0) = 0$) and the function f_i is assumed to be increasing in p.

1. The co-metabolite/co-factor acts on the first enzyme, $\mathbb{E}_1$. The maximal flux of the metabolic pathway is given by $\bar{v}_{n,\max}(\bar{p}) \triangleq \dfrac{g(0)}{\mu} f_1(\bar{x}_1, 0, \bar{p})$, where the co-metabolite or the co-factor reaches its steady-state regime $\bar{p}$. If the factor decreases the activity of the first enzyme, then x_n is decreasing and E_1 is increasing.
2. The co-metabolite/co-factor acts on the last enzyme, $\mathbb{E}_n$. By definition, $f_1(\bar{x}_1, \bar{x}_n) g(\bar{x}_n) = \mu E_n f_n(\bar{x}_n, \bar{p})$. The limitation of the concentration of the co-metabolite/co-factor leads to a decrease in the flux demand. Therefore, $\bar{x}_n$ and $\bar{E}_1$ are increasing and decreasing functions of $\bar{p}$, respectively.
3. The co-metabolite/co-factor acts on an intermediate enzyme, $\mathbb{E}_2, \ldots, \mathbb{E}_{n-1}$. Remarkably, as long as variations of p do not lead to enzyme saturation, the steady states of the main components, ($\bar{v}_n$, $\bar{x}_n$ and $\bar{E}_1$), remain unchanged.

5.3.2.2 Role of an Isoenzyme

Isoenzymes are enzymes that catalyse the same chemical reaction. Let the isoenzyme $\mathbb{E}_1^*$, as represented in Fig. 5.4, catalyse the same irreversible reaction as $\mathbb{E}_1$ (the first reaction). $\mathbb{E}_1^*$ is not regulated by any intermediate metabolite of the metabolic pathway (neither at the genetic level or at the enzymatic level), which leads to the flux $E_1^* f_1^*(x_1)$ for $x_1, E_1^* \geq 0$.

Fig. 5.4 Presence of an isoenzyme in the metabolic pathway

Fig. 5.5 Initial-product control structure

The steady-state regime satisfies the following equation:

$$f_1(\bar{x}_1, \bar{x}_n)\frac{g(\bar{x}_n)}{\mu} + E_1^* f_1^*(\bar{x}_1) = E_n f_n(\bar{x}_n). \tag{5.19}$$

Moreover, the maximal capability of the flux through the metabolic pathway is also modified:

$$\bar{\nu}_{n,\max} = f_1(\bar{x}_1, 0)\frac{g(0)}{\mu} + E_1^* f_1^*(\bar{x}_1).$$

From Eq. (5.19), as long as the intermediate enzymes do not saturate, the increase of the flux $\bar{\nu}_n$ can be obtained either by a decrease in the end product $\bar{x}_n$ or an increase in the concentration of the isoenzyme E_1^*.

5.4 Other Control Structures

The other elementary module [see Fig. 5.1 (top)], which is named initial-product control structure, usually corresponds to the control structure of degradation pathways. Enzyme synthesis is controlled by the concentration of the first metabolite, x_1. Due to lack of space, we will only give the condition of existence of the steady-state regime and the qualitative behaviour of the module components to deduce the rules that dictate the connections between modules.

5.4.1 Initial-Product Control Structure

We will consider the linear pathway that is shown in Fig. 5.5, which consists of n metabolites $(X_1, \ldots, X_n)$ and $n-1$ irreversible enzymes $(\mathbb{E}_1, \ldots, \mathbb{E}_{n-1})$ for which the encoding genes are organised in a single operon. The enzyme synthesis is induced

when the concentration of the first metabolite increases. The behaviour of this pathway obeys the following system of differential equations:

$$\begin{cases} \dot{x}_1 = \nu_0 - E_1 f_1(x_1) \\ \vdots \\ \dot{x}_i = E_1(\alpha_{i-1} f_{i-1}(x_{i-1}) - \alpha_i f_i(x_i)) \\ \vdots \\ \dot{x}_n(t) = \alpha_{n-1} E_1 f_{n-1}(x_{n-1}) - E_n f_n(x_n) \\ \dot{E}_1 = g(x_1) - \mu E_1 \end{cases} \tag{5.20}$$

where f_i has the same characteristics as in Sect. 5.3 and g is a positive, continuous and increasing function of x_1 such that for all $x_1 > 0$, $g(x_1) > 0$, and $g(0) = 0$. We also assume that there exists $P_{\max} > 0$ such that $\lim_{x \to +\infty} g(x) = P_{\max}$.

Proposition 4 *For all $\mu > 0$, $\bar{x}_1 > 0$ and $E_n > 0$, there exists a unique steady-state regime $(\bar{x}_2, \ldots, \bar{x}_n)$ and $(\bar{E}_1, \ldots, \bar{E}_{n-1})$ to the system (5.20) such that*

$$\begin{cases} \bar{E}_1 = \dfrac{g(\bar{x}_1)}{\mu} \\ \nu_0 = \dfrac{g(\bar{x}_1)}{\mu} f_1(\bar{x}_1) \\ \bar{x}_i = f_i^{-1}\left(\frac{\alpha_{i-1}}{\alpha_i} f_{i-1}(\bar{x}_{i-1})\right) \quad \textit{for } i = \{2, \ldots, n\} \end{cases} \tag{5.21}$$

if and only if, for all $i \in \{2, \ldots, n-1\}$, $M_1 < \alpha_i M_i$.
Moreover, the functions $\bar{x}_i = \bar{x}_i(\nu_0)$ for $i = 1, \ldots, n$ are increasing in ν_0, the input flux is bounded and the maximal value of $\bar{x}_1 > 0$ is $\nu_{0,\max} \triangleq \dfrac{P_{\max}}{\mu} M_1$.

Proof The proof of this proposition is straightforward through the writing of the steady-state regime, which, by definition, corresponds to $\nu_0 = \dfrac{g(\bar{x}_1)}{\mu} f_1(\bar{x}_1)$, and because of the monotonicity of the functions. The existence of the steady-state regime is achieved if and only if the enzymes of the pathway are not saturated. This means that the maximum capacity of each enzyme must be greater than ν_0.

