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Getting to the inside of cells using metabolic control analysis

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Abstract

Metabolic control analysis can relate control properties of an intact system to kinetic properties (elasticity coefficients) of the enzymes within that system. The method formulating the former as matrix inverse of the latter is elaborated here for the general case and founded in standard metabolic control theory. Then a method is developed that accomplishes the reverse: it is shown that a matrix containing all elasticity coefficients and information concerning the pathway structure equals the inverse of a matrix containing flux and concentration control coefficients. As a consequence, by measuring the control properties of an intact system, one is able to deduce its *in situ* pathway structure and enzyme kinetic properties: This solves the ever-present question of whether the kinetic properties of enzymes in their isolated state differ from those under the conditions prevailing in the cell.

1. Introduction

Metabolic control theory and the parallel control analysis have helped to elucidate the distribution of flux and concentration control among enzymes in metabolic pathways [1]. First, they have led to operational definition of the extent to which an enzyme controls a flux or concentration, and to experimental determination of the corresponding control coefficients. Second, theorems have been derived and applied, such as the summation theorem stating that the sum of the flux control coefficients over all enzymes should amount to 1 [2–5]. And then, the theory has been implemented so as to uncover the basis for the control pattern observed in a metabolic pathway. Various methods were developed that express the control coefficients in terms of kinetic properties of the pathway enzymes, the so-called elasticity coefficients [6–11], cf. [12] for an evaluation. For a number of systems, experimentally observed distributions of control could indeed be accounted for in terms of experimentally determined elasticity coefficients, e.g. [1,13,14]. Control coefficients being properties of the system as a whole and elasticity coefficients relating to enzyme kinetic properties, this amounted to understanding properties of the system as a whole on

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the basis of the properties of its elements. We shall call this approach forward control analysis.

In biology it is also a challenge to investigate the properties of the elemental processes that are responsible for cell metabolism, as they are *in situ*. It has been the great success of biochemistry and molecular biology to isolate cellular components and then study their properties. For enzymes this amounts to the study of the kinetics of the purified enzymes. However, this endeavour often meets with the problem that, in order to obtain experimental access to the elemental processes, one must disrupt the cell. Consequently, it remains necessary to check whether the properties measured for the isolated enzymes are the same as the properties of those enzymes *in vivo*. Methods to determine the *in vivo* properties of enzymes are scarce.

Where the forward control analysis is able to deduce the (control) properties of the intact system from the properties of the individual enzymes, it would seem that the aim of deriving properties of the intracellular processes from the control properties of the intact cellular system corresponds to some sort of *reverse* control analysis. Such a procedure would partly share the aims of a recent elaboration of metabolic design [15].

This paper elaborates this suggestion. It first generalizes an existing method of forward control analysis which writes a matrix of control coefficients as the inverse of a square matrix containing the relevant enzyme properties, called elasticity coefficients [7,16–19]. Then it shows how inversion of this equation accomplishes inverse control analysis, i.e. the calculation of *in situ* enzyme properties (elasticity coefficients) on the basis of control coefficients referring to the control of fluxes and concentrations. As a bonus, the pathway structure can be obtained. The methods are illustrated by two examples.

2. Results

2.1. Definitions and established control theory

Let us consider a system in which there are r processes (reactions, each catalyzed by an enzyme e_i at a rate v_i) and m ($<r$) internal metabolites, each present at a concentration X_j. For simplicity we shall consider the system to be "ideal" in the sense that channelling and enzyme–enzyme interaction are absent. Systems with channelling can be dealt with analogously by combining the approach developed here with the control analysis for channelled systems developed recently [19], cf. [20]. The rates and concentrations constitute vectors (column matrices) $\boldsymbol{v}$ ($r \times 1$) and $\boldsymbol{X}$ ($m \times 1$), respectively. The stoichiometry ($m \times r$) matrix $\mathbf{N}$ defines how the processes affect the metabolite concentrations through:

$$\mathrm{d}\boldsymbol{X}/\mathrm{d}t = \mathbf{N} \cdot \boldsymbol{v}. \tag{1}$$

In general, the rank of $\mathbf{N}$ may be smaller than m. Then the concentrations of the metabolites are dependent on one another and $\mathbf{N}$ can be reduced to a maximum rank matrix by a link matrix $\mathbf{L}$ [9]. Here we shall assume that such reduction has already been accomplished and that the concentrations $\boldsymbol{X}$ are linearly independent. The control over the steady-state properties of the system by the enzyme activities is given by the flux control matrix $\phi(r \times r)$ and the concentration control matrix $\gamma(m \times r)$. The element in the ith row and the kth column of ϕ, ϕ_{ik}, quantifies the control exerted by enzyme k on steady state rate v_i,

$$\phi_{ik} = \left[(\mathrm{d}\ln|v_i|)/(\mathrm{d}\ln|e_k|)\right]_{\mathrm{sys}}, \tag{2}$$

where e_k is the activity of enzyme k, often equal to enzyme concentration expressed in terms of turnover rate. The subscript sys refers to the differentiation conditions requiring that the system remains at steady state throughout the differentiation. Similarly, γ_{jk} quantifies the control exerted by enzyme k on the steady state concentration of metabolite j,

$$\gamma_{jk} = C^{Xj}_{e_k} = \left[(\mathrm{d}\ln|X_j|)/(\mathrm{d}\ln|e_k|)\right]_{\mathrm{sys}}. \tag{3}$$

These control properties are log–log derivatives.

