Determination of Flux Control Coefficients from transient metabolite concentrations

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Flux Control Coefficients have been used in the analysis of metabolic regulation for quantifying the effect of an enzyme on the overall steady-state flux. However, the experimental determination of these coefficients is very time-consuming, involving either determining the individual enzyme kinetics or perturbing the enzyme activity by genetic or other means. We developed a methodology that enables the determination of the Flux Control Coefficients from transient metabolite concentrations without knowing kinetic parameters. The transient states can be generated by changing the incubation conditions or adding the initial substrate. This approach is suitable for investigating metabolic regulation in vivo or multiple enzyme systems in vitro. It is particularly helpful if used in conjunction with n.m.r. measurements. The approach is based on a relationship between transient metabolite concentrations and the Flux Control Coefficients. The methodology has been improved from our previous results, and it is illustrated by three examples with simple pathway topologies.

INTRODUCTION

The quantitative analysis of metabolic regulation requires simultaneously considering most, if not all, of the variables involved in the system. In this regard, the Flux Control Coefficients (Kacser & Burns, 1973; Heinrich & Rapoport, 1974) provide a useful basis for quantifying metabolic regulations: the higher the Flux Control Coefficient, the more controlling the enzyme is to the steady-state flux. Although the Control Coefficients reveal only the effect of infinitesimal changes in parameters (such as enzyme concentrations or $V_{max}$) on the overall fluxes, they are conceptually useful in biochemical research. If all the enzyme kinetics or the Elasticity Coefficients are available, one can calculate the Flux Control Coefficients without much difficulty (e.g. Groen et al., 1986; Fell & Snell, 1988). However, in most cases, enzyme kinetics are not available, and determining individual enzyme kinetics is very time-consuming.

On the other hand, direct experimental determination of Flux Control Coefficients involves altering enzymic activity or kinetic parameters by genetic means or by the use of inhibitors and measuring the change in the steady-state flux. Several examples of the experimental determination of the Flux Control Coefficients have been reported (e.g. Flint et al., 1981; Groen et al., 1982; Mazat et al., 1986; Salt et al., 1986; Torres et al., 1986, 1989; Brand et al., 1988; Kruckeberg et al., 1989). However, these approaches are not generally applicable to all the systems of interest. As an alternative, we proposed using the transient metabolite concentrations to calculate the Control Coefficients (Delgado & Liao, 1991). This methodology provides a useful tool to extract information from transient metabolic data, and can simplify the experiments required to determine the Control Coefficients. As measurement techniques such as n.m.r. in vivo and h.p.l.c. become more accurate and user-friendly, it is possible that the measurement of transient metabolite concentrations will eventually become a routine task. Therefore the approach based on the transient metabolic data is promising.

In this paper we present a refined version of the methodology, which greatly improves the accuracy of the estimated Flux Control Coefficients. For simplicity, we discuss only the linear and branched pathways without substrate cycles or conserved metabolites, and we assume that the metabolites are homogeneously distributed in the system. Extension to more complex systems will need to be discussed elsewhere. The derivations of the theory are presented in Appendixes, as they are not critical for understanding the essence of the approach.

BACKGROUND

For definition purposes, let us consider the pathway depicted in Fig. 1, where $X_i$ and $X_{i+1}$ are extracellular substrate and product respectively. Although several representations have been used, the Flux Control Coefficient has been defined as (Burns et al., 1985):

$$C_{ij}^\prime = \left[ \frac{\partial J_i}{\partial e_j} \right]_{ss}$$

(1)

where $C_{ij}^\prime$ is the steady-state Flux Control Coefficient of enzyme $i$, $J_i$ is the steady-state flux through the pathway and $e_i$ is the concentration of enzyme $i$. If one is interested in the effect of activity change in enzyme $i$ then $e_i$ can be defined as $V_{max}$. The subscript 'ss' denotes that the coefficient is evaluated at the steady state. This definition quantifies the fractional change in the steady-state flux per unit fractional change in enzyme concentration, if the latter change is infinitesimal. If the change is large, it does not necessarily provide precise information because of the non-linear nature of the kinetics.

Note that in order for the definition of Flux Control Coefficients to be meaningful, the internal metabolite concentrations have to be able to reach a unique non-trivial stable steady or quasi-steady state. Therefore it is commonly assumed that $X_i$

$$X_1 \xrightarrow{\epsilon_1} X_2 \xrightarrow{\epsilon_2} X_3 \xrightarrow{\epsilon_3} \ldots \xrightarrow{\epsilon_{n-1}} X_n \xrightarrow{\epsilon_n} X_{n+1}$$

Fig. 1. Metabolic pathway for definition purposes

$X_i$ and $X_{i+1}$ are extracellular substrate and product respectively; $v_i$ is the flux through reaction $i$ catalysed by enzyme $E_i$.