Thus, when $\bar{x}_1$ is increasing, the flux ν_0 and the concentrations of the downstream metabolites are increasing. The IPCS module has also specific properties that can be directly obtained by following the line of the analysis of the EPCS module. The proofs of all of these results are straightforward and are easily deduced from the previous proofs.

5.4.1.1 Comparison Between the Different Control Structures

The two control structures that have been analysed have common characteristics, which were obtained under the assumption that none of the intermediate enzymes saturate:

- the steady-state regime is determined by the characteristics of the first enzyme and its genetic control;
- the maximum capacity of the pathway is limited;
- the co-metabolites of the intermediate enzymes have no impact on the input/output flux or on the genetic control; and
- the presence of an irreversible enzyme prevents the direct spread of the information that is carried by the concentrations of downstream metabolites to the upstream metabolites.

However, there are also notable differences. The EPCS module is inherently driven by the downstream flux demand through $\bar{x}_n$, whereas the IPCS module is driven by the upstream flux through $\bar{x}_1$. Moreover, the characteristics f_n of the enzyme $\mathbb{E}_n$ do not affect the existence of a steady-state regime of the IPCS if the control structure is monotonic. The function f_n can be increasing or decreasing in x_n. In other words, a metabolic pathway that is controlled by this type of control structure cannot accommodate a final flux demand of ν_n.

5.4.2 *Not Controlled Structure*

We also introduce a third module, which is named NCS (Not Controlled Structure). This module consists of enzymes that are not genetically or enzymatically controlled by a metabolite in the pathway. The input/output feature of the NCS module at steady state is obtained under the assumption that none of the enzymes of the module saturate and that the first enzyme is irreversible:

$$E_1 f_1(\bar{x}_1) = E_n f_n(\bar{x}_n). \tag{5.22}$$

It follows that the steady-state regime is determined by the concentration of the initial metabolite $\bar{x}_1$ and by the enzymatic characteristics f_1 and f_n.

5.5 Coordination Between Modules

The mathematical properties that are associated with the two main types of modules have been characterised in the previous sections. We will now discuss the methods by which these modules can be coordinated: global regulations [see Fig. 5.1 (bottom)] and direct connections.

5.5.1 *Impact of a Global Regulator*

In this section, we investigate the impact of a global regulator on the EPCS module. The results for the other structure can be easily deduced. Let us consider that the

synthesis of the first enzyme is also controlled by a global regulator, which leads to

$$\dot{E}_1(t) = g(x_n(t), q(t)) - \mu E_1(t)$$

where $q(t)$ is the effect of the global regulator. This parameter can also represent any factor that could impact the synthesis of enzyme $\mathbb{E}_1$.
Assuming that the global regulator reaches its own steady-state regime $\bar{q}$, we can deduce from the above results that the global regulator changes the relationship between the concentration of the final product $\bar{x}_n$, the flux demand and the enzyme concentration:

$$f_1(\bar{x}_1, \bar{x}_n)g(\bar{x}_n, \bar{q}) = \mu E_n f_n(\bar{x}_n).$$

As long as none of the intermediate enzymes saturate, the global regulator changes the steady-state regime at the level of

- the enzyme concentrations (if the genes are in the same operon):

$$\bar{E}_1 = \frac{g(\bar{x}_n, \bar{q})}{\mu} \quad \text{and} \quad \bar{E}_i = \alpha_i \frac{g(\bar{x}_n, \bar{q})}{\mu} \text{ for } i \in \{2, \dots, n-1\},$$

- the end-product concentration: $f_1(\bar{x}_1, \bar{x}_n)g(\bar{x}_n, \bar{q}) = \mu E_n f_n(\bar{x}_n)$,
- the maximal flux capability of the metabolic pathway: $\bar{\nu}_{n,\max}(\bar{q}) \triangleq \frac{g(0, \bar{q})}{\mu} f_1(\bar{x}_1, 0)$,
- the concentrations of the intermediate metabolites: $\bar{x}_i = f_i^{-1}\left(\frac{\mu E_n f_n(\bar{x}_n)}{\alpha_i g(\bar{x}_n, \bar{q})}\right)$.

A global regulator changes the maximum capability of the metabolic pathway directly through the modulation of the concentration of different enzymes in the pathway. Moreover, the flux demand ν_n adapts itself in agreement with the variations induced by the effect of q on the production function g.

5.5.2 Interconnections Between Modules

In this section, we investigate the conditions of existence and uniqueness of a structural steady-state regime for different configurations of connected modules. Therefore, we analysed two modules that are connected in series and in parallel. We will first introduce a generic result for modules that are connected in series and will then provide the rules that define the connection between modules in the summary tables.

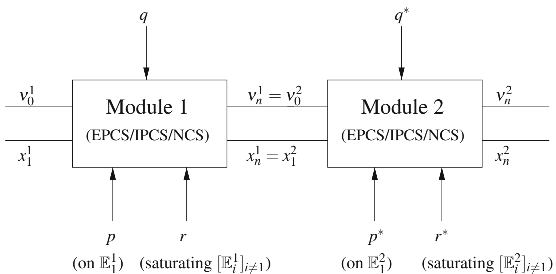
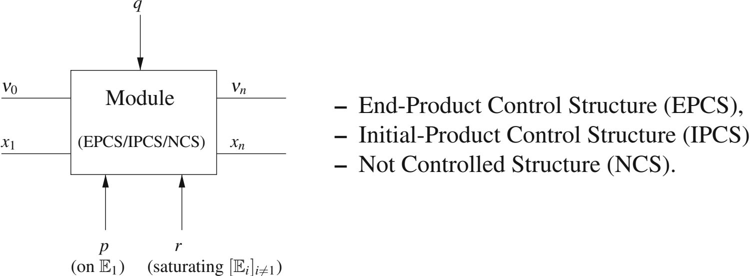


Fig. 5.6 Connection between two modules in series

5.5.2.1 The Input/Output Representation of a Module



In steady state, a module is characterised by its input/output characteristics (displayed in the above figure and see Remark 4) whose existence is conditioned by the assumption that the enzymes do not saturate. In the remainder of this section, we assume that this condition of existence is always satisfied. The input/output notations of flux and metabolites are in agreement with systems (5.5), (5.20). We recall the following input/output characteristics, which were obtained for the three types of modules:

- EPCS module: $\bar{x}_n = H_{pf}(\bar{x}_1)$ and $\nu_0 = \nu_n$, (the consequences of Corollary 2 are extended for the case of (a) only the first enzyme is irreversible and (b) the genes are not in the same operon), where H_{pf} is increasing in its argument;
- IPCS module: $\bar{x}_n = H_{pi}(\bar{x}_1)$ and $\nu_0 = \nu_n$ is defined in Proposition 4, which was extended for the same conditions as the EPCS module, where H_{pi} is increasing in its argument;
- NCS module: $\bar{x}_n = H_{ncs}(\bar{x}_1)$ and $\nu_0 = \nu_n$, where H_{ncs} is increasing in its argument.