The properties of the individual enzymes that are relevant for control are the elasticity coefficients, summarized in the ($r \times m$) matrix ϵ. Each is defined as the partial log–log derivative of a reaction rate with respect to one of the metabo-

lite concentrations, at constant magnitudes of the other metabolite concentrations,

$$\epsilon_{kj} = e^{vk}_{Xj} = \left[\left(\partial \ln|v_k|\right)/\left(\partial \ln[X_j]\right)\right]_{enz}. \quad (4)$$

Summarizing preceding metabolic control theory, Reder [9] has shown that the control coefficients can be expressed in terms of the non-normalized elasticity coefficients by:

$$\mathbf{\Gamma} = -(\mathbf{N}\cdot\mathbf{D})^{-1}\cdot\mathbf{N} \quad (5)$$

and

$$\mathbf{\Phi} = \mathbf{I}_r + \mathbf{D}\cdot\mathbf{\Gamma}. \quad (6)$$

$\mathbf{\Phi}$ and $\mathbf{\Gamma}$ represent the non-normalized flux and concentration control coefficients, respectively (the analogues of $\boldsymbol{\phi}$ and $\boldsymbol{\gamma}$, respectively). $\mathbf{D}$ is the matrix of non-normalized elasticity coefficients (the analogue of $\boldsymbol{\epsilon}$). $\mathbf{I}_r$ is the $r\times r$ identity matrix.

Although this is not essential we shall formulate this paper in terms of the normalized control coefficients. Accordingly we reformulate Eqs. (5) and (6) as,

$$\boldsymbol{\gamma} = -(\mathbf{n}\cdot\boldsymbol{\epsilon})^{-1}\cdot\mathbf{n} \quad (7)$$

and

$$\boldsymbol{\phi} = \mathbf{I}_r + \boldsymbol{\epsilon}\cdot\boldsymbol{\gamma}. \quad (8)$$

Here the following transformation equations were used:

$$\boldsymbol{\phi} = \mathbf{v}_d^{-1}\cdot\mathbf{\Phi}\cdot\mathbf{v}_d, \quad (9)$$

$$\boldsymbol{\gamma} = \mathbf{X}_d^{-1}\cdot\mathbf{\Gamma}\cdot\mathbf{v}_d, \quad (10)$$

$$\boldsymbol{\epsilon} = \mathbf{v}_d^{-1}\cdot\mathbf{D}\cdot\mathbf{X}_d, \quad (11)$$

$$\mathbf{n} = \mathbf{N}\cdot\mathbf{v}_d, \quad (12)$$

where $\mathbf{v}_d$ $(r\times r)$ and $\mathbf{X}_d$ $(m\times m)$ are diagonal matrices containing the steady-state rates and concentrations. The summation theorems can be readily derived from these equations,

$$\boldsymbol{\gamma}\cdot\boldsymbol{\kappa} = 0, \quad (13)$$

$$\boldsymbol{\phi}\cdot\boldsymbol{\kappa} = \boldsymbol{\kappa}, \quad (14)$$

where $\boldsymbol{\kappa}$ is an $[r\times(r-m)]$ matrix consisting of any set of $r-m$ linearly independent vectors out of the Kernel of $\mathbf{n}$, i.e.

$$\mathbf{n}\cdot\boldsymbol{\kappa} = 0. \quad (15)$$

A well known such vector is the column of r 1's, leading to the best known summation theorem for flux (Eq. (14)) and concentration (Eq. (13)) control coefficients. Similarly the connectivity theorems are derived from Eqs. (7) and (8)

$$\boldsymbol{\gamma}\cdot - \boldsymbol{\epsilon} = \mathbf{I}_m, \quad (16)$$

$$\boldsymbol{\phi}\cdot - \boldsymbol{\epsilon} = \mathbf{0}. \quad (17)$$

For sample pathways, Sauro et al., Cascante et al. and Westerhoff and Kell [6,7,16–18,21] have proven that normalized control coefficients can be expressed into the elasticity coefficients using the matrix equation

$$\mathbf{C}\cdot\mathbf{E} = \mathbf{I}_r. \quad (18)$$

Matrix $\mathbf{C}$ $(r\times r)$ contains both the $m\times r$ concentration control coefficients and a subset of $(r-m)\times r$ flux control coefficients. Matrix $\mathbf{E}$ $(r\times r)$ contains $r\times(r-m)$ elasticity coefficients and information related to the networking of the system. Now we shall prove Eq. (18) and provide systematic ways of formulating $\mathbf{C}$ and $\mathbf{E}$ for the general case. This will be necessary in order to derive *inverse* control analysis.