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and $X_{n+1}$ are sufficiently buffered or that they do not affect the intracellular enzyme kinetics. This assumption can be relaxed as long as there is a non-trivial quasi-steady state for the internal metabolite concentrations.

If the enzyme kinetics around the steady state are known, one can calculate the Elasticity Coefficients, which are defined as:

$$c_{ik} = \left[ \frac{x_k}{v_i} \frac{\partial v_i}{\partial x_k} \right]_{ss}$$

(2)

where $x_k$ is the concentration of metabolite $k$ and $v_i$ is the flux through enzyme $i$. The Flux Control Coefficients can then be obtained by solving a set of linear algebraic equations, known as the Summation and Connectivity Theorems (e.g. Fell & Sauro, 1985; Westerhoff & Kell, 1987).

In most cases, however, the enzyme kinetics or the Elasticity Coefficients are not readily available, or not accurate enough. This situation is one of the major difficulties in determining Flux Control Coefficients in practical systems.

**THEORY**

We proposed a methodology using the transient metabolite concentrations to calculate the Flux Control Coefficients without using the Elasticity Coefficients (Delgado & Liao, 1991). The methodology requires measuring the metabolite concentrations in a transient state, and it is derived with the use of four assumptions: (1) as in other metabolic control analyses, the external (pool) metabolites do not affect the pathway kinetics or these metabolites are buffered; (2) the kinetic rate laws are sufficiently linear around the steady state of interest; (3) the calculation of the transient fluxes from the measurement of metabolite concentrations for every reaction in the pathway must be theoretically possible; (4) the metabolites are homogeneously distributed in the system. The first assumption makes the definition of the Control Coefficients mathematically meaningful. These conditions enable the internal metabolite concentrations to reach a unique non-trivial stable steady or quasi-steady state. The second assumption is in effect equivalent to the use of Elasticity Coefficients evaluated at the steady state. Although most of the enzyme kinetics are non-linear, the approximation by the above equation is satisfactory for practical purposes. The third assumption is necessary because the methodology implicitly converts the transient metabolite concentrations into the transient flux through each enzyme. Such conversion will not be possible if the number of metabolites is less than the number of enzymes. The fourth assumption is necessary because the cases of enzyme–enzyme complex and substrate channeling are not investigated in the present paper. If there are any enzyme–enzyme complexes, each of them is treated as a single step.

Using these assumptions, we derived a relationship between the Flux Control Coefficients and the transient flux through each enzyme (Delgado & Liao, 1991):

$$\sum_{i=1}^{n} C_{ii}^{(t)} \left( \frac{v_i(t)}{J_i} \right) = 1$$

(3)

where $v_i(t)$ and $J_i$ are the transient and steady-state fluxes through enzyme $i$ respectively. Although the original derivation of eqn. (3) was based on simple pathways, it is applicable to any pathway topology as long as the assumptions stated above are valid. A derivation based on the approach developed by Reder (1988) for an arbitrary pathway configuration is presented in Appendix A.

Note that, although the Flux Control Coefficient has been defined for the steady state, transient reaction rates or fluxes are used in the above equation. This equation states that the ratios between the transient and the steady-state fluxes through each reaction are related to each other by the Flux Control Coefficients. Therefore the reactions with larger Flux Control Coefficients have more significant effects on the other reactions, even in the transient state. The reactions with small Flux Control Coefficients can fluctuate significantly during the transient state without affecting others, whereas reactions with large Flux Control Coefficients must be held relatively constant. This can be seen in the computer simulation of human erythrocyte glycolysis (Schauer et al., 1981): the fluxes through controlling enzymes, hexokinase, phosphofructokinase and pyruvate kinase, stay relatively constant, whereas the fluxes through the ‘fast-equilibrium’ enzymes can adjust very rapidly (within a few seconds) to dissipate the free energy.

The direct application of eqn. (3) has been illustrated (Delgado & Liao, 1991). However, the calculation of the transient fluxes involves differentiation of the transient metabolite concentrations, and is highly sensitive to experimental error. Here we present an integral form of eqn. (3), where transient metabolite concentrations are used directly to calculate the Flux Control Coefficients without differentiation. The derivation is shown in Appendix B. The reader who is more interested in the applications does not have to attend to the detailed derivations. Rather, the results in the following should be emphasized.

The integral form of eqn. (3) is:

$$\sum_{i=1}^{n+1} \alpha_i [x_i(t) - x_i(0)] = t$$

(4)

where $\alpha_i$ are coefficients to be determined. Note that both internal and external metabolites are considered in eqn. (4). This equation states that the transient metabolite concentrations are not completely independent: they are related by a linear constraint. The coefficients in the constraint are related to the Flux Control Coefficients, steady-state fluxes and the stoichiometric coefficients [Appendix B, eqn. (B-9)]. Note that the stoichiometric coefficients of the pathway are usually known and that the steady-state fluxes can be measured experimentally. In practice, we first determine $\alpha_i$ from the transient metabolite concentrations, and then calculate the Flux Control Coefficients as discussed below.