We can deduce the following consequences for two modules that are connected in series (see Fig. 5.6):

- the connection of EPCS modules in series leads to a system with a unique steady-state regime. For the ith EPCS module, all of the upstream EPCS modules are reduced through the increasing characteristics $\hat{H}_{pf}$ such that $\bar{x}_n^i = \hat{H}_{pf}(\bar{x}_1^1)$ and $\nu_0^1 = \nu_n^i$ by using $\bar{x}_1^{k+1} = \bar{x}_n^k$ and $\bar{x}_n^k = H_{pf}^k(\bar{x}_1^k)$ for $k \in \{1, \ldots, i-1\}$;

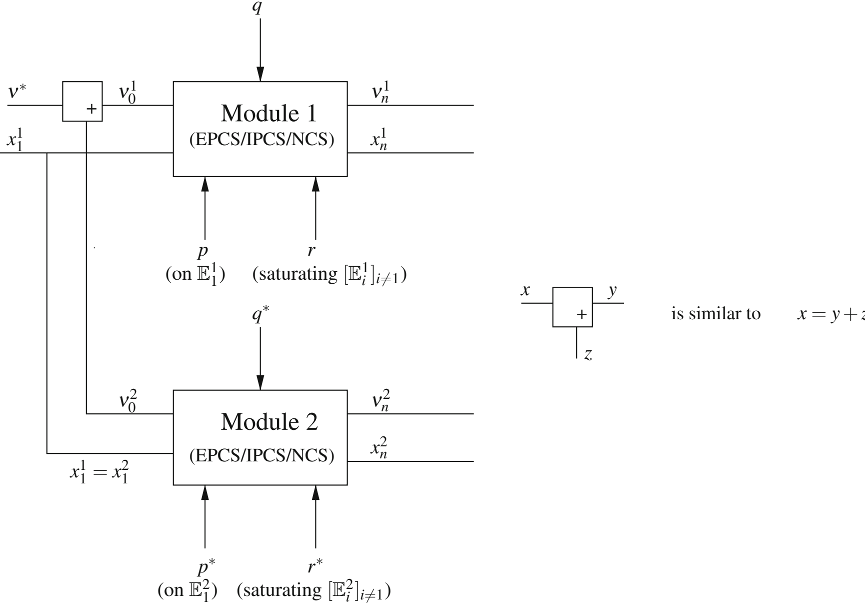


Fig. 5.7 Two modules connected in parallel

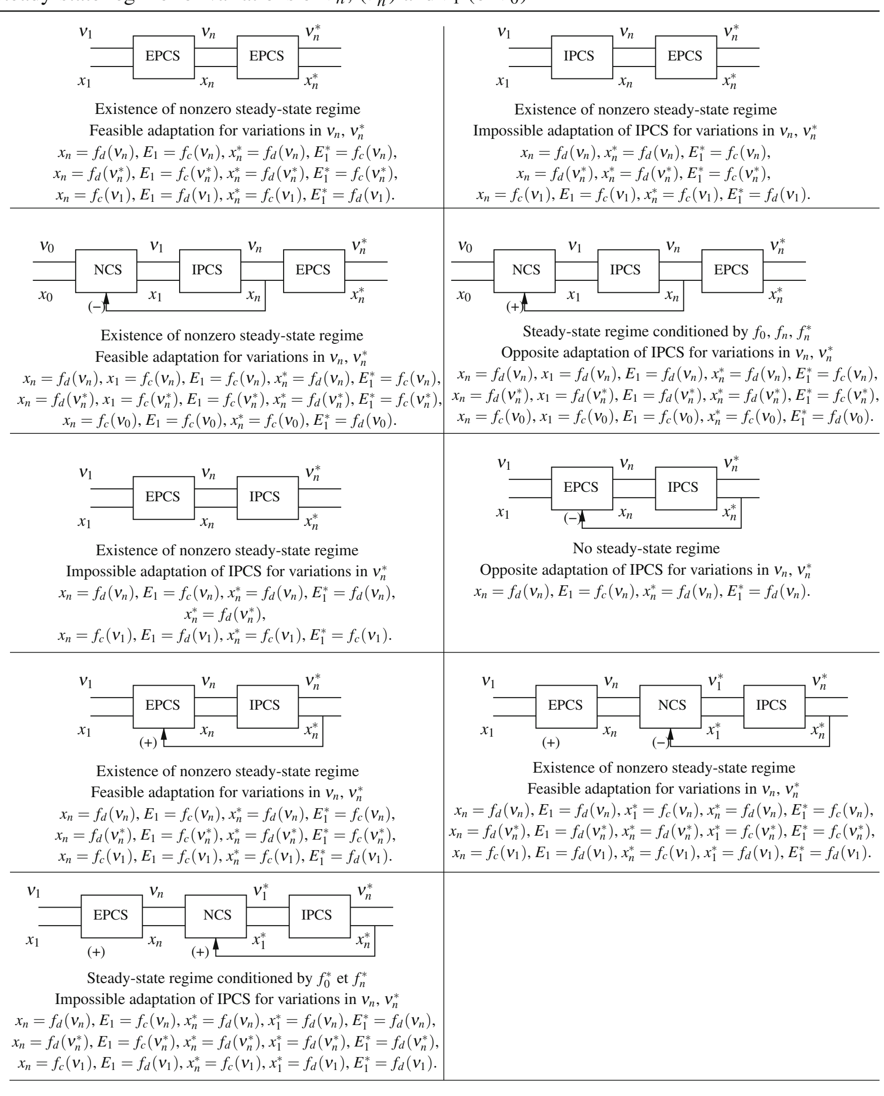
- the connection of IPCS modules in series leads to a system that has a unique steady-state regime. For the ith IPCS module, all of the upstream IPCS modules are reduced through the increasing characteristics $\hat{H}_{pi}$ such that $\bar{x}_n^i = \hat{H}_{pi}(\bar{x}_1^1)$ and $\nu_0^1 = \nu_n^i$ by using $\bar{x}_1^{k+1} = \bar{x}_n^k$ and $\bar{x}_n^k = H_{pi}^k(\bar{x}_1^k)$ for $k \in \{1, \ldots, i-1\}$.

