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Identifying and characterising regulatory metabolites with generalised supply–demand analysis ☆

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Abstract

We present the framework of generalised supply–demand analysis (SDA) of a kinetic model of a cellular system, which can be applied to networks of arbitrary complexity. By fixing the concentrations of each of the variable species in turn and varying them in a parameter scan, rate characteristics of supply–demand are constructed around each of these species. By inspecting the shapes of the rate characteristic patterns and comparing the flux–response coefficients of the supply and demand blocks with the elasticities of the enzymes that interact directly with the fixed metabolite, regulatory metabolites in the system can be identified and characterised. The analysis provides information on whether and where the system is functionally differentiated and which of its species are homeostatically buffered. The novelty in our proposed method lies in the fact that all metabolites are considered for SDA (hence the term ''generalised''), which removes investigator bias. It supplies an entry point for the further analysis and detailed characterisation of large models of cellular systems, in which the choice of metabolite around which to perform a SDA is not always obvious.

Keywords: Rate characteristics; Homeostasis; Control analysis; Functional differentiation; Elasticity

1. Introduction

While the 20th century can be regarded as the era of structural characterisation in biochemistry and molecular biology, the 21st century is rapidly emerging as an era of integration and synthesis. The burgeoning field of systems biology has developed out of the realisation that biological systems cannot be understood from reductionist characterisation of their components alone, but that their interactions have to be put together in a ''systems'' framework. This has led to experimentation on a system-wide scale in the various omics fields, and to coordinated efforts through large international alliances (Kitano, 2005) for constructing large-scale computer models of living systems. The number of kinetic models of cellular pathways grows weekly, as any survey of the JWS Online (Olivier and Snoep, 2004) and BioModels (le Novère et al., 2006) databases will show. Increasing model sizes have resulted in the development of standards such as SBML for model representation and visualisation (Hucka and Finney, 2005). Such models provide powerful tools that are often more accessible to query and interrogation than experimental systems. Yet, without proper frameworks of analysis, these models, albeit big and comprehensive, remain little more than collections of data.

The framework of supply–demand analysis (SDA), developed by Hofmeyr and Cornish-Bowden (2000), has proved useful in studying the regulation of cellular pathways within the metaphor of an economy controlled by supply and demand. It has become a reference framework for analysing metabolic pathways by teaching scientists to look for flux control beyond the scope of what has traditionally been called the pathway, i.e. in the demand for its end-product, a view that has subsequently been corroborated by experimental data (e.g. Koebmann et al., 2002).

☆This paper is dedicated to the memory of Reinhart Heinrich, one of the founders of control analysis and pioneer in systems biology.

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While SDA can provide useful results, its application to large kinetic models of cellular pathways is hampered by the problem that their complexity may preclude us from finding a "natural" subdivision of the system into supply and demand blocks. With this in mind, the present paper aims to generalise SDA so that it can be applied to models of arbitrary size and complexity in a systematic, computer-driven way. We will show that this enables us to identify and characterise regulatory metabolites in the system and affords an entry point for further detailed investigation of the model.

It is appropriate that this work appears in a memorial issue dedicated to the memory of Reinhart Heinrich. He was together with Kacser and Burns (1973), one of the fathers of the field of control analysis (Heinrich and Rapoport, 1974; Heinrich et al., 1977). For textbook review of this field, see Fell (1996) and Heinrich and Schuster (1996). Control analysis lies at the foundation of SDA. Heinrich was one of the pioneers to realise the power of analysing cellular systems through computer modelling, arguably the most well-known example of this is his work on erythrocytes (e.g. Heinrich, 1985). Until his death, he was never shy to develop new concepts to aid in understanding cellular function; most recently, these included expanding metabolic networks (Ebenhöh et al., 2004) and scopes of compounds (Handorf et al., 2005). We hope that by developing the concept of generalised supply–demand analysis (GSDA) here, we will be able to stand on the shoulders of this scientific giant.

2. The concept: Generalised supply–demand analysis

We begin by considering a system of coupled enzyme-catalysed reactions constituting a cellular network. Such a system can be described (and simulated) with a kinetic model, $\mathrm{d}\mathbf{s}/\mathrm{d}t = \mathbf{N}\mathbf{v}$, where $\mathbf{s}$ and $\mathbf{v}$ are vectors of variable species concentrations and rates, respectively, and $\mathbf{N}$ is the stoichiometric matrix of the system. In order for a steady state to obtain in this system, the inputs and outputs have to be maintained at constant levels (i.e. "clamped"); in practice this means that the pathway substrate has to be continually replenished and its product continually removed. In kinetic models of such a system, these external/terminal species are therefore also kept fixed.