It is interesting to note that, even if one deletes some of the metabolite concentrations, there still exists a constraint in the form of eqn. (4). This is because metabolite concentrations are used implicitly to calculate the flux through each enzyme. In pathways without direct substrate cycling, such calculation is possible if the number of measured metabolites is greater than or equal to the number of enzymes. Therefore, for pathways without cycling, $n + 1$ metabolites can be deleted if there are $n$ branch points. We take advantage of this result in the examples.

**Linear pathways**

To determine the Flux Control Coefficients experimentally, one first measures the metabolite concentrations $x_i(t)$ in a transient state. The transient state can be generated by shifting incubation conditions for the whole cell, or adding initial substrate to the pathway reconstituted in vitro. These transient metabolite data are then regressed to obtain the coefficients $\alpha_i$. Knowing $\alpha_i$, the stoichiometric coefficients and the steady-state fluxes, one can then calculate the Flux Control Coefficients with:

$$[C_{C_1}^{s}, C_{C_2}^{s}, \ldots, C_{C_n}^{s}] = [\alpha_1, \alpha_2, \ldots, \alpha_{n+1}] A J$$

(5)

where $J$ is the steady-state flux through the pathway and $A$ is the stoichiometric matrix of the pathway. The element of $A$ in the $i$th row and $j$th column is the stoichiometric coefficient of metabolite
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Coefficients. We therefore measure the metabolite concentrations as a function of time. This is done here by computer simulations, and we take ten points (with three significant digits) before the system reaches the quasi-steady state (Fig. 3).

In this example, eqn. (4) takes the following form:

\[
\sum_{i=1}^{n} \alpha_i \left( x_i(t) - x_i(0) \right) = t
\]

We then determine \( \alpha_i \) from these simulated data. Here one may be tempted to use \( x_i(t) - x_i(0) \) as regressors and \( t \) as the dependent variable and apply linear-regression techniques to estimate \( \alpha_i \). However, one has to be extremely careful because linear-regression techniques assume that the regressors are independent. In fact, the regressors here are not independent: they are related by the mass-balance equation as discussed above. Although the mass-balance equation will not cause a problem in most cases, it is advisable to avoid this additional collinearity by deleting one variable from the equation. One usual way to select the best variables to be deleted from the set of regressors is to choose those with large standard errors or large variance inflation factors (Myers, 1990). Another possibility is to use the variance-decomposition proportions matrix discussed by Belsley et al. (1980). In may cases, however, a few trial deletions will yield satisfactory results. In this case, we delete \( X_1 \), and use \( X_4 \) to \( X_6 \) as regressors. The resulting constraint is:

\[
t = 6.66(x_4(t) - x_4(0)) + 10.92(x_5(t) - x_5(0)) + 19.25(x_6(t) - x_6(0)) + 26.95(x_7(t) - x_7(0)) + 41.38(x_8(t) - x_8(0))
\]

The coefficients of the above equations are the values of \( \alpha_2 \), \( \alpha_4 \), \( \alpha_5 \), and \( \alpha_6 \) respectively. The first coefficient, \( \alpha_2 \), is equal to zero since the variable \( x_2(t) - x_2(0) \) does not appear in the above equation. The steady-state flux, \( J \), can be calculated from the steady-state accumulation of the product, \( X_8 \). In this case, \( J = 0.0242 \). Using eqn. (5) and the stoichiometric matrix, one obtains:

\[
[C_{1f} C_{2f} C_{3f} C_{4f} C_{5f} C_{6f}] = [0.16 0.10 0.20 0.19 0.35]
\]

Fig. 3. Metabolite concentration profiles for Example 1.

Vertical axis is \( \Delta x / \Delta t \), where \( \Delta x = x - x_{\text{initial}} \) and \( \Delta t = t_{\text{final}} - t_{\text{initial}} \). Symbols represent hypothetical experimental data points: ■, \( X_1 \); □, \( X_2 \); ▲, \( X_3 \); △, \( X_4 \); ○, \( X_6 \); ●, \( X_8 \).

Example 1. Consider the linear pathway with feedback inhibition depicted in Fig. 2. The stoichiometric matrix is given by:

\[
A = \begin{bmatrix}
-1 & 0 & 0 & 0 & 0 \\
1 & -1 & 0 & 0 & 0 \\
0 & 1 & -1 & 0 & 0 \\
0 & 0 & 1 & -1 & 0 \\
0 & 0 & 0 & 1 & 0 \\
0 & 0 & 0 & 0 & 1
\end{bmatrix}
\]

(7)