5.5.2.2 The Rules That Define the Connection of Modules

The rules for the interconnection of modules can easily be deduced from the proofs of Propositions 3, 4, 5, which are, respectively, shown in pages 12, 17 and 25, for the connection of modules in series (see Fig. 5.6) or in parallel (see Fig. 5.7) under the assumption that none of the enzymes are saturated. Tables 5.1 and 5.2 summarise the rules of interconnection between modules in series and in parallel, respectively. Specifically, for each of the different connections, these tables show if there exists a structural nonzero steady-state regime and how changes in ν_1, ν_n and ν_n^* results in variations in x_n, x_n^*, E_1, E_1^*, x_1, and x_1^*. In both tables, for the sake of readability, we use the following notations: f_c for increasing functions and f_d for decreasing functions to describe the monotonicity.

The existence of the equilibrium state is always inferred through the monotonicity of the functions and by assuming a final demand for all last connected modules ($\nu_n = E_n f_n(x_n)$). In some cases, such as in a connection of NCS/IPCS/EPCS modules in

Table 5.1 Rules for the interconnection of several modules in series and characteristics of the steady-state regime for variations of ν_n, (ν_n^*) and ν_1 (or ν_0)



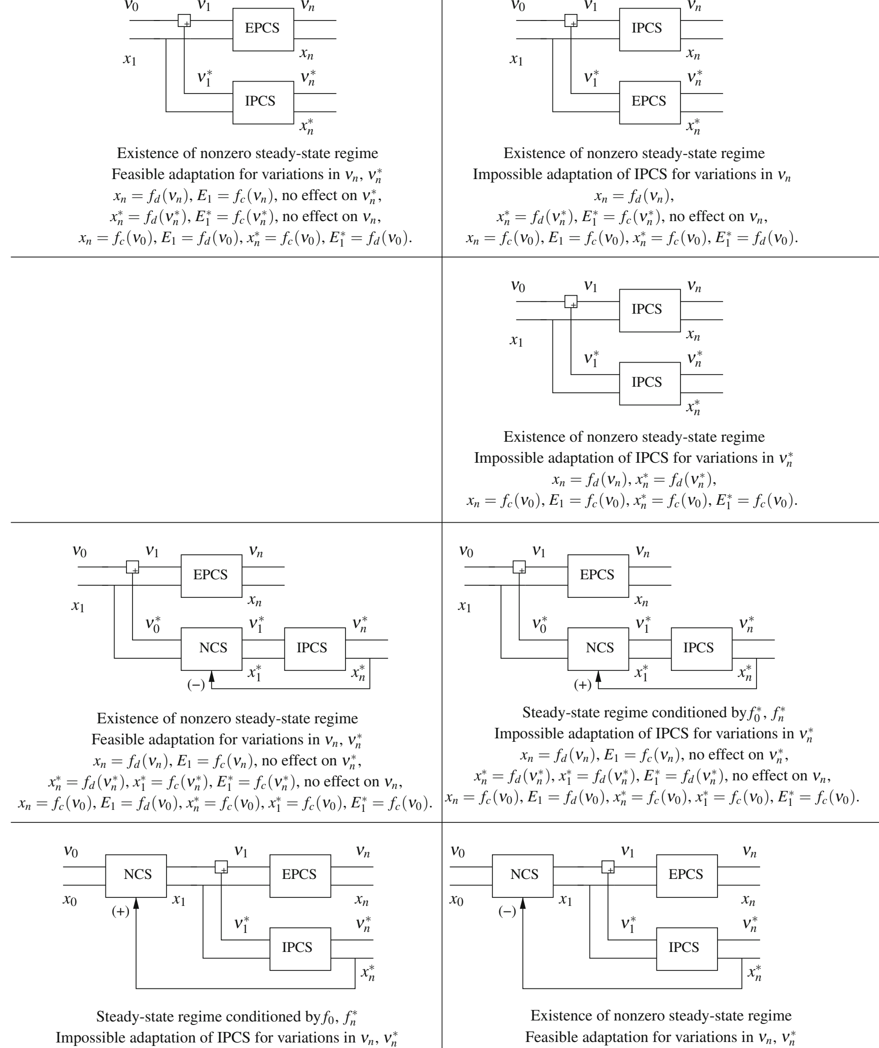
Existence of nonzero steady-state regime Feasible adaptation for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_c(\nu_n)$, $x_n = f_d(\nu_n^*), E_1 = f_c(\nu_n^*), x_n^* = f_d(\nu_n^*), E_1^* = f_c(\nu_n^*)$, $x_n = f_c(\nu_1), E_1 = f_d(\nu_1), x_n^* = f_c(\nu_1), E_1^* = f_d(\nu_1)$.	Existence of nonzero steady-state regime Impossible adaptation of IPCS for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_c(\nu_n)$, $x_n = f_d(\nu_n^*), x_n^* = f_d(\nu_n^*), E_1^* = f_c(\nu_n^*)$, $x_n = f_c(\nu_1), E_1 = f_c(\nu_1), x_n^* = f_c(\nu_1), E_1^* = f_d(\nu_1)$.
Existence of nonzero steady-state regime Feasible adaptation for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), x_1 = f_c(\nu_n), E_1 = f_c(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_c(\nu_n)$, $x_n = f_d(\nu_n^*), x_1 = f_c(\nu_n^*), E_1 = f_c(\nu_n^*), x_n^* = f_d(\nu_n^*), E_1^* = f_c(\nu_n^*)$, $x_n = f_c(\nu_0), E_1 = f_c(\nu_0), x_n^* = f_c(\nu_0), E_1^* = f_d(\nu_0)$.	Steady-state regime conditioned by f_0, f_n, f_n^* Opposite adaptation of IPCS for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), x_1 = f_d(\nu_n), E_1 = f_d(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_c(\nu_n)$, $x_n = f_d(\nu_n^*), x_1 = f_d(\nu_n^*), E_1 = f_d(\nu_n^*), x_n^* = f_d(\nu_n^*), E_1^* = f_c(\nu_n^*)$, $x_n = f_c(\nu_0), x_1 = f_c(\nu_0), E_1 = f_c(\nu_0), x_n^* = f_c(\nu_0), E_1^* = f_d(\nu_0)$.
Existence of nonzero steady-state regime Impossible adaptation of IPCS for variations in ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_d(\nu_n)$, $x_n^* = f_d(\nu_n^*)$, $x_n = f_c(\nu_1), E_1 = f_d(\nu_1), x_n^* = f_c(\nu_1), E_1^* = f_c(\nu_1)$.	No steady-state regime Opposite adaptation of IPCS for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_d(\nu_n)$.
Existence of nonzero steady-state regime Feasible adaptation for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_c(\nu_n)$, $x_n = f_d(\nu_n^*), E_1 = f_c(\nu_n^*), x_n^* = f_d(\nu_n^*), E_1^* = f_c(\nu_n^*)$, $x_n = f_c(\nu_1), E_1 = f_c(\nu_1), x_n^* = f_c(\nu_1), E_1^* = f_d(\nu_1)$.	Existence of nonzero steady-state regime Feasible adaptation for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), E_1 = f_d(\nu_n), x_1^* = f_c(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_c(\nu_n)$, $x_n = f_d(\nu_n^*), E_1 = f_d(\nu_n^*), x_n^* = f_d(\nu_n^*), x_1^* = f_c(\nu_n^*), E_1^* = f_c(\nu_n^*)$, $x_n = f_c(\nu_1), E_1 = f_d(\nu_1), x_n^* = f_c(\nu_1), x_1^* = f_d(\nu_1), E_1^* = f_d(\nu_1)$.
Steady-state regime conditioned by f_0^* et f_n^* Impossible adaptation of IPCS for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n), x_n^* = f_d(\nu_n), x_1^* = f_d(\nu_n), E_1^* = f_d(\nu_n)$, $x_n = f_d(\nu_n^*), E_1 = f_c(\nu_n^*), x_n^* = f_d(\nu_n^*), x_1^* = f_d(\nu_n^*), E_1^* = f_d(\nu_n^*)$, $x_n = f_c(\nu_1), E_1 = f_d(\nu_1), x_n^* = f_c(\nu_1), x_1^* = f_d(\nu_1), E_1^* = f_d(\nu_1)$.	