2.2. *Forward control analysis: the square matrix method for relating control coefficients to elasticity coefficients and pathway structure; the general case*

If only Eq. (14) read that the product $\boldsymbol{\phi}\cdot\boldsymbol{\kappa}$ equalled an $(r-m)\times(r-m)$ identity matrix, rather than the $r\times(r-m)$ matrix $\boldsymbol{\kappa}$, Eqs. (13), (14) and (15), (17) could be arranged into the form of Eq. (18). Consequently, our task is to find a transformed matrix $\boldsymbol{\kappa}'$ and a transformed matrix $\boldsymbol{\phi}'$, such that

$$\boldsymbol{\phi}'\cdot\boldsymbol{\kappa}' = \mathbf{I}_{r-m}. \quad (19)$$

If $\mathbf{S}$ is an arbitrary $(r-m)\times r$ matrix of rank $r-m$, then $\boldsymbol{\phi}'$ and $\boldsymbol{\kappa}'$ defined by the following equations fulfil this requirement:

$$\boldsymbol{\phi}' = \mathbf{S}\cdot\boldsymbol{\phi} \quad (20)$$

and

$$\boldsymbol{\kappa}' = \boldsymbol{\kappa}\cdot(\mathbf{S}\cdot\boldsymbol{\kappa})^{-1}. \quad (21)$$

Importantly, these ϕ' and κ' also satisfy Eqs. (13), (15) and (17),

$$\gamma \cdot \kappa' = \gamma \cdot \kappa \cdot (S \cdot \kappa)^{-1} = 0 \cdot (S \cdot \kappa)^{-1} = 0, \quad (22)$$

$$\phi' \cdot -\epsilon = S \cdot \phi \cdot -\epsilon = 0, \quad (23)$$

$$n \cdot \kappa' = n \cdot \kappa \cdot (S \cdot \kappa)^{-1} = 0. \quad (24)$$

It may be noted that

$$S \equiv (\kappa')_{\text{left inverse}} \quad (25)$$

is the left-inverse matrix of κ', i.e. the product of S and κ' equals the $r-m$ identity matrix.

A special set of possible choices for matrix S is that of the $(r-m)\times r$ left-inverse of matrix κ itself. Then κ equals κ' and ϕ' equals the left inverse of κ times ϕ.

Matrix $\kappa_{\text{left inverse}}$ may be obtained by the algorithm more generally used for obtaining a matrix inverse, i.e. by (i) joining an $r\times r$ identity matrix to the left of κ, which produces matrix $A_{r\times(2r-m)} = \{I_r \mid \kappa_{r\times(r-m)}\}$, (ii) linearly recombining rows until at the $(r-m)\times(r-m)$ upper right-hand quadrant of the matrix A the identity matrix appears, (iii) taking the $(r-m)\times r$ left-hand upper part of the resulting matrix as $\kappa_{\text{left inverse}}$. Alternatively, one may add independent columns to the right-hand side of κ, with only 0's in the upper $r-m$ rows, until the resulting matrix becomes square $(r\times r)$, invert the result, and use the upper left, $(r-m)\times r$, part of that inverse for matrix A. A third method renumbers the rows of κ in such a manner that the first $r-m$ rows are independent. The upper $r-m$ by $r-m$ uppermost part of the resulting matrix is then inverted and m columns of zeros are juxtaposed on the right-hand side. The result is a left inverse of κ. Here the general matrix algebra property is used that

$$\begin{pmatrix} P \\ Q \end{pmatrix}(R \mid S) = \begin{pmatrix} PR & PS \\ QR & QS \end{pmatrix} \quad (26)$$

when P, Q, R, and S are arbitrary matrices of appropriate dimensions.

If the total product is the identity matrix and P, Q, R, S are dimensioned appropriately, then all the sub products will be identity and null matrices, and vice versa.

Eq. (26) allows us to reformulate Eqs. (13), (19), (22), and (23), as Eq. (18), with the following definitions for the control and elasticity matrices $C\ (r\times r)$ and $E\ (r\times r)$, respectively:

$$C = \begin{vmatrix} \phi' \\ \gamma \end{vmatrix} \quad (27)$$

and

$$E = (\kappa' \mid -\epsilon), \quad (28)$$

i.e. the upper $r-m$ rows of C are equal to matrix ϕ', whereas the lower m rows are equal to matrix γ, and the left-hand $r-m$ columns of E are equal to κ', whereas the subsequent m columns are equal to minus the matrix of elasticity coefficients. Matrix C contains the control coefficients, hence the systemic information about the system. Matrix E contains the relevant kinetic information of the enzymes in the system plus (in its submatrix κ') the information on how the enzymes are networked in the system.