The kinetic rate laws are approximated by:

\[
\begin{align*}
\ell_1 &= -0.4x_1 + 0.1 & (8a) \\
\ell_2 &= 0.5x_2 - 0.8x_3 - 0.3x_5 + 0.01 & (8b) \\
\ell_3 &= 0.6x_3 - 0.2x_5 + 0.01 & (8c) \\
\ell_4 &= 0.15x_4 - 0.12x_5 + 0.01 & (8d) \\
\ell_5 &= 0.2x_5 + 0.01 & (8e)
\end{align*}
\]

Knowing the above equations, one can calculate the steady-state concentrations for all the metabolites and all the Elasticity Coefficients. We can then use the Summation and Connectivity Theorems to calculate the Flux Control Coefficients (Fell & Saha, 1985; Westerhoff & Kell, 1987). The results are:

\[
[C_{1f} C_{2f} C_{3f} C_{4f} C_{5f} C_{6f}] = [0.16 0.13 0.17 0.22 0.32]
\]

Note that this calculation is possible only if we know the kinetic rate laws or the Elasticity Coefficients.

In practice, however, one does not have the kinetic rate laws
which is in good agreement with the true values [eqn. (9)] calculated from the known enzyme kinetics. For practical purposes, the differences between the estimates and the true values are negligible. In this case, if one did not measure the steady-state flux, $J = 1$ can be used in eqn. (5). The resulting coefficients are then normalized so that their summation is unity.

Note that, if the original method proposed by Delgado & Liao (1991) were used, the Flux Control Coefficients determined would be

$$[C'_{e_1} C'_{e_2} C'_{e_3} C'_{e_5} C'_{e_6}] = [-10.9 41.1 -38.6 21.7 -12.3]$$ (13)

which are not satisfactory compared with the theoretical values. The poor performance of the original method is due to the differentiation of the transient metabolite concentrations, which requires much more information (in the form of more data points and more significant digits) to be successful. Here we used only ten data points in the transient state, and three significant digits for the estimation.

**Example 2.** In this case we consider again the pathway shown in Fig. 2, but now assume that one knows a priori that reactions 3 and 4 are fast-equilibrium reactions and that they are not rate-controlling. It is shown below how one can incorporate this additional information into the analysis.

The reaction rates are approximated by:

$$v_1 = -1.1x_1 + 0.01$$ (14a)

$$v_2 = 0.5x_2 - 0.8x_3 - 0.3x_4 + 0.00001$$ (14b)

$$v_3 = 6.0x_3 - 2.0x_4 + 0.00001$$ (14c)

$$v_4 = 10.0x_4 - 7.0x_5 + 0.00001$$ (14d)

$$v_5 = 0.6x_5 + 0.00001$$ (14e)

The Flux Control Coefficients calculated from the above rate laws are:

$$[C'_{e_1} C'_{e_2} C'_{e_3} C'_{e_4} C'_{e_5}] = [0.19 0.41 0.06 0.01 0.33]$$ (15)

Now assume that the kinetic parameters are unknown, but it is known that enzymes 3 and 4 are not rate-controlling so that the Flux Control Coefficients are close to zero compared with the others. To determine the Flux Control Coefficients for the other enzymes, we measure the metabolite concentrations in a transient state. Again, this is done by using computer simulation, and the data are shown in Fig. 4. It has to be noted that this time we used only two significant digits in the simulation, and again ten data points.

Since reactions 3 and 4 are fast, they reach quasi-equilibrium before the first measurement. Therefore if we use $X_1$ to $X_4$ as regressors, there will be three collinearities present among the transient metabolite concentrations: a mass-balance constraint and two others due to the quasi-equilibrium of reactions 3 and 4. Regression using all the variables or even deleting one variable will still encounter difficulties. To solve this problem, we take advantage of the known fact that the reactions 3 and 4 are mediated by fast-equilibrium enzymes, and postulate that the transients between $X_3$, $X_4$, and $X_5$ are not important. Therefore, we can lump $X_3$, $X_4$, and $X_5$ into a common pool, which is denoted by $P$. The lumped pathway then is shown in Fig. 5.

One can now treat this case as in Example 1: use $X_2$, $P$ ($= X_3 + X_4 + X_5$) and $X_6$ as regressors, and obtain:

$$t = 103.31[x_2(t) - x_2(0)] + 331.41[p - p(0)] + 537.4[x_6(t) - x_6(0)]$$ (16)

Note that $X_6$ was deleted in order to avoid collinearity due to the mass balance. Moreover, because of the lumping the stoichiometric matrix $A$ is now:

$$A = \begin{bmatrix} -1 & 0 & 0 \\ 1 & -1 & 0 \\ 0 & 1 & 1 \\ 0 & 0 & 1 \end{bmatrix}$$ (17)

The Flux Control Coefficients for enzymes 1, 2 and 5 can now be calculated by using eqn. (5):

$$[C'_{e_1} C'_{e_2} C'_{e_5}] = [0.19 0.43 0.39]$$ (18)

which are very good estimations for practical purposes. Note that $C'_{e_1}$ and $C'_{e_5}$ are determined to be zero, reflecting the assumptions made a priori. The estimation would deteriorate as the values of $C'_{e_1}$ and $C'_{e_5}$ differ from zero.