We assume that (i) the input flux ν_1 (or ν_0) is able to maintain the concentration of the first metabolite x_1 (or x_0) constant and (ii) the enzymes of the modules do not saturate. f_c increasing function and f_d decreasing function

series that is associated with positive feedback (see Table 5.1), we cannot directly conclude the existence of a steady-state regime. Typically, we obtain a necessary condition of intersection between two increasing functions:

$$E_0 f_0(x_0, \bar{x}_n) = E_n f_n(\bar{x}_n),$$

Table 5.2 Rules for the interconnection of several modules in parallel and characteristics of the steady-state regime for variations of ν_n, (ν_n^*) and ν_1 (or ν_0)



Existence of nonzero steady-state regime Feasible adaptation for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n)$, no effect on ν_n^*, $x_n^* = f_d(\nu_n^*), E_1^* = f_c(\nu_n^*)$, no effect on ν_n, $x_n = f_c(\nu_0), E_1 = f_d(\nu_0), x_n^* = f_c(\nu_0), E_1^* = f_d(\nu_0)$.	Existence of nonzero steady-state regime Impossible adaptation of IPCS for variations in ν_n $x_n = f_d(\nu_n)$, $x_n^* = f_d(\nu_n^*), E_1^* = f_c(\nu_n^*)$, no effect on ν_n, $x_n = f_c(\nu_0), E_1 = f_c(\nu_0), x_n^* = f_c(\nu_0), E_1^* = f_d(\nu_0)$.
	Existence of nonzero steady-state regime Impossible adaptation of IPCS for variations in ν_n^* $x_n = f_d(\nu_n), x_n^* = f_d(\nu_n^*)$, $x_n = f_c(\nu_0), E_1 = f_c(\nu_0), x_n^* = f_c(\nu_0), E_1^* = f_c(\nu_0)$.
Existence of nonzero steady-state regime Feasible adaptation for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n)$, no effect on ν_n^*, $x_n^* = f_d(\nu_n^*), x_1^* = f_c(\nu_n^*), E_1^* = f_c(\nu_n^*)$, no effect on ν_n, $x_n = f_c(\nu_0), E_1 = f_d(\nu_0), x_n^* = f_c(\nu_0), x_1^* = f_c(\nu_0), E_1^* = f_c(\nu_0)$.	Steady-state regime conditioned by f_0^*, f_n^* Impossible adaptation of IPCS for variations in ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n)$, no effect on ν_n^*, $x_n^* = f_d(\nu_n^*), x_1^* = f_d(\nu_n^*), E_1^* = f_d(\nu_n^*)$, no effect on ν_n, $x_n = f_c(\nu_0), E_1 = f_d(\nu_0), x_n^* = f_c(\nu_0), x_1^* = f_c(\nu_0), E_1^* = f_c(\nu_0)$.
Steady-state regime conditioned by f_0, f_n^* Impossible adaptation of IPCS for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n), x_1 = f_d(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_d(\nu_n)$, $x_n = f_d(\nu_n^*), E_1 = f_c(\nu_n^*), x_1 = f_d(\nu_n^*), x_n^* = f_d(\nu_n^*), E_1^* = f_d(\nu_n^*)$, $x_1 = f_c(\nu_0), x_n = f_c(\nu_0), E_1 = f_d(\nu_0), x_n^* = f_c(\nu_0), E_1^* = f_c(\nu_0)$.	Existence of nonzero steady-state regime Feasible adaptation for variations in ν_n, ν_n^* $x_n = f_d(\nu_n), E_1 = f_c(\nu_n), x_1 = f_d(\nu_n), x_n^* = f_d(\nu_n), E_1^* = f_d(\nu_n)$, $x_n = f_c(\nu_n^*), E_1 = f_d(\nu_n^*), x_1 = f_c(\nu_n^*), x_n^* = f_d(\nu_n^*), E_1^* = f_c(\nu_n^*)$, $x_1 = f_c(\nu_0), x_n = f_c(\nu_0), E_1 = f_d(\nu_0), x_n^* = f_c(\nu_0), E_1^* = f_c(\nu_0)$.