2.3. The relationship between the matrix of flux control coefficients and the matrix κ'

There is a relationship between the fluxes of which the controls are addressed in matrix ϕ' and the columns in matrix κ'. Matrix $S = \kappa'_{\text{left inverse}}$ maps the logarithms of the steady state reaction rates $\ln|v| = (\ln|v_1| \ln|v_2| \ldots \ln|v_r|)^T$ to the logarithms of independent steady state fluxes $\ln|J| = (\ln|J_1| \ln|J_2| \ldots \ln|J_{r-m}|)^T$:

$$\ln|J| = S \cdot \ln|v|. \quad (29)$$

For modulations around the steady state of interest, S is constant. Consequently,

$$d\ln|J| = S \cdot d\ln|v| = S \cdot v_d^{-1} \cdot dv \quad (30)$$

Each of the $r-m$ columns of κ' represents an independent vector of the Kernel of n (cf., Eq. (15)). This implies that each column represents one of a set of $r-m$ independent steady-state fluxes through the system. This representation is such that the ith element of such a column represents the fraction of v_i that is contributed by that steady-state flux J_i:

$$v = v_d \cdot \kappa' \cdot \lambda, \quad (31)$$

where λ is a $1\times(r-m)$ matrix. If κ'_j represents the jth column of κ', then we may consider a

change in enzyme activities parameterized by $d\lambda_j$, such that

$$de = e_d \cdot \kappa' \cdot 1_j \cdot d\lambda_j, \quad (32)$$

where e_d is the diagonal matrix of enzyme activities. 1_j is a column vector of zero's except for a 1 in the jth row. Such a change corresponds to an increase in the independent flux represented by column j, at constant magnitudes of the other independent fluxes *as defined by* κ'. Consequent to this change, steady state is maintained and the rates are increased by

$$dv = v_d \cdot \kappa' \cdot 1_j \cdot d\lambda_j. \quad (33)$$

Using Eqs. (25) and (30) we find that the consequent change in steady-state flux J_j amounts to

$$d \ln |J_j| = d\lambda_j, \quad (34)$$

whereas all other fluxes remained untouched. Clearly, the jth flux considered in the matrix of control coefficients ϕ' (the jth row of ϕ') corresponds to the flux represented by the jth column of κ'.

We shall now consider a some possible choices for the independent fluxes considered in the control analysis, i.e. some choices for J, κ' and ϕ'.

2.4. Choices for the matrix κ and for the flux control coefficients considered

The above assumes one knows a suitable matrix κ. The sole requirement for κ is that its columns be independent vectors in the Kernel of n. These can be found by writing an $m \times 1$ column vector $(1, x, y, z, u, \ldots, w)^T$ and solving the r linear equations defined by $n \cdot (1, x, y, z, u, \ldots, w)^T = 0$. There are many possible solutions, but $r - m$ independent such solutions may be chosen, because all others are linear combinations of these solutions. These solutions are then written as column vectors and juxtaposed so as to yield the $r \times (r - m)$ matrix κ.

If one uses the left-inverse of this matrix κ to obtain the matrix of flux control coefficients through Eqs. (20) and (25), one may end up with a rather arbitrary set of flux properties the controls of which are considered. The control properties of all other flux properties can then be calculated subsequently.

Once the arbitrary κ is available, one may however select the flux properties of which one wishes to determine the control, formulate the corresponding matrix S, and then transform κ to κ' following Eq. (21).

There is a procedure for constructing a κ that leads to flux control properties of immediate interest: it defines one of the fluxes into the system as the input flux, then identifies all other points of entry or exit, as (positive or negative, respectively) "exit" points. From the entry point a pathway is then drawn to each exit point and the rate at each exit point is equated to a corresponding flux. For each of the resulting fluxes, the path is traced back to the input and at every enzyme the ratio between the flux and the local steady state rate is entered as the corresponding element of the column vector k. For p exit fluxes, one thus obtains p columns for k. If p equals $r - m$, this provides a matrix κ of interest. Along the lines discussed above, the flux control coefficients defined by Eqs. (25) and (20) are those with respect to these exit fluxes.

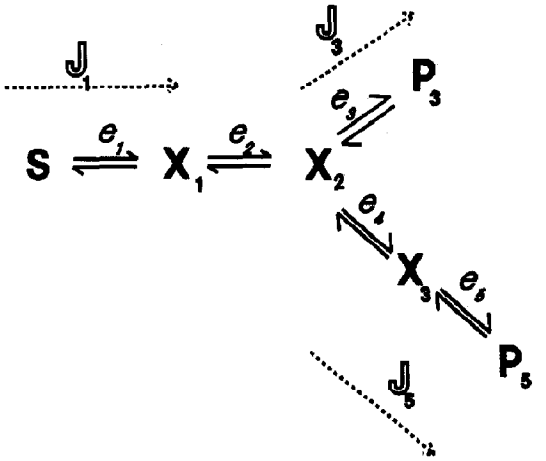

Fig. 1. Diagram of a simple metabolic pathway. Pathway flux J_1 branches into output flows J_3 and J_5.