SDA (Hofmeyr and Cornish-Bowden, 2000) considers the subdivision of a pathway around a central intermediate, with the block or blocks of reactions contributing to the production of the intermediate constituting the "supply", and those that contribute to its consumption, the "demand". The behaviour of the system around the steady-state point is assessed with a so-called combined rate characteristic (Hofmeyr, 1995), which depicts how the rates of supply and demand vary with changes in the concentration of the intermediate. The intersection of the supply and demand rate characteristics signifies the steady-state point. If the rate characteristic is drawn in double-logarithmic space, the elasticities of supply and demand towards the intermediate can be read off directly as slopes of the tangents to the supply and demand curves at the steady-state point, enabling the calculation of control coefficients.

One of the main tenets of SDA is that when one of the two blocks controls the flux, the other one determines the degree of homeostasis in the intermediate; such a system has been termed *functionally differentiated*. Thus, in a classical biosynthetic pathway of an amino acid, for example, if the demand for the amino acid controls the flux, the kinetic properties of the supply will set the steady-state concentration of the amino acid, and the supply elasticity will determine the degree of homeostasis in its concentration (the larger the absolute value of the supply elasticity, the smaller the absolute value of the concentration–control coefficients of both supply and demand—they are numerically equal and opposite in sign—and the better the homeostasis in the amino acid concentration).

In silico SDA with a kinetic model is extremely easy. The intermediate around which the rate characteristic is to be constructed, is made into a fixed (clamped) species of the model, thus turning it into a model parameter (and effectively decoupling supply and demand). This parameter is then varied over a wide range through a parameter scan of the model; the fluxes of supply and demand are calculated at each value. An implicit assumption of this approach is that the system has been or can readily be partitioned into supply and demand, and that there is no communication between supply and demand other than through the intermediate. However, when faced with the complexity of cellular pathways or of large models of such pathways, the choice of intermediate around which to perform the SDA is often far from obvious. The aim of this paper is therefore to generalise SDA in such a way that it can easily be performed on kinetic models of any cellular system, large or small, without requiring prior knowledge of its regulatory structure.

GSDA works in the following way: *each* of the variable intermediates is *clamped in turn* and thus made into a parameter of the system. Its concentration is then varied above and below the reference steady-state value in the original system through a parameter scan, and the fluxes through the supply and demand reactions that are directly connected to the intermediate are plotted on a log–log rate characteristic. Every flux that directly produces the intermediate is a separate supply flux, and likewise, each flux that directly consumes it is a separate demand flux. There will thus be as many rate characteristics as there are reactions that produce or consume the intermediate. It should be emphasised that this procedure is valid for arbitrary models and certainly not limited to linear pathways. Neither does it presuppose a subdivision of the system into supply and demand blocks; rather, the analysis is performed for every intermediate. Moreover, the restriction in ordinary SDA that prohibits communication between the blocks other than through the linking intermediate is removed (e.g. it is permissible for a

metabolite in a supply block to affect a reaction in a demand block directly through allosteric interaction).

GSDA yields as many combined rate characteristic graphs as there are variable species in the system. As will be shown below, the following important features about the regulation of the system can be identified from the shapes of the curves and associated elasticities and response coefficients:

(1) potential sites of regulation;
(2) regulatory metabolites;
(3) the quantitative relative contribution of different routes of interaction from an intermediate to a supply or demand block;
(4) sites of functional differentiation where one of the supply or demand blocks predominantly controls the flux, and the other determines the degree of homeostatic buffering of the intermediate.

At this point, the above considerations may seem abstract and theoretical. We therefore exemplify GSDA in the next section with a model of a linear 5-enzyme pathway containing a feedback loop and with a branched model.

3. Example: Generalised supply–demand analysis of two small metabolic pathways

3.1. Computational methods

All simulations presented in this paper use variants of a kinetic model of a simple linear 5-step pathway (Fig. 1) or a branched 6-step pathway (Fig. 2). Steady-state calculations were performed with the PySCeS software developed in our group (Olivier et al., 2005), using an IBM-compatible PC. PySCeS is written in the Python programming language (`http://www.python.org`) and makes use of the SciPy library of numerical routines (`http://scipy.org`). Graphs were prepared with the Matplotlib (`http://matplotlib.sourceforge.net`) plotting library for Python. An add-on Python package, ratechar, was developed for PySCeS, which automatically performs the GSDA for a given PySCeS model by clamping each variable species in turn, varying its concentration in a parameter scan and plotting the resulting graphs. The code is available from the authors under the open-source GNU general public licence.

Detailed model descriptions for all the models, as well as model definition files in PySCeS input file (Olivier et al., 2005) and SBML (Hucka et al., 2004) formats, are available in Supplementary data accompanying this paper.