We assume that (*i*) the input flux ν_1 (or ν_0) is able to maintain the concentration of the first metabolite x_1 (or x_0) constant and (*ii*) the enzymes of the modules do not saturate. f_c increasing function and f_d decreasing function

where f_0 and f_n are both increasing functions of $\bar{x}_n$. By convention, the condition of existence of the steady-state regime in these cases is dependent, which is in contrast to those cases in which the existence of the steady state was achieved structurally.

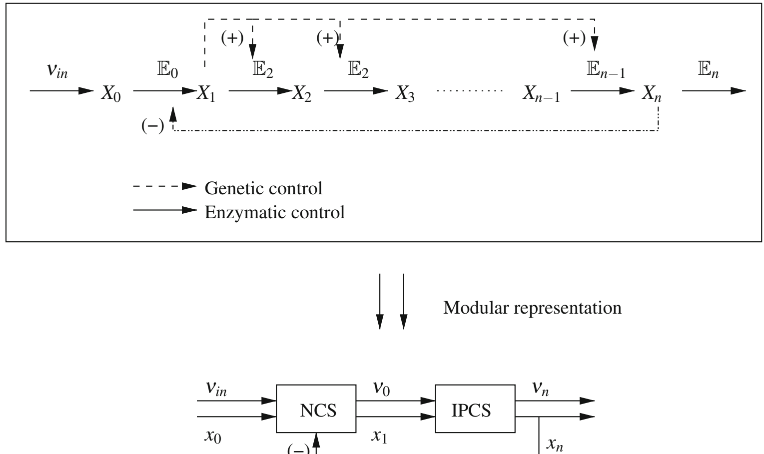


Fig. 5.8 The modular decomposition of the synthesis of purines: NCS and IPCS modules are connected in series and combined with a negative feedback

5.5.2.3 An Example: The Synthesis of Purines

Purines are the main precursors of RNA and DNA synthesis. Thus, one could expect that the control of the synthesis pathway of purines would be driven by the downstream flux demand, i.e., an end-product control structure, such as with amino acids. Surprisingly, the control structure corresponds to an IPCS module that is coupled to an enzymatic inhibition of the upstream enzyme $\mathbb{E}_0$, which produces the initial metabolite X_1, by the final metabolite X_n [27, 30, 39]. We will now prove that, contrary to an IPCS module alone, this control structure is able to cope with a final flux demand. Schematically, the combination corresponds to a NCS module and an IPCS module that are connected in series; these connected modules are combined with negative feedback (see Fig. 5.8). This combination will be referred to as IPCS$^{(-)}$ in the next section. Moreover, all the genes involved in the purine synthesis are in operon [39].

The steady-state output flux ν_0 of the NCS module is given by:

$$\nu_0 = E_0 f_0(x_0, x_n), \tag{5.23}$$

where $E_0 > 0$ is fixed and f_0 satisfies the characteristics of an irreversible enzyme that is inhibited by a metabolite and is decreasing (resp. increasing) in x_n (resp. x_0). The flux ν_0 is the input flux of module IPCS.

Proposition 5 *For all $\mu > 0$, $E_0 > 0$, $E_n > 0$ and $x_0 > 0$, there exists a unique steady-state regime $(\bar{x}_1, \ldots, \bar{x}_n)$ and $(\bar{E}_1, \ldots, \bar{E}_{n-1})$ to system (5.20), which is associated with Eq. (5.23), such that*

$$\begin{cases} E_0 f_0(x_0, \bar{x}_n) = E_n f_n(\bar{x}_n) \\ \nu_0 = E_0 f_0(x_0, \bar{x}_n) \\ \nu_n = E_n f_n(\bar{x}_n) \end{cases} \tag{5.24}$$

if and only if $\nu_0 < E_1 M_1$ *and for all* $i \in \{2, \ldots, n-1\}$, *we have* $\nu_0 < \alpha_i E_1 M_i$. *Moreover,*

- $\bar{x}_n = \bar{x}_n(x_0)$ *is increasing in* x_0.
- $\bar{x}_n = \bar{x}_n(E_n)$ *is decreasing in* E_n *and* $\bar{x}_i = \bar{x}_i(E_n)$ *for* $i = 1, \ldots, n-1$ *are increasing in* E_n.
- $\bar{x}_i = \bar{x}_i(E_0)$ *for* $i = 1, \ldots, n$ *are increasing in* E_0.

Proof The proof is achieved by writing the input/output characteristics of the modules. The connection between the NCS and IPCS in series is direct and the associated characteristics is $\bar{\nu}_{n-1} = H_*(\bar{x}_0, \bar{x}_n)$, where H_* is increasing in $\bar{x}_0$ and decreasing in $\bar{x}_n$. Then it remains to connect this characteristics with the final flux demand $E_n f_n(\bar{x}_n) = \bar{\nu}_n$, which is increasing in $\bar{x}_n$. Due to the monotonicity of the functions H_* and f_n with respect to $\bar{x}_n$, we conclude the existence and uniqueness of the steady state (under the assumption that the enzymes do not saturate). The behaviour of the module components are deduced from the individual module properties.