For the example of Fig. 1, this procedure yields for κ:

$$\kappa = \begin{pmatrix} v_3/v_1 & v_5/v_1 \\ v_3/v_1 & v_5/v_1 \\ 1 & 0 \\ 0 & 1 \\ 0 & 1 \end{pmatrix}. \tag{35}$$

Using the first of the three procedures sketched to obtain a left inverse for κ, one possible such left inverse is:

$$\kappa_{\text{left inverse}} = \begin{pmatrix} \frac{v_1}{v_3} & 0 & 0 & 0 & -\frac{v_5}{v_3} \\ 0 & \frac{v_1}{v_5} & -\frac{v_3}{v_5} & 0 & 0 \end{pmatrix}. \tag{36}$$

Using Eqs. (20) and (25), this leads to the following form for ϕ':

$$\phi' = \begin{pmatrix} C_{e_1}^{v_3} & C_{e_2}^{v_3} & C_{e_3}^{v_3} & C_{e_4}^{v_3} & C_{e_5}^{v_3} \\ C_{e_1}^{v_5} & C_{e_2}^{v_5} & C_{e_3}^{v_5} & C_{e_4}^{v_5} & C_{e_5}^{v_5} \end{pmatrix}. \tag{37}$$

Clearly the two independent flux properties considered (J_1 and J_2) correspond to the steady-state rate through e_3 and the steady-state rate through e_5, respectively.

If the number of fluxes connecting the system with its environment is smaller than $1+r-m$, there are independent fluxes internal to the system. These correspond to (or can be constructed so as to correspond to) cyclic fluxes. If these cyclic fluxes are not immediately obvious, one may start from the stoichiometry matrix $\mathbf{n}$, in order to find independent fluxes: by Gaussian elimination one reduces $\mathbf{n}$ to the row echelon form. The $r-m$ columns that do not correspond to elements at the right-hand end of a step of the echelon may be used to refer to independent fluxes. For the system of Fig. 1, $\mathbf{n}$ already has the echelon form,

$$\mathbf{n} = \begin{pmatrix} v_1 & -v_2 & 0 & 0 & 0 \\ 0 & v_2 & -v_3 & -v_4 & 0 \\ 0 & 0 & 0 & v_4 & -v_5 \end{pmatrix}. \tag{38}$$

Columns 2 and 4 of this matrix are not at the right-hand end of a step, suggesting to choose v_2 and v_4 as the independent fluxes. Because at steady state these fluxes are equal to v_3 and v_5 respectively, this result corresponds to that obtained above and κ may be constructed in the same manner.

The flux control summation theorem is of great interest. The product $\phi' \cdot \kappa$ for the matrices given by Eqs. (36) and (37) does not lead to summation theorems; for that κ should contain a column of 1's. Indeed, the column vector consisting of all 1's is always part of the Kernel of $\mathbf{n}$, precisely because it is a steady-state solution if all reaction rates assume their steady-state value. For the system of Fig. 1 one may take a flux of magnitude v_3 running through enzymes 1, 2, and 3 as the second independent steady state flux, which leads to a formulation for κ of:

$$\kappa = \begin{pmatrix} 1 & v_3/v_1 \\ 1 & v_3/v_1 \\ 1 & 1 \\ 1 & 0 \\ 1 & 0 \end{pmatrix}. \tag{39}$$

A left inverse of this matrix is

$$\kappa_{\text{left inverse}} = \begin{pmatrix} \frac{v_1}{v_5} & 0 & -\frac{v_3}{v_5} & 0 & 0 \\ 0 & \frac{v_1}{v_3} & 0 & 0 & -\frac{v_1}{v_3} \end{pmatrix}. \tag{40}$$

Multiplication from the left with the matrix ϕ shows that ϕ' considers the control of $J_1 = v_5$ and $J_2 = v_3/v_5$, which is the flux ratio of the two effluxes. This is what should be expected; the two columns of κ correspond to the following two independent fluxes: (1) a flux through all branches equal to v_1 and distributing to v_3 and v_5 (closely corresponding to the steady state situation) and (2) a flux of magnitude v_3 flowing through enzyme 1, 2, and 3. Considering the two fluxes independently, implies that the former flux only affects v_5 and not v_3. The latter flux should not affect v_1, hence merely redistribute the flux from v_5 to v_3.

Inspecting Fig. 1, one may decide to be primarily interested in the control of the input flux

and in the control of the fraction of the input flux that exits the system through enzyme 3, i.e. in the flow ratio v_3/v_1. That is:

$$\phi' = \begin{pmatrix} C^{J_1}_{e_1} & C^{J_1}_{e_2} & C^{J_1}_{e_3} & C^{J_1}_{e_4} & C^{J_1}_{e_5} \\ C^{j_3}_{e_1} & C^{j_3}_{e_2} & C^{j_3}_{e_3} & C^{j_3}_{e_4} & C^{j_3}_{e_5} \end{pmatrix}, \quad (41)$$

where the flow ratio j_3 is defined by:

$$j_3 = J_3/J_1. \quad (42)$$

This implies that the matrix **S** should read:

$$\mathbf{S} = \begin{pmatrix} 1 & 0 & 0 & 0 & 0 \\ -1 & 0 & 1 & 0 & 0 \end{pmatrix} \quad (43)$$

Starting from the κ of Eq. (39) (or from the k of Eq. (35)), Eq. (21) leads to:

$$\kappa' = \begin{pmatrix} 1 & 0 \\ 1 & 0 \\ 1 & 1 \\ 1 & -v_3/v_5 \\ 1 & -v_3/v_5 \end{pmatrix}. \quad (44)$$

3. Inverse control analysis: from control coefficients to elasticity coefficients.

3.1. The method

Eq. (18) can be used to express the control coefficients into the elasticity coefficients, cf. [6,7,9,16–18,21,22]. The control theoretic equations of Reder [9,23] offer the same possibility. Eq. (18) also has the inverse application, cf. [22], which is not immediately obtainable from the equations of Reder [9], i.e. that of calculating the elasticity coefficients from the control coefficients,

$$\mathbf{E} = \mathbf{C}^{-1}. \quad (45)$$

Because (above) the present paper provides a general proof for Eq. (18), it also proves Eq. (45). The implication is that one can calculate the *in situ* enzyme properties called elasticity coefficients from the global properties which are the control coefficients through a single matrix inversion step. When the structure of the network, rather than its elasticity coefficients are of interest, one may apply Eq. (45) and obtain (cf. Eq. (28)) a Kernel of the stoichiometry matrix **n**.