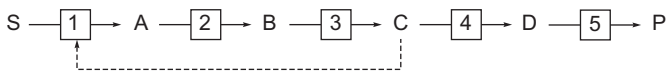

Fig. 1. A five-enzyme linear pathway converting substrate S to product P. In model III, the first enzyme is allosterically inhibited by intermediate C (see main text).

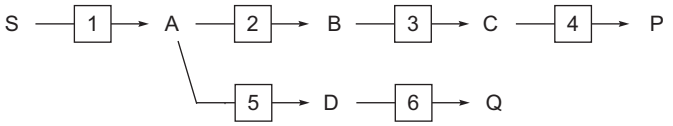

Fig. 2. A six-enzyme branched pathway converting substrate S to products P and Q.

3.2. The procedure of generalised supply–demand analysis

To illustrate the procedure of GSDA, three variants of the model in Fig. 1 were considered (models I–III), each with different kinetics to give different dynamic behaviour that could be classified as "regulated" vs. "non-regulated" or "functionally differentiated" vs. "undifferentiated".

Furthermore, to illustrate that the analysis is not restricted to linear pathways, the procedure was applied to the branched model in Fig. 2 (model IV).

The models all have four variable metabolites (A–D) around which the GSDA is performed. The main features of the models are the following:

Model I: This is the base-line, undifferentiated version of the linear model (Fig. 1). All five enzymes have identical kinetic parameters and are modelled with reversible Michaelis–Menten kinetics (with the exception of enzyme 5, which is modelled with irreversible Michaelis–Menten kinetics). There is no allosteric feedback from C to enzyme 1.

Model II: This is the same as model I, except that reaction 4 has been made insensitive to changes in the concentration of C by lowering the limiting rate of enzyme 4 (V_{f4}) and its K_M for C.

Model III: In this model, enzyme 1 is inhibited allosterically by C and is modelled with reversible Hill kinetics (Hofmeyr and Cornish-Bowden, 1997). The limiting rates of enzymes 2 and 3 have been increased so that they are close to equilibrium. Enzymes 4 and 5 together have almost complete control over the flux through the pathway.

Model IV: This is branched model of Fig. 2.

As explained in Section 2, a GSDA is performed by clamping each variable species of the model in turn and varying its concentration to generate the supply and demand rate characteristics. This yields graphs such as in Fig. 3, which shows the GSDA around metabolite B in model I. To facilitate the interpretation of such graphs, this specific case will be discussed in detail before presenting the GSDA for all metabolites of all four models. Fig. 3 shows the log–log rate characteristics of supply and demand, with their intersection marking the steady-state point. The supply rate characteristic is drawn in light grey and the demand rate characteristic in medium grey. Rate characteristics are extremely useful for visualising supply–demand control analysis. For example, the slopes of the tangents to the rate characteristics (indicated by dashed

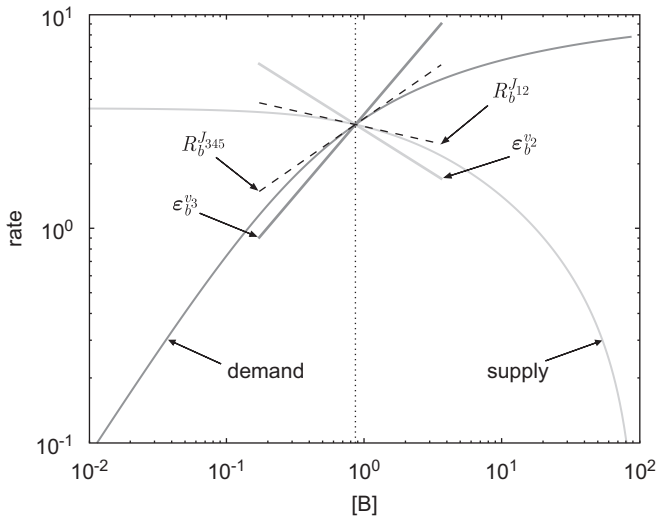

Fig. 3. Supply–demand analysis around metabolite B for model I. The concentration of B was clamped and varied to generate the supply and demand rate characteristics, as described in the text. The steady-state concentration is indicated by a vertical dotted line. The rate characteristics, response coefficients (tangents to the rate characteristics at the steady-state point) and elasticities of the supply and demand enzymes directly connected to B are labelled on the graph.

lines on the graph) equal the flux–response coefficients of supply and demand towards B (in Fig. 3, J_{12} signifies the flux through the supply block and J_{345} that through the demand block). These response coefficients quantify how sensitively the supply and demand fluxes respond towards changes in b. Note that they are equivalent to "block-elasticities" (Fell and Sauro, 1985) or co-response coefficients (Hofmeyr et al., 1993; Hofmeyr and Cornish-Bowden, 1996) in the complete system where B is not clamped, e.g.