Remarkably, the steady-state concentration $\bar{x}_n$ of the final metabolite is completely determined by the concentrations and the characteristics of the enzymes $\mathbb{E}_0$ and $\mathbb{E}_n$ and not by the enzymes of the IPCS module. For fixed E_0 and x_0, the input flux ν_0 is directly determined as a function of ν_n and $\bar{x}_n$. The other components of the IPCS module, $(\bar{E}_i, \bar{x}_i)$ for $i \in \{1, \ldots, n-1\}$, are adjusted to cope with the flux demand. In contrast with the case of the IPCS module alone, this module combination is able to cope with the final flux demand.

5.6 Decomposition of the Metabolic Network into Modules

5.6.1 The Main Identified Combinations

Tables 5.1 and 5.2 show the rules that define the interconnection between modules, regardless of their actual presence in an organism. Using the knowledge-based model of *B. subtilis* [17], we can indicate the actual combination of modules that are present in this organism (and in *E. coli*).

Connection of EPCS-EPCS modules in series: This motif, which corresponds to the series of two EPCS modules with an intermediate branching point, occurs in (*a*) the synthesis of glutamate and glutamine [8, 14, 26, 41, 43], (*b*) the synthesis of glutamate and proline [7, 8, 26], and (*c*) the synthesis of S-adenosyl-methionine and cysteine [4, 25]. In *E. coli*, the regulation of the amino acid synthesis pathways have been deeply characterised; therefore, we found that the synthesis of threonine and isoleucine can also be represented by a connected EPCS-EPCS motif [20].

Connection of IPCS-EPCS modules in series: We could not identify this type of connection in the metabolic model. However, if we consider that the initial-product control structure is associated with the inhibition of enzyme $\mathbb{E}_0$ (IPCS$^{(-)}$ in Fig. 5.8), the IPCS$^{(-)}$-EPCS connection can be used to represent the connection between the glycolysis pathway and the syntheses of isoleucine, leucine and valine [9, 32, 33, 35, 37].
Connection of EPCS-IPCS modules in series: The EPCS/IPCS connection is the standard configuration that is used to connect the synthesis and degradation pathways of amino acids, such as arginine [15, 23] and most likely histidine [12, 13, 40, 42]. Unfortunately, the regulation of the synthesis of histidine is unknown. In *E. coli*, the synthesis of histidine is controlled by histidine through the corresponding charged-tRNA and thus by an EPCS module [20]. Usually, a global regulation is present on the connected IPCS module to prevent the simultaneous induction of both modules [6, 12, 13, 42].
Connection of IPCS-IPCS modules in series: We identified the presence of the IPCS-IPCS connection at the level of the synthesis and degradation of fatty acids [22, 31]. The global regulator CcpA prevents the degradation of the fatty acids in glycolytic conditions [22, 24]. Moreover, the IPCS/IPCS$^{(-)}$ connection connects the degradation of carbohydrates with the glycolysis pathways (see references in [17] and [9]). The IPCS$^{(-)}$-IPCS$^{(-)}$ connection has not yet been identified. However, it could exist because the regulatory network is only partially known.

The conditions of existence and uniqueness of the steady-state regime and the qualitative evolution of the main module components can be deduced for all types of these realistic combinations. Remarquably, in most of cases, the steady-state regime exists structurally. Therefore, the existence of steady state only depends on the concentrations of enzymes, which have to be high enough to avoid intermediate enzyme saturation. Finally, the prediction of the qualitative evolution of the main module components has been successfully used to analyse the consistency of datasets (transcriptome, fluxome and metabolome) (see [16] for details).

5.6.2 An Example: The Synthesis of Lysine

In this section, we used our results to compare a specific metabolic pathway, the synthesis of lysine, under two distinct physiological conditions: steady-state growth in glucose and in malate. Both of these growth conditions result in similar growth rate values. Therefore, we used two datasets that were produced in the European project BaSysBio (LSHG-CT-2006-037469). Using our approach, we explained the unexpected repression of the lysine pathway that occurs under malate conditions and not in glucose. As will be shown in the rest of the section, this effect is most likely a direct consequence of the high level of aspartate (the first metabolite of the pathway) that is accumulated under malate growth conditions.

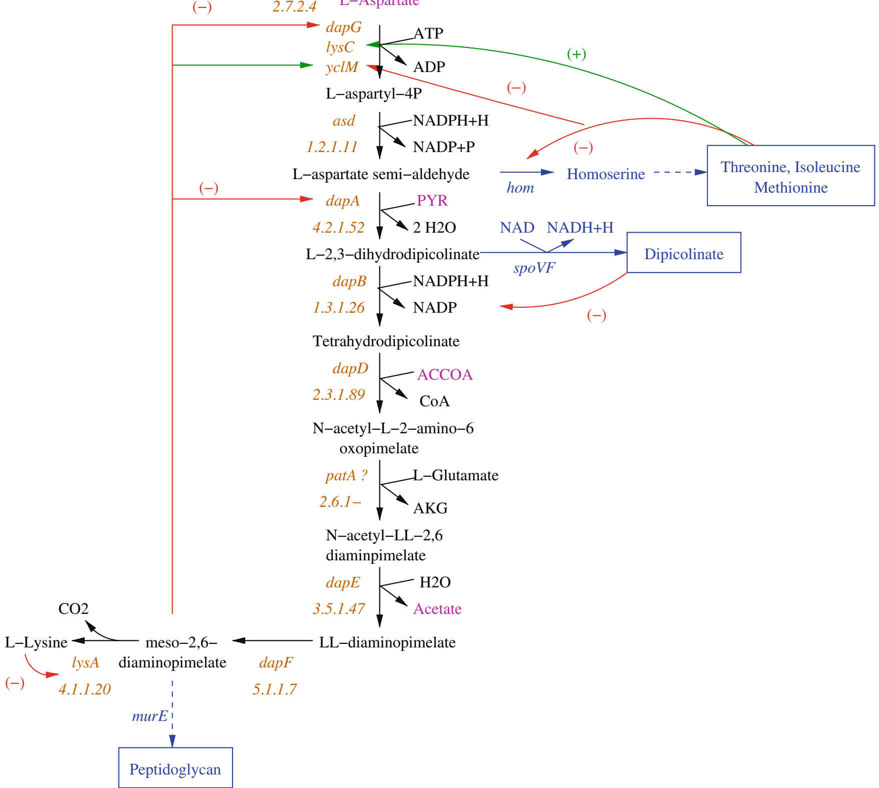


Fig. 5.9 The synthesis pathway of lysine

Figure 5.9 describes the lysine synthesis pathway and its connections with other essential pathways, whereas Fig. 5.10 highlights the key elements that are involved in the regulation of the lysine synthesis pathway:

- the feedback inhibition of the first enzyme of the pathway, which is encoded by the *lysC* gene, by the end product (lysine) and
- the genetic regulation of the same gene by an L-box mechanism.