When the network structure of the system is known any of the methods used to find proper formulations for κ' and ϕ' described above, may be used. Eq. (27) then leads to matrix **C**. Using Fig. 3, we now illustrate one method of formulating a matrix **C** when the network structure of the system is not known. We assume that for the system one has identified all the independent metabolite concentration (-variables), all the enzymes, as well as all the fluxes in which matter is exchanged between the system and its environment. One now selects one flux as the input flux and normalizes all other exchange fluxes by this input flux. The first row of **C** then should contain the coefficients quantifying the controls exerted by the respective enzymes on this input flux. The second row contains the control coefficients with respect to one of the normalized output fluxes. The subsequent rows contain the control coefficients with respect to any of the other normalized exchange fluxes. This continues, until all but one exchange flux has been accommodated. Then the subsequent row contains all the control coefficients with respect to any of the metabolite concentrations. Subsequent rows contain the control coefficients with respect to the other independent concentrations. The resulting matrix **C** should be square and nonsingular by virtue of the independence of the concentrations and fluxes.

3.2. Examples

As a first example we consider the pathway of Fig. 1. We suppose that the pathway structure is known and that, e.g., by way of genetic manipulation, the enzyme activities can be modulated and the consequent changes in fluxes J_1 and J_3, and in concentrations X_1, X_2 and X_3 can be measured. Let the upper matrix in Fig. 2 contain the magnitudes of the control coefficients determined in this manner. Then the inverse of this matrix yields the elasticity coefficients, given as the lower matrix in Fig. 2.

Our second example addresses the case where we are not so much interested in the elasticity coefficients, as we are in the pathway structure.

$C^{J_1}_{e_1}$	$C^{J_1}_{e_2}$	$C^{J_1}_{e_3}$	$C^{J_1}_{e_4}$	$C^{J_1}_{e_5}$	0.62	0.15	0.05	0.17	0.0
$C^{J_3}_{e_1}$	$C^{J_3}_{e_2}$	$C^{J_3}_{e_3}$	$C^{J_3}_{e_4}$	$C^{J_3}_{e_5}$	-0.25	0.0	0.88	-0.51	0.0
$C^{X_1}_{e_1}$	$C^{X_1}_{e_2}$	$C^{X_1}_{e_3}$	$C^{X_1}_{e_4}$	$C^{X_1}_{e_5}$ =	2	-0.78	-0.22	-0.85	0
$C^{X_2}_{e_1}$	$C^{X_2}_{e_2}$	$C^{X_2}_{e_3}$	$C^{X_2}_{e_4}$	$C^{X_2}_{e_5}$	1.9	0.5	-.45	-1.7	-0.17
$C^{X_3}_{e_1}$	$C^{X_3}_{e_2}$	$C^{X_3}_{e_3}$	$C^{X_3}_{e_4}$	$C^{X_3}_{e_5}$	0.71	0.17	-0.2	0.3	-0.96

1	0	$-\varepsilon^1_{X_1}$	$-\varepsilon^1_{X_2}$	$-\varepsilon^1_{X_3}$	0.98	-0.01	0.19	0.00	0.00
1	0	$-\varepsilon^2_{X_1}$	$-\varepsilon^2_{X_2}$	$-\varepsilon^2_{X_3}$	1.09	-0.03	-0.78	0.49	-0.09
1	1	$-\varepsilon^3_{X_1}$	$-\varepsilon^3_{X_2}$	$-\varepsilon^3_{X_3}$ =	0.90	0.99	0.04	-0.22	0.04
1	$\frac{J_3}{J_5}$	$-\varepsilon^4_{X_1}$	$-\varepsilon^4_{X_2}$	$-\varepsilon^4_{X_3}$	1.07	-0.25	-0.02	-0.38	0.07
1	$\frac{J_3}{J_5}$	$-\varepsilon^5_{X_1}$	$-\varepsilon^5_{X_2}$	$-\varepsilon^5_{X_3}$	1.07	-0.29	-0.01	0.02	-1.04

Fig. 2. Example of inverse control analysis. It is supposed that the control coefficients quantifying the control by the five enzymes in the pathway of Fig. 1 on the flux J_1 and on the flux ratio J_3/J_1, as well as on the concentrations of the three metabolites X_1, X_2, and X_3, have been measured and have the value indicated in the matrix of control coefficients. The second matrix was calculated by inverting this matrix of control coefficients, **C**. In this manner the elasticity coefficients of the enzymes in the pathway can be calculated from the control properties.