$$R_b^{J345}(\text{B clamped}) = \varepsilon_b^{v345}(\text{whole system}) = O_{12}^{J345:b}(\text{whole system}). \quad (1)$$

SDA as originally described (Hofmeyr and Cornish-Bowden, 2000) assumes that the only communication between supply and demand is via the linking intermediate. In this situation, the supply–demand block control coefficients of the complete pathway can be directly calculated from the supply and demand block elasticities:

$$C_{v_{12}}^J = \frac{\varepsilon_b^{v345}}{\varepsilon_b^{v345} - \varepsilon_b^{v12}}, \quad C_{v_{345}}^J = \frac{-\varepsilon_b^{v12}}{\varepsilon_b^{v345} - \varepsilon_b^{v12}},$$

$$C_{v_{12}}^b = \frac{1}{\varepsilon_b^{v345} - \varepsilon_b^{v12}}, \quad C_{v_{345}}^b = \frac{-1}{\varepsilon_b^{v345} - \varepsilon_b^{v12}}. \quad (2)$$

Eq. (2) shows that the distribution of flux control is determined by the ratio of the block elasticities ($C_{v_{12}}^J / C_{v_{345}}^J = -\varepsilon_b^{v345}/\varepsilon_b^{v12}$), while the magnitude of concentration control is determined by the sum $\varepsilon_b^{v345} - \varepsilon_b^{v12}$. GSDA relaxes the condition that the only communication between supply and demand is through the linking metabolite. As a consequence, the control analysis of Eq. (2) will be invalid for A and B in model III (Fig. 4c), as the feedback loop introduces an additional link from their demand to their supply blocks.

Fig. 3 also shows graphically the elasticities of the enzymes that produce or consume B. Elasticities are local properties of enzymes and quantify how sensitively an enzyme's local rate responds to changes in a substrate, product or effector. In this case B is a product of v_2 and a substrate for v_3, so Fig. 3 shows $\varepsilon_b^{v_2}$ (thick light grey line) and $\varepsilon_b^{v_3}$ (thick medium grey line).

The crux of GSDA now lies in the comparison of the values of the response coefficients with the elasticities of the enzymes that are directly connected to the clamped metabolite. In Fig. 3, these values differ, i.e. $R_b^{J12} \neq \varepsilon_b^{v_2}$ and $R_b^{J345} \neq \varepsilon_b^{v_3}$. In other cases, they will be seen to agree. However, before comparing them in detail, first we have to present the GSDA of all metabolites for the four models.

The graphs in Fig. 4 present the results of the GSDA on models I–IV. To avoid clutter, the graphs are not annotated but they follow the same convention as Fig. 3: light grey is used for supply and medium grey for demand, response coefficients are drawn with dashed lines and elasticities with thick lines. The only additional piece of information required is that of an allosteric modifier elasticity ($\varepsilon_c^{v_1}$ in Fig. 4c with C clamped, as only model III has the feedback loop). This is drawn in a thick dark grey line to set it apart from the supply and demand elasticities. At a branch point, where there is more than one supply or demand flux (e.g. for metabolite A in Fig. 4d), their sum is indicated by a dash-dotted line; here, the intersection of the total supply and total demand rate characteristics determines the steady-state concentration of the intermediate.

The graphs in Fig. 4 contain a wealth of information. Having shown how to create them using GSDA, the next section deals with their interpretation.

4. Interpretation of generalised supply–demand analysis graphs

Rate characteristics generated by GSDA can be interpreted on four levels, i.e. differences in the rate characteristic shapes as one proceeds from one metabolite to the next in the pathway, comparison of elasticity and response slopes, identification of points of functional differentiation and homeostasis, and finally, refined analysis through partial response coefficients.

4.1. Differences in rate characteristic shapes

The first assessment criterion of GSDA merely looks at the general shapes of the supply and demand rate characteristics and is not yet concerned with elasticities and response coefficients. In model I (Fig. 4a) all enzymes