The L-box is a RNA riboswitch that involves lysine. Lysine binds directly to the lysC nascent mRNA, which causes a structural shift that ends the transcription. The regulation of the *lysA* gene by the same L-box mechanism remains elusive and it is therefore not considered further in the analysis (to maintain the explanation as simple as possible). This structure is classical in metabolic networks and corresponds to the end-product control structure that was described in this chapter. We can directly characterise the properties of the pathway at steady state. The regulation of lysine synthesis satisfies all of the assumptions that are explained in Corollary 4 because

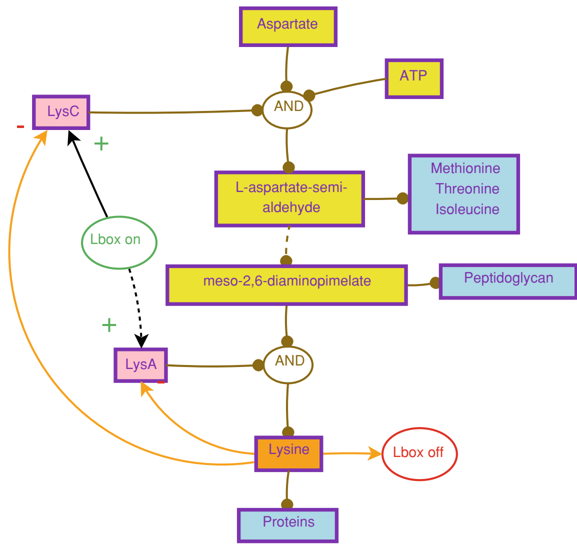


Fig. 5.10 Regulatory network of lysine synthesis

- LysC is irreversible due to the hydrolysis of ATP,
- The activity of the first enzyme is inhibited by the end product (lysine), and
- The transcription of the first enzyme is repressed by the end-product through an L-box mechanism.

Based on the results described in Sect. 5.3, the expression of the gene *lysC* depends on various factors:

1. metabolites, other than lysine, that act on the first enzyme of the pathway, such as aspartate,
2. flux demand, which is defined mainly by the activity of the tRNA synthase LysS, and
3. external factors that modulate the transcription and translation of the first gene, such as the activity of the RNA polymerases and/or the ribosomes.

The qualitative prediction of the system behaviour with respect to the evolution of the first metabolite (aspartate) and the flux demand (the activity of tRNA synthase LysS) can be predicted (see Table 5.3). The predictions that are shown in Table 5.3 can be extended to any other compatible combinations of conditions. Nevertheless, some qualitative predictions are not possible for some combinations due to their contradictory effects on the system. A contradictory combination, such as an increase in both the flux demand and the aspartate pool, could only be solved if the relative effect of the different factors that act on the regulation is known. Obviously, the knowledge of these factors is related to the identification of system. Because the growth rate between the malate and glucose experiments is similar, the impact of the growth rate on (i) the enzyme synthesis and (ii) the amino acid flux demand by the ribosomes is limited by these two conditions. We thus identified the different

Table 5.3 Qualitative prediction of the lysine pathway behaviour under various conditions

Considered conditions		Predictions		
LysS ($\mathbb{E}_n$)	Aspartate	Lysine evolution	Flux evolution	*lysC*-mRNA
+	Constant	−	+	+
−	Constant	+	−	−
Constant	+	+	+	−
Constant	−	−	−	+

Table 5.4 Variation of the lysine module components. gdwc = gram of cell dry weight

Module components	Glucose	Malate
Aspartate (μmol/gdwc)	1.4	10.5
Lysine (μmol/gdwc)	0.1	0.2
mRNA-$lysC$(log)	14.3	12.3

predictions for a constant flux demand under the two conditions. The concentration of lysine is then an increasing function of the aspartate concentration, and in contrast, the expression of *lysC* is a decreasing function of the aspartate concentration. These predictions are in agreement with the experimental data (see Table 5.4), which led us to conclude that the increasing value of the lysine concentration under malate conditions is most likely due to the increasing aspartate concentration.

5.7 Conclusion

The framework that was proposed in this chapter is dedicated to the formal definition and characterization of modules in metabolic pathways. This framework is general enough to study the existence and uniqueness of a structural steady state in any metabolic pathway, including complete metabolic networks. Combined with our results in [17], this is the first report, to the best of our knowledge, of a global-scale analysis of the systematic exploration of all configurations in a realistic biological model. Remarkably, most of the steady-state regimes of realistic metabolic configurations exist structurally. More globally, the local properties of modules have important consequences on the entire metabolic network. Indeed, despite the high coupling that exists in the metabolic pathways (and its associated genetic regulatory network), the steady-state regime of the entire metabolic network is dramatically decoupled. In terms of control, this property is highly expected. Otherwise small variations in a specific module could constantly lead to global genetic adaptations of the entire metabolic network. Beyond the aspects of controllability of the metabolic pathways, we recently shown that the sparing management of resources between the intracellular biological processes of the cell leads to define structural constraints, whose one of their consequences is the emergence of a modular organisation in the metabolic network [18, 19]. An interesting perspective of this framework is the study

of the stability of the elementary modules and their interconnection. The analysis of the stability of metabolic pathways is an open area of research given the very large diversity of configurations and systems and the non-linearity of the equations. Some results have been obtained for linearised systems of specific metabolic pathways [1–3, 36]. Nevertheless, the obtaining of results on the global stability of nonlinear biological system even for one single module remains an open question.

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