The way metabolites 3, 4 and 5 were lumped is no coincidence. It has been shown that the presence of non-controlling enzymes in metabolic pathways is related to the characteristic reaction path and that lumping has to be done in a stoichiometric fashion (Liao & Lightfoot, 1988). Non-controlling enzymes result in the relaxation of modes of the system. If these non-controlling enzymes are not identified a priori (i.e. they are not lumped) an increased number of linear dependencies will appear among the transient metabolite concentrations, and this situation would complicate the regression analysis.

**Branched pathways**

Consider now the branched pathway shown in Fig. 6. There are three sets of Flux Control Coefficients, one for each of the three branches:

$$C'_{e_k} = \frac{c_k}{J_k} \left( \frac{\partial J_k}{\partial x_{e_k}^{\text{ss}}} \right) K = A, B, C$$ (19)

where the index K denotes on which branch the Flux Control Coefficients are based.

Since the derivation of eqn. (5) is not restricted to any of the
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Flux Control Coefficients, one can write an equation for each of them:

\[ \sum a_i^a[x_i(t) - x_i(0)] = t \]  \hspace{1cm} (20a)

\[ \sum a_i^b[x_i(t) - x_i(0)] = t \]  \hspace{1cm} (20b)

and

\[ \sum a_i^c[x_i(t) - x_i(0)] = t \]  \hspace{1cm} (20c)

where \( a_i^a \), \( a_i^b \) and \( a_i^c \) are coefficients leading to the Flux Control Coefficients of branches A, B and C respectively.

However, only two of these three constraints are linearly independent, as one can show that:

\[ J_A C_i^a = J_B C_i^b + J_C C_i^c \]  \hspace{1cm} (21)

where \( J_A \), \( J_B \) and \( J_C \) are the steady-state fluxes through branches A, B and C respectively.

Therefore, if we use all the variables in the pathways as regressors and \( t \) as the independent variable to estimate the coefficients \( C_i^a \), \( C_i^b \) and \( C_i^c \), one will find three collinearities: a mass-balance constraint and two out of eqns. (20a) to (20c). To avoid the problem caused by the multicollinearities, one has to delete variables in the regression. If the Flux Control Coefficients based on branch A are to be calculated, one possibility is to delete the terminal metabolites in the other two branches, namely \( X_5 \) and \( X_6 \). If branch B is of interest, one deletes \( X_1 \) and \( X_4 \). Similarly, if one wants to estimate the coefficients based on branch C, \( X_1 \) and \( X_4 \) are to be deleted. The procedure is illustrated in Example 3 below and the reasons why these particular deletions yield the desired Flux Control Coefficients are shown in Appendix D.

Once the coefficients in eqns. (20a) to (20c) are determined, the corresponding Flux Control Coefficients can be determined from the following equations:

\[ [C_i^a C_i^b C_i^c C_i^d C_i^e C_i^f C_i^g C_i^h] = [x_i^2 x_i^2 x_i^2 x_i^0 x_i^0 0 0] AJ \]  \hspace{1cm} (22a)

\[ [C_i^a C_i^b C_i^c C_i^d C_i^e C_i^f C_i^g C_i^h] = [x_i^2 x_i^2 x_i^2 x_i^0 x_i^0 0 0] AJ \]  \hspace{1cm} (22b)

\[ [C_i^a C_i^b C_i^c C_i^d C_i^e C_i^f C_i^g C_i^h] = [x_i^2 x_i^2 x_i^2 x_i^0 x_i^0 0 0] AJ \]  \hspace{1cm} (22c)

In this case, since the steady-state fluxes in different branches are different, \( J \) is now a diagonal matrix:

\[ J = \begin{bmatrix} J_A & 0 & 0 & 0 & 0 & 0 \\ 0 & J_B & 0 & 0 & 0 & 0 \\ 0 & 0 & J_C & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & J_D \end{bmatrix} \]  \hspace{1cm} (23)

where \( J_i \) is the steady-state flux though reaction \( i \).

Note that, when one calculates the Flux Control Coefficients based on branch A, \( x_i^0 = x_i^2 = 0 \) since \( X_4 \) and \( X_6 \) were deleted for the regression analysis. The same argument holds when one calculates the Flux Control Coefficients based on branches B and C.