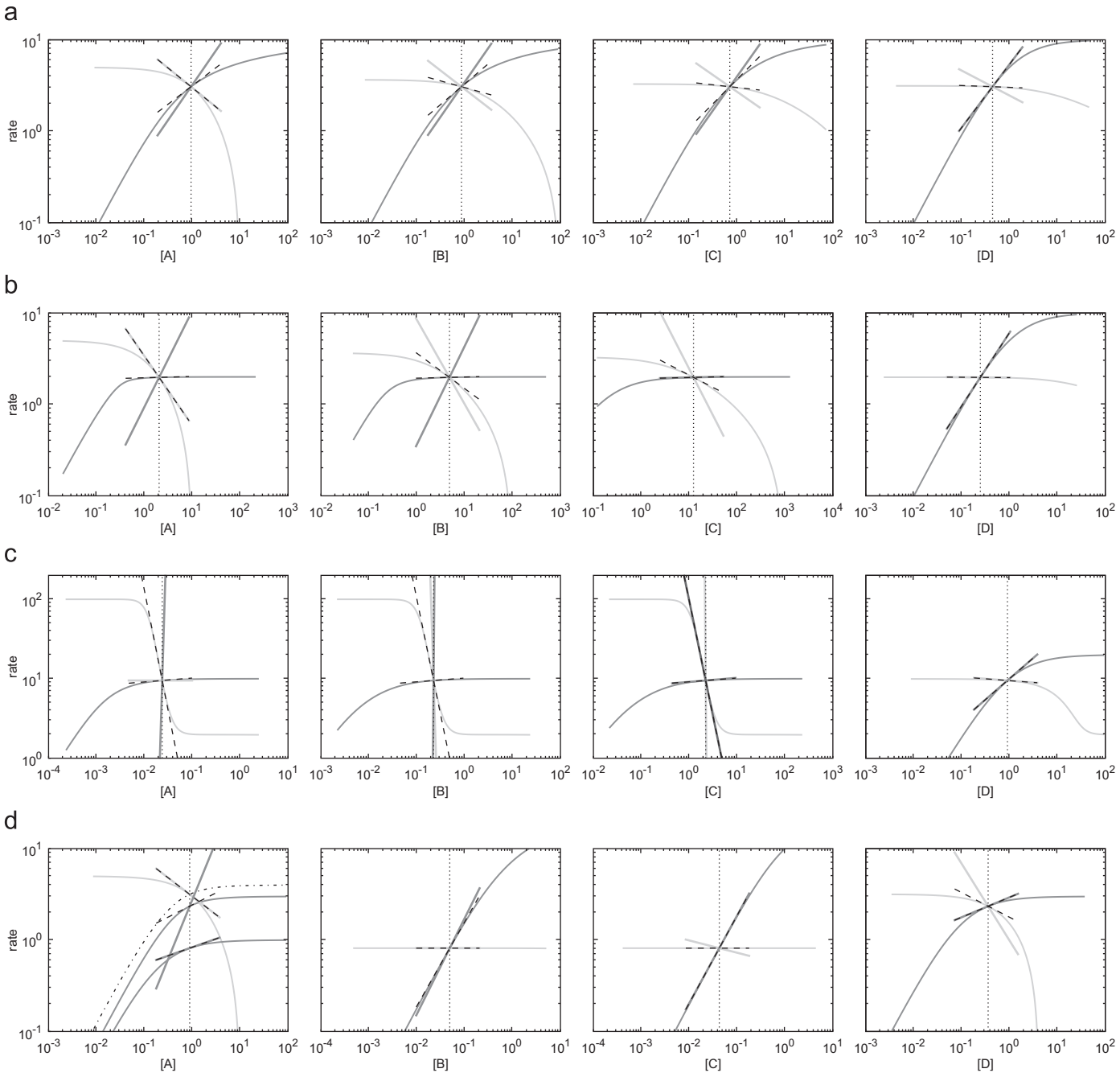

Fig. 4. Generalised supply–demand analysis of the systems depicted in Figs. 1 and 2. The concentrations of A–D were clamped in turn and varied to generate the supply and demand rate characteristics, as described in the text. The supply rate characteristic is drawn in light grey, that for the demand in medium grey. The steady-state concentration of the clamped metabolite is indicated by a vertical dotted line. The response coefficients of the supply and demand blocks are indicated by black dashed lines. The elasticities of the supply and demand enzymes for the clamped intermediate they are directly connected to are indicated by thick lines of the same colour as the rate characteristic. Model variants: (a) model I, (b) model II, (c) model III and (d) model IV (see main text). In (c), the allosteric elasticity $\varepsilon_c^{v_1}$ is indicated by a dark grey thick line. For the branch point at A in (d), the rate characteristics, elasticities and response coefficients are shown separately for each of the two demand fluxes; the dash-dotted line indicates their sum and its intersection with the supply rate characteristic determines the steady-state concentration of A.

have identical kinetics and the overall shapes of the rate characteristics are similar for metabolites A–D. In models II and III (Fig. 4b and c), however, the pattern for D is different from those for A–C (which are still similar). This means that the kinetic properties of enzyme 4 are such that a site of regulation has been introduced into the system. In this specific case the reason is that enzyme 4 has been made insensitive to changes in the concentration of C ($\varepsilon_c^{v_4} \approx 0$). In general, such zero elasticities, whether towards substrate or product, induce a change in the rate characteristic shape because they shift the flux control to demand or supply, respectively.