Fig. 3 illustrates this case: we have a black box of which we know that there is one entry flux and two exit fluxes and three variable metabolite concentrations. In addition there are five enzymes

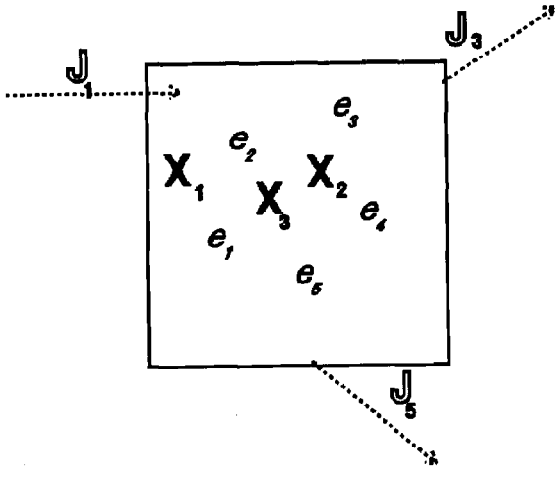

Fig. 3. Diagram of a metabolic pathway of unknown structure. What is known is that there are three fluxes related by one steady state relation ($J_1 = J_3 + J_5$) by which the system exchanges matter with the outside world, three independent metabolites (X_1, X_2, and X_3) and five enzymes (e_1 through e_5).

$C^{J_1}_{e_1}$	$C^{J_1}_{e_2}$	$C^{J_1}_{e_3}$	$C^{J_1}_{e_4}$	$C^{J_1}_{e_5}$	0.66	0.16	-0.09	0.25	0.03
$C^{J_3}_{e_1}$	$C^{J_3}_{e_2}$	$C^{J_3}_{e_3}$	$C^{J_3}_{e_4}$	$C^{J_3}_{e_5}$	-0.26	-0.06	0.92	-0.54	-0.05
$C^{X_1}_{e_1}$	$C^{X_1}_{e_2}$	$C^{X_1}_{e_3}$	$C^{X_1}_{e_4}$	$C^{X_1}_{e_5}$ =	1.73	-0.82	0.47	-1.26	-0.13
$C^{X_2}_{e_1}$	$C^{X_2}_{e_2}$	$C^{X_2}_{e_3}$	$C^{X_2}_{e_4}$	$C^{X_2}_{e_5}$	1.98	0.49	-.89	-1.44	-0.14
$C^{X_3}_{e_1}$	$C^{X_3}_{e_2}$	$C^{X_3}_{e_3}$	$C^{X_3}_{e_4}$	$C^{X_3}_{e_5}$	0.72	0.18	-0.32	0.39	-0.96

1	?	$-\varepsilon^1_{X_1}$	$-\varepsilon^1_{X_2}$	$-\varepsilon^1_{X_3}$	0.99	0.00	0.20	0.00	0.00
1	?	$-\varepsilon^2_{X_1}$	$-\varepsilon^2_{X_2}$	$-\varepsilon^2_{X_3}$	0.97	0.99	-0.80	0.50	0.01
1	?	$-\varepsilon^3_{X_1}$	$-\varepsilon^3_{X_2}$	$-\varepsilon^3_{X_3}$ =	0.98	0.99	0.00	-0.20	0.01
1	?	$-\varepsilon^4_{X_1}$	$-\varepsilon^4_{X_2}$	$-\varepsilon^4_{X_3}$	0.99	-0.25	0.00	-0.40	0.10
1	?	$-\varepsilon^5_{X_1}$	$-\varepsilon^5_{X_2}$	$-\varepsilon^5_{X_3}$	1.00	-0.25	-0.00	0.00	-1.00

Fig. 4. Example of the use of inverse control analysis to determine the network structure of a metabolic network. Fig. 3 indicates what is supposed to be known of the system: an entry flux J_1, two exit fluxes J_2 and J_3 and the presence of three independent metabolites and five enzymes. The control coefficients can be measured and are assumed to have the values indicated in the matrix of control coefficients. What is unknown is how the enzymes are networked. The second column of the inverse of the matrix of control coefficients after rounding off reveals the networking: 0,1,1,-0.25,-0.25, suggesting that the pathway branches after enzyme 1, 20% of the flux leading to one branch and 80% to the other branch. It should be noted that this matrix differs from that in Fig. 2 precisely in that second column, as does the network found here from that in Fig. 1 to which Fig. 2 applies.

(five reading frames in the DNA). The question is how these enzymes are networked. Obviously, one possibility is the one given in Fig. 1. Let the values measured for the control coefficients amount to those given in Fig. 4. Then the inverse of the matrix is given by the lower matrix in Fig. 4. The first two columns of this matrix contain κ', a kernel of the normalized stoichiometry matrix. The structure of this κ' proves that the pathway in the black box does not have the structure of Fig. 1, but instead branches after the first metabolite.