**Example 3.** Consider the branched pathway shown in Fig. 6. The stoichiometric matrix is:

\[ A = \begin{bmatrix} -1 & 0 & 0 & 0 & 0 \\ 1 & -1 & 0 & 0 & 0 \\ 0 & 1 & -1 & 0 & 0 \\ 0 & 0 & 1 & -1 & 0 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \]  \hspace{1cm} (24)

![Fig. 6. Branched pathway](image)

X_1 is an extracellular substrate and X_5 and X_6 are extracellular products.

and the reaction rate laws are approximated by:

\[ v_1 = -0.8 x_2 + 0.1 \]  \hspace{1cm} (25a)

\[ v_2 = x_3 - x_4 + 0.001 \]  \hspace{1cm} (25b)

\[ v_3 = 0.1 x_1 - 0.15 x_4 + 0.001 \]  \hspace{1cm} (25c)

\[ v_4 = 0.1 x_2 + 0.001 \]  \hspace{1cm} (25d)

\[ v_5 = 0.18 x_3 + 0.001 \]  \hspace{1cm} (25e)

The theoretical values for the Flux Control Coefficients are:

\[ [C_i^a C_i^b C_i^c C_i^d C_i^e C_i^f C_i^g C_i^h] = [0.18 0.15 0.06 0.09 0.53] \]  \hspace{1cm} (26a)

\[ [C_i^a C_i^b C_i^c C_i^d C_i^e C_i^f C_i^g C_i^h] = [0.16 0.13 0.38 0.56 -0.22] \]  \hspace{1cm} (26b)

\[ [C_i^a C_i^b C_i^c C_i^d C_i^e C_i^f C_i^g C_i^h] = [0.19 0.15 -0.03 -0.04 0.73] \]  \hspace{1cm} (26c)

To estimate these Flux Control Coefficients without knowing the kinetic parameters, one again measures the metabolite concentrations in a transient state. The computer-simulated data are shown in Fig. 7. The steady-state fluxes in the three branches are: \( J_A = 0.0199 \), \( J_B = 0.00425 \) and \( J_C = 0.0156 \), so the steady-state flux matrix \( J \) is given by:

\[ J = \begin{bmatrix} 0.0199 & 0 & 0 & 0 & 0 \\ 0 & 0.00425 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0.0156 \end{bmatrix} \]  \hspace{1cm} (27)

We first use \( X_1 \) to \( X_4 \) as regressors and obtain:

\[ t = -50.29 [x_1(t) - x_1(0)] - 41.38 [x_2(t) - x_2(0)] - 33.13 [x_3(t) - x_3(0)] - 20.12 [x_4(t) - x_4(0)] \]  \hspace{1cm} (28)

Substituting these coefficients into eqn. (22a) yields:

\[ [C_i^a C_i^b C_i^c C_i^d C_i^e C_i^f C_i^g C_i^h] = [0.18 0.16 0.06 0.09 0.52] \]  \hspace{1cm} (29)

![Fig. 7. Metabolite concentration profiles for Example 3](image)

Vertical axis is \( \Delta x/\Delta x_i \), where \( \Delta x = x - x_{initial} \) and \( \Delta x_i = x_{final} - x_{initial} \). Symbols represent hypothetical experimental data points: ■: \( X_1 \); □: \( X_2 \); △: \( X_3 \); ▲: \( X_4 \); ○: \( X_5 \); ●: \( X_6 \).
Similarly, we use $X_4$ to $X_7$ as the regressors to obtain:

$$t = 7.54[x_4(t) - x_4(0)] + 14.93[x_5(t) - x_5(0)]$$

$$+ 100.67[x_6(t) - x_6(0)] + 235.68[x_7(t) - x_7(0)]$$ (30)

Substituting these values into eqn. (22b) gives:

$$[C^0_t C^1_t C^2_t C^3_t C^4_t C^5_t C^6_t] = [0.15 0.15 0.36 0.57 -0.23]$$ (31)

If the Flux Control Coefficients for branch C are to be calculated, one uses $X_2$, $X_3$, $X_4$ and $X_5$ as the regressors to obtain:

$$t = 9.56[x_2(t) - x_2(0)] + 17.38[x_3(t) - x_3(0)]$$

$$+ 10.91[x_4(t) - x_4(0)] + 63.96[x_5(t) - x_5(0)]$$ (32)

and the Flux Control Coefficients for branch C can be calculated from eqn. (22c) as:

$$[C^0_t C^1_t C^2_t C^3_t C^4_t C^5_t C^6_t] = [0.19 0.16 -0.03 -0.05 0.73]$$ (33)

Note that the estimated Control Coefficients are very close to the theoretical values.

**DISCUSSION**

The Flux Control Coefficient is a useful index for determining the rate-controlling capacity of an enzyme in a pathway. In theory, it quantifies the change in the steady-state flux caused by an infinitesimal change in enzyme concentration or related kinetic parameter. However, no practical method exists to change enzyme concentration or kinetic parameter infinitesimally. Existing approaches for determining this coefficient can be roughly classified into the following categories: (1) direct perturbation of enzyme activity or concentration and measuring the change in the steady-state flux (e.g. Flint et al., 1981; Groen et al., 1982; Mazat et al., 1986; Salter et al., 1986; Torres et al., 1986, 1989; Brand et al., 1988; Kruckenberg et al., 1989), and (2) calculation from the Elasticity Coefficients, which in turn require comprehensive information on individual enzyme kinetics (e.g. Groen et al., 1986; Fell & Snell, 1988). We have now presented a methodology that enables one to determine the Flux Control Coefficients from transient metabolite concentrations. This approach can potentially reduce the amount of experimentation by an order of magnitude, if it is used in conjunction with n.m.r. measurements. Although the accuracy of the methodology has been greatly improved from our previous results, this approach still requires relatively accurate measurements (about 5–10% maximum error).