Overall, changes in the rate characteristic shapes thus pin-point potential sites of regulation.

4.2. Comparison of elasticities and response coefficients

GSDA can be extended to a second level by comparing the values of the elasticities and flux–response coefficients at the steady-state point for each metabolite. From the partitioned response property of control analysis (Kacser and Burns, 1973)

$$R_p^J = \varepsilon_p^{v_i} \times C_{v_i}^J \tag{3}$$

it follows that $R_p^J = \varepsilon_p^{v_i}$ if $C_{v_i}^J = 1$. This means that the enzyme on which the intermediate acts directly must have full control over its own flux. Note that in the context of GSDA these fluxes and response coefficients are defined in the modified system with the intermediate clamped, and not for the complete (original) system. The reason for this is that the rate characteristics themselves are generated with such models that in turn clamp each of the intermediates.

Fig. 4 shows that in general response and elasticity coefficients differ. There are, however, a few notable exceptions. The first of these is the trivial case of the first and last metabolites in the chain (A and D for models I–III; A, C and D for model IV), which take part in a reaction that either consumes a clamped source metabolite or produces a clamped sink metabolite. Consider models I–III (Fig. 4a–c), for example, where $\varepsilon_a^{v1} = R_a^{J1}$ and $\varepsilon_d^{v5} = R_d^{J5}$. This is understandable because the supply block for A and demand block for D each consist only of a single enzyme. Similarly, in model IV (Fig. 4d), $\varepsilon_a^{v1} = R_a^{J1}$, $\varepsilon_c^{v4} = R_c^{J4}$ and $\varepsilon_d^{v6} = R_d^{J6}$. That $\varepsilon_a^{v1} \neq R_a^{J1}$ in Fig. 4c has to do with the feedback loop and will be further discussed in Section 4.4.

Aside from the trivial case, any agreement between elasticity and response coefficient points to a site of regulation. Eq. (3) shows that the response coefficient can equal the elasticity either if the control coefficient is one (as discussed above), or if the elasticity is zero (which effectively makes the value of the control coefficient irrelevant). The first case obtains, for example, in Fig. 4c with C clamped, where $\varepsilon_c^{v1} = R_c^{J123}$ (feedback loop with $C_{v_1}^{J123} = 1$). Here, C can be classified as a "regulatory metabolite" with respect to its supply block because the flux response of this supply towards the clamped metabolite concentration is exactly the same as the activity response (i.e. elasticity) of the enzyme directly affected by the clamped metabolite. The flux–control coefficient of one causes the flux response to be transmitted fully through the block. That metabolites such as C in this example could also be regarded as "regulated" in the context of the whole pathway is discussed in Section 4.3.

The second case (zero elasticity) obtains, for example, in Fig. 4b and c, where $\varepsilon_c^{v4} = R_c^{J45} \approx 0$. Such a zero elasticity confers flux control (in the complete system) to that particular block and results in functional differentiation of the system, which is further discussed in Section 4.3.

When a branch point exists (such as for A in model IV), the elasticities and response coefficients are compared for each branch flux *separately*. Thus, Fig. 4d (metabolite A) shows that for flux J_{234} (the lower of the two demand fluxes), $\varepsilon_a^{v2} = R_a^{J234}$, while for flux J_{56} (the higher of the demand fluxes), $\varepsilon_a^{v5} \neq R_a^{J56}$. This means that A is a regulatory metabolite for the branch J_{234} (the effect of a change in its concentration is transmitted perfectly along the branch since $C_{v_2}^{J234} \approx 1$), while this is not the case for the other branch J_{56}.

4.3. Functional differentiation and homeostasis

SDA has shown that when one block (say, demand) controls the flux through a pathway, the other (say, supply) will determine and control the concentration of the intermediate (Hofmeyr and Cornish-Bowden, 2000). Such a pathway has been termed "functionally differentiated" as flux and concentration control are functions of different blocks. Complete flux control by a supply or demand block (over the whole pathway) can easily be identified by a zero response coefficient (i.e. block elasticity) of that block towards the intermediate (e.g. R_c^{J45} in Fig. 4b or c). The response coefficient of the other block (R_c^{J123}) will then determine the degree of homeostasis in the intermediate: the larger its numerical value, the better the homeostatic buffering.

Model III (Fig. 4c) has been discussed in detail as an example of a functionally differentiated system in the context of SDA (Hofmeyr and Cornish-Bowden, 1991, 2000), and the arguments will not be repeated here. Suffice it to say that the properties of the feedback elasticity ε_c^{v1}, which equals R_c^{J123} here, set the steady-state concentration of C and determine its degree of homeostatic buffering. In this sense, the steady-state concentration of C can be regarded as "regulated". Why C can be considered a "regulatory" metabolite when considering the supply block in isolation has been discussed above.