4. Discussion

In this paper the matrix inverse method [6,7,16] of metabolic control theory, which calculates the flux and concentration control coefficients in a single step by inverting the matrix of elasticity coefficients, has been generalized to pathways of arbitrary complexity. Neither an earlier general-

ization [17], nor the general control analysis method in terms of absolute control coefficients developed by Reder [9], resulted in the formulation of a control matrix equalling the simple inverse of an elasticity matrix. Hence, the latter methods were not directly suitable for the inverse approach. Cascante and co-workers [18] did generalize the earlier method, without explicit proof of the general case however. The present linking of our method with the metabolic control theory (e.g., through Eqs. (5)–(12)) may help to further complete it. The parallel study by Acerenza [15] of "metabolic control design" has a similar aim and provides a different algorithm for performing "inverse" control analysis. It explicity treats systems with moiety-conserved cycles and attempts to extrapolate from elasticity coefficients to rate equations and their kinetics constants. We find the nonmechanistic formulation of control theory in terms of Eq. (18) attractive, as it indicates that the systemic control coefficients are nothing but the inverse of the local enzyme properties (elasticity coefficients), be it that all elasticity coefficients must be inverted simultaneously, in the context provided by the networking of the system (which defines the κ' part of the matrix **E**).

By contrast, the forward control analysis method as generalized here, does formulate that the matrix of control coefficients is the matrix inverse of the matrix of elasticity coefficients and is hence directly amenable to inverse application. This paper uses the traditional matrices out of the forward control analysis method [6,7] and obtains elasticity coefficients and network information from concentration and control coefficients.

On purpose, our method of constructing the control matrix **C** for the cases where the system's network structure is unknown, treats the system as a black box; it only addresses exchange fluxes with the system's environment and control coefficients thereupon. In some cases the number of external fluxes minus 1 will equal the number of independent fluxes in the black box. Then it will be sufficient to measure the external fluxes in addition to the internal metabolite concentrations, for inverse control analysis to be carried out. In other cases, 1 plus the number of reactions minus the number of independent metabolites exceeds the number of external fluxes. Then the system harbours parallel or cyclic fluxes (where the former may be formally reduced to the latter). Above we have indicated how the independent fluxes could be identified in such a case. If the independent cyclic flux can be measured (e.g., because it is coupled to another output chemical reaction; many cycles are coupled to net ATP hydrolysis), matrix **C** can still be formulated and *inverse* control analysis applied.

A simple example is where there are two enzymes catalysing the same reaction in the system converting metabolite X to metabolite Y. When the cyclic flux cannot be measured, these two enzymes may be treated as a unit, and *inverse* control analysis may still be applied provided that the two enzymes are identified. Such identification is possible because all control coefficients of the two enzymes should be interdependent; their ratio should amount to the ratio of the two reaction rates.

A more complex case is that where the reaction from X to Y may have a metabolite Z as an intermediate in one of the two branches. In case this metabolite does not have elasticities with respect to any other reaction in the system than for the two reactions it is a substrate and a product of in this branch, the metabolite and the three enzymes involved can still be identified (again because their control coefficients except with respect to metabolite Z are dependent; which then identifies this metabolite). Subsequently, this three enzyme subsystem may be treated as a module [24–26].

The actual application of the method developed here requires the determination of the control coefficients of all concentrations by all enzymes. Such a determination could be performed by genetic manipulation of the enzymes [27–32] and measurements of the resulting intracellular metabolite concentrations. We note that, although the latter may involve disruption of the cell, it may be achieved by an immediate quenching method. Then, the method developed here allows the measurement of the elasticity coefficients of the enzymes *in situ*, in contrast to measuring the enzyme kinetic properties of the puri-

fied enzymes, or of enzymes in permeabilized cells. Of course, this method is similar in some aspects to other methods which measure input and output flows and metabolite concentrations.

In practice, the measurement of all metabolite concentrations and the manipulation of all enzymes is not required: First, there are usually "remote" parts of metabolism that do not control the metabolism of interest [33]. Second, metabolism may be split up into a limited number of modules and control can be analyzed in terms of the control in and between these [24,26,34]. Also, the method developed here may be applied to smaller metabolic networks, which are of interest of their own.

We here elaborated *inverse control analysis* on the basis of the standard version of metabolic control analysis, which is applicable to "ideal" systems. Here "ideal" systems are defined as those in which channelling is absent, there is no regulated gene expression and moiety conserved concentrations far exceed enzyme concentrations. However, *inverse control analysis* should be equally applicable to the more complex system, especially after combination with the appropriate extended metabolic control analysis for such channelling [19], group-transfer pathways [20] and regulated gene expression [35,36]. Combination to recent work on the topic of regulation [36,37] should then also allow inverse analysis of metabolic regulation to these more complex systems.

As noted earlier [21,38–40], metabolic control analysis and especially the *inverse* control analysis developed here, should be of use to the biotechnologist. It is a rational method for devising the enzyme and network properties one should engineer into a microorganism in order to bestow it with the desired control properties; after formulating the desired control properties, one only has to invert the matrix of control coefficients to obtain the network structure and enzyme kinetic properties necessary to obtain the desired control structure. Especially for the second generation engineered organisms, which should not just produce much of the desired product, but should also be amenable to regulation of its rate of production, this should be important.

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