The central concept of our approach is that the transient metabolite concentrations are determined by the enzyme kinetics, and that the transient state contains much more information than the steady state. It is therefore possible, in principle at least, to extract kinetic information from the transient state. However, such practice is so sensitive to measurement noise that it becomes almost impossible to extract all the kinetic parameters from the transient data. Instead of estimating all the kinetic parameters, here we limit the scope to extract some useful information, namely the Flux Control Coefficients, from the transient metabolite data.

The theoretical basis of our approach is presented by eqns. (3) and (4), which can be applied to pathways of any topology as long as (1) the internal metabolite concentrations are able to reach a unique and stable steady or quasi-steady state, (2) the calculation of the transient fluxes through each reaction from the transient metabolite concentration data must be theoretically possible, and (3) the metabolites are homogeneously distributed in the system. The first assumption makes the definition of the steady-state Flux Control Coefficients meaningful. The second assumption is necessary as the methodology implicitly converts the transient metabolite concentrations into transient fluxes.

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APPENDIX A

This Appendix presents the derivation of eqn. (3) based on previous results reported by Reder (1988). The complete derivation of the fundamental equations is rather technical and mathematically intensive. Therefore only a brief summary useful for our derivation and to define the nomenclature is presented. Complete details of the basic equations can be found in the original paper (Reder, 1988).

Consider a biochemical system with m internal metabolites and r reactions described by:

$$\frac{dy}{dt} = Nv$$  \hspace{1cm} (A-1)

where y is an m x 1 column vector containing the internal metabolite concentrations, N is the m x r stoichiometric matrix where the element in the ith row and jth column is the stoichiometric coefficient of internal metabolite i in reaction j, and v is an r x 1 column vector containing the rates of reaction of fluxes for each reaction of the system under analysis. Note that the stoichiometric matrix N is different from the full stoichiometric matrix A used in the main text, as A contains additional rows that include the external (pool) metabolites.

The stoichiometric matrix N can be decomposed as:

$$N = LN_R$$  \hspace{1cm} (A-2)

where $N_R$ is an m x r matrix formed by the first m_r rows of N that constitute a basis for its row space. L is an m x m_o matrix that has the form:

$$L = \begin{bmatrix} I_{m_o} \\ L_o \end{bmatrix}$$  \hspace{1cm} (A-3)

where $I_{m_o}$ is the m_o x m_o identity matrix and L_o is (m - m_o) x m_o.

It has been shown that the following relation is satisfied (Theorem 5 of Reder, 1988):

$$ZD_y L = 0$$  \hspace{1cm} (A-4)

Z is defined as the r x r simple steady-state flux-control matrix that satisfies (Proposition 4 of Reder, 1988):

$$D_J = ZD_y$$  \hspace{1cm} (A-5)

where $D_J$ and $D_y$ are defined as:

$$[D_J]_{ij} = \frac{\partial J_i}{\partial y_j}$$  \hspace{1cm} (A-6a)

and

$$[D_y]_{ij} = \frac{\partial y_i}{\partial \lambda_j}$$  \hspace{1cm} (A-6b)

The parameter $\lambda_j$ may represent any external parameter (such as a fixed extracellular substrate or enzyme concentration). Note that $\lambda_j$ cannot normally be an internal variable for any useful purpose. $J_i$ is the steady-state flux through enzyme i and $y_i$ is the velocity through enzyme i (note that Z was denoted as C by Reder, 1988). The relation between the Z matrix and the conventional Flux Control Coefficients is shown later below. The element of the ith row and jth column of $D_y$ is given by:

$$[D_y]_{ij} = \frac{\partial y_i}{\partial y_j}$$  \hspace{1cm} (A-7)

where the derivative is evaluated at the steady state.

Combining eqns. (A-1), (A-2) and (A-3) and partitioning the metabolite concentration vector y accordingly, one obtains:

$$\frac{dy}{dt} = \begin{bmatrix} \frac{d}{dr} [y_R] \\ \frac{d}{dr} [y_R'] \end{bmatrix} = \begin{bmatrix} I_{m_o} \\ L_o \end{bmatrix} N_R y$$  \hspace{1cm} (A-8)

where $y_R$ is m_o x 1 and $y_R'$ is (m - m_o) x 1.