4.4. Multiple routes of interaction and partial response coefficients

When two or more direct routes of interaction exist from a clamped metabolite to a particular supply or demand block, GSDA can be further refined by dissecting the response coefficient into partial response coefficients. An example is the GSDA around metabolite C in Fig. 1, where C can affect both enzymes 1 and 3 directly (the former through allosteric inhibition, the latter through product inhibition). Here the total response coefficient can be written as

$$R_c^{J123} = \varepsilon_c^{v1} C_{v_1}^{J123} + \varepsilon_c^{v3} C_{v_3}^{J123} = {}^1R_c^{J123} + {}^3R_c^{J123}. \tag{4}$$

The terms on the right-hand side of Eq. (4) are known as *partial response coefficients* and quantify the contribution of each interaction to the total response coefficient.

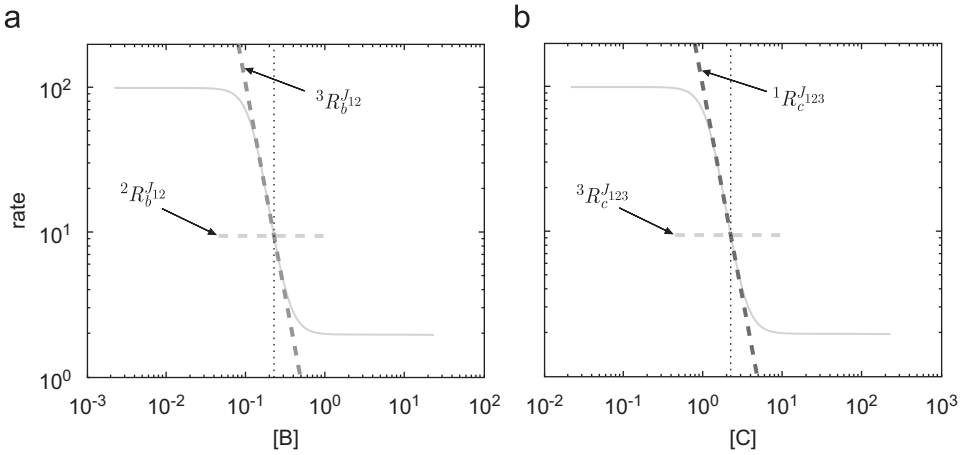

Fig. 5. Partial supply flux–response coefficients for model III. Supply rate characteristics are drawn around metabolite B (a) and metabolite C (b), cf. Fig. 4c. The partial response coefficients shown correspond to two different routes of interaction (see text) and add up to the total response coefficient.

Fig. 5 now illustrates how the supply–response coefficient is split up into partial response coefficients for rate characteristics around B and C in model III. First consider C (Fig. 5b). Comparison with Fig. 4c shows that the supply response coefficient is completely dominated by $^1R_c^{J123}$, i.e. all of the regulation occurs through the feedback loop and inhibition along the main chain of the pathway is negligible.

A more interesting scenario unfolds for B (Fig. 5a). A comparison with the total response coefficient in Fig. 4c reveals that this time the response coefficient is dominated by $^3R_b^{J12}$ (i.e. the route of interaction on the *demand side* of B)! This means that the inhibition of the supply flux in the face of increasing B is not transmitted along the main chain through E2 and A to E1, but rather along E3, C, via ε_c^{v1} to E1. The scenario for A is similar to that of B, in that $R_a^{J1}=^2R_a^{J1}$ and $^1R_a^{J1}\approx 0$.

An important conclusion from this section is that in all cases where multiple routes of interaction exist from an intermediate to a supply or demand block, GSDA can quantify and graphically visualise the importance of each of these routes. Moreover, if for the flux–response coefficient of a particular block with respect to an intermediate, any partial response coefficient *via another block* is non-zero, this indicates that there is additional communication between the supply and demand blocks, and consequently Eq. (2) cannot be used to calculate the block control coefficients. Likewise, the response coefficients generated by GSDA (see dashed lines in Fig. 4c for A and B) do not correspond to block elasticities.

5. Discussion

This paper has described GSDA as a method for identifying and characterising regulatory metabolites in kinetic models of cellular pathways. The method can be generally applied to complex networks. The approach involves clamping each of the variable species of the model in turn and varying their concentration over a range in a parameter scan. The rate characteristics of supply and demand of that particular species are then generated and plotted, together with straight lines representing the elasticities of the enzymes directly connected to the clamped intermediate, and the response coefficients of the supply and demand blocks. GSDA can provide the following information about a pathway:

- Any change in the overall shape of the rate characteristics as one proceeds downstream along a pathway identifies a potential site of regulation (Section 4.1).
- Regulatory metabolites can be identified by comparing the values of the flux–response coefficient of supply or demand towards the intermediate concentration with the elasticity of the enzyme(s) in the supply or demand block that directly sense the intermediate concentration. When these values agree and are non-zero, the metabolite is termed "regulatory" because the flux–control coefficient in the block of the enzyme on which the clamped intermediate acts is equal to one (Section 4.2). Such metabolites can be identified by visual inspection of the rate characteristics (see Fig. 4), or programmatically by searching for species where the difference between the two coefficients (expressed as an angle in polar coordinates) is less than a small threshold value.
- When both the flux–response coefficient of a particular supply or demand block and the elasticity of the corresponding supply or demand enzyme for the intermediate become very small, this leads to functional differentiation. The result is a shift of flux control (in the complete system) to that block, and the magnitude of concentration control is then determined by the properties of the other block (Section 4.3). Also, the sum of the absolute values of the supply and demand elasticities determines the degree of homeostasis of the intermediate in the face of varying supply and demand—the greater the sum, the smaller the concentration–control coefficient, and the better the homeostatic buffering (see extensive discussion in Hofmeyr and Cornish-Bowden,

2000). Again, both functional differentiation and homeostasis can be identified by inspection of the rate characteristics.

- Finally, when more than one direct route of interaction exists from an intermediate to a supply or demand block, the quantitative importance of each of these routes can be assessed by calculating the partial response coefficients (Section 4.4).

There are obvious inter-relations between GSDA and the "modular" (Schuster et al., 1993) or "top-down" (Brown et al., 1990; Quant, 1993) approaches to control analysis. As explained in Section 3.2, the response coefficients generated by clamping an intermediate are actually equivalent to "block" (Fell and Sauro, 1985) or "overall" (Westerhoff et al., 1983) elasticities in the complete system. Moreover, if there are no additional routes of communication between the supply and demand blocks other than through the intermediate, all enzymes belonging to a particular block (say, supply) form a "monofunctional unit" (Rohwer et al., 1996), which means that the co-response coefficient of the intermediate and the flux through the demand block (see Eq. (1)) will not depend on the position in the supply block where the system is perturbed. In the context of the present paper, however, the advantages of GSDA are twofold: first, by considering the behaviour of the system over a wide range, a broader picture of its control and regulation (e.g. in the face of varying demand loads) is obtained than from a mere set of control and elasticity coefficients at a single steady-state point; and second, the rate characteristics and associated elasticity slopes provide a visual picture that allows easy inference of which block controls the flux, to what extent the intermediate is homeostatically buffered, etc.

Several papers from the group of Westerhoff have investigated the problem of how different routes of regulation can be dissected in cellular systems. Bruggeman et al. (2005) have developed a method for quantifying the time-dependent contributions of parallel regulatory pathways affecting activity of glutamine synthetase in *Escherichia coli*. For steady-state systems, the method of regulation analysis (ter Kuile and Westerhoff, 2001; Rossell et al., 2006) dissects how much of the change in the flux through an enzyme is due to metabolic and how much due to hierarchical (i.e. transcriptional and translational) regulation. These approaches have in common that they focus on multiple routes of regulation of a *single step*. They contrast with our approach of GSDA, which focuses in turn on every *metabolite* in the system and the extent to which it affects all the reactions or reaction blocks that produce and consume it.

While SDA has been around for some seven years, the novelty of GSDA lies in its application to every variable species of a kinetic model of a cellular system. For large models, there may not be a logical choice of regulatory metabolite around which to perform an SDA, or it may not be obvious. Regulatory metabolites identified with the method presented here provide a point of entry for further detailed analysis of large models. Moreover, in addition to identifying regulatory metabolites, GSDA characterises them in terms of flux control and homeostasis in the system and provides a useful visualisation in terms of rate characteristics.

We are fully aware that a method such as GSDA cannot claim to be "general" by only addressing small models with four intermediates. We have tested the initial scalability of the method to networks comprising 15–20 reactions. The method currently works with linear and branched pathways, but in future will also have to deal with moiety-conserved cycles. Since control analysis of conserved cycles has been worked out (Hofmeyr et al., 1986; Sauro, 1994; Kholodenko et al., 1994), the problem should be tractable in principle. The aim of this paper is thus to present the idea; for lack of space, fleshing out the details is left for further work.

In conclusion, the strength of GSDA lies in the fact that it provides a computational tool for the systematic functional analysis of large "silicon-cell"-type kinetic models. The tool has been implemented in the ratechar module of the PySCeS software. By including all model species in the analysis, human bias is removed and regulatory metabolites can be readily identified. In subsequent refined analyses, the modeller can then focus on and zoom in on those parts of the model exhibiting regulatory behaviour.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtbi.2007.10.032.

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