It can be shown that (Theorem 1 of Reder, 1988):

$$\frac{d}{dr} (y_R' - L_y y_R) = 0$$  \hspace{1cm} (A-9)

Integrating between two arbitrary time points, one gets:

$$\Delta y_R' = L_y \Delta y_R$$  \hspace{1cm} (A-10)

Eqn. (A-9) or eqn. (A-10) describes all the m - m_o structural conservation relationships existing among the internal metabolite concentrations.

Multiplying eqn. (A-4) by $\Delta y_R$:

$$ZD_y L \Delta y_R = 0$$  \hspace{1cm} (A-11)

Using the partitioned form of L [eqn. (A-3)]:

$$ZD_y \begin{bmatrix} \Delta y_R \\ L_y \Delta y_R \end{bmatrix} = 0$$  \hspace{1cm} (A-12)

By eqn. (A-10):

$$ZD_y \begin{bmatrix} \Delta y_R \\ \Delta y_R' \end{bmatrix} = 0$$  \hspace{1cm} (A-13)

or

$$ZD_y \Delta y = 0$$  \hspace{1cm} (A-14)

The first-order approximation of the kinetic rate laws around the steady state is:

$$\Delta y_i = \sum_{i=1}^{m} \frac{\partial y_i}{\partial y_j} \Delta y_j$$ \hspace{1cm} (A-15)

or in matrix form:

$$\Delta v = D_y \Delta y$$  \hspace{1cm} (A-16)

Note that in eqn. (A-15) it has been assumed that the fluxes $y_i$ do not depend on the external (pool) metabolites. Otherwise, the
external metabolites that do not comply with this assumption have to be kept constant during the transient state.

One can substitute the term $D_y \Delta y$ by $\Delta v$ in eqn. (A-14) and obtain:

$$Z \Delta v = 0 \quad \text{(A-17)}$$

Eqn. (A-17) is the generalized version of eqn. (3). The significance of the elements of $Z$ and their relation to the conventional Flux Control Coefficients defined in eqn. (1) can be seen if some simplifying assumptions are made. First, assume there are $r$ parameters $\lambda_j$ that act specifically on the rates $v_j$. Then, $Z$ can be normalized as:

$$C = J^{-1}ZJ \quad \text{(A-18)}$$

where $J$ is the steady-state flux diagonal matrix and $C$ is the Flux Control Coefficients matrix, in which the elements are:

$$[C]_{ij} = \frac{\partial J}{\partial \lambda_i} \left( \frac{\partial \lambda_j}{\partial \lambda_i} \right)^{-1} \quad i = 1, 2, \ldots, r; j = 1, 2, \ldots r \quad \text{(A-19)}$$

Substituting eqn. (A-18) into eqn. (A-17):

$$Z \Delta v = JCJ^{-1} \Delta v = 0$$

or

$$C J^{-1} \Delta v = 0 \quad \text{(A-20)}$$

which is the matrix version of eqn. (3). Note that in a linear (unbranched) pathway $K = J = \ldots = J_c$, and all the rows in the matrix $C$ are the same. Eqn. (A-20) becomes a single equation, reducing to eqn. (3). Eqns. (A-17) and (A-20) are valid for any pathway configuration as long as the system under analysis possesses a unique stable steady or quasi-steady state for the internal metabolite concentrations. This condition is usually fulfilled if the pathway kinetics do not depend on the external (pool) metabolite concentrations, or, if they do, these concentrations are buffered.

Finally, if $\lambda_j$ denotes enzyme concentration, $\lambda_j = e_j$ and $v_j$ is proportional to $e_j$, then the generalized Flux Control Coefficient defined in eqn. (A-19) reduces to the conventional Flux Control Coefficient defined in eqn. (1).

**APPENDIX B**

This Appendix presents the derivation of eqns. (4), (20a), (20b) and (20c), the integral versions of eqn. (3). Substituting $\Delta v = v - v_s$ into eqn. (A-17) yields:

$$Zv = Zv_s \quad \text{(B-1)}$$

where $v_s$ is the $r \times 1$ steady-state flux vector.

The fluxes $v$ can be expressed in terms of the metabolite concentrations (internal and external) by using the pseudo-inverse of the (full) stoichiometric matrix $A$:

$$v = A^+ \frac{dx}{dt} \quad \text{(B-2)}$$

Here the concentration vector $x$ includes both internal and external (pool) metabolites as they are needed to obtain the transient fluxes $v$. Matrix $A^+$ denotes the pseudo-inverse of $A$. Eqn. (B-2) is also known as the least-squares solution for $v$. For general $p \times q$ matrix $A$, the pseudo-inverse $A^+$ is the $q \times p$ matrix whose $j$th column $a_j$ is the unique minimum-length solution of the least-squares problem $Ax_j \approx e_j$, where $e_j$ is the $j$th column of the identity matrix $I_p$. Further details can be found in Lawson & Hanson (1974).

Substituting eqn. (B-2) into eqn. (B-1):

$$ZA^+ \frac{dx}{dt} = Zv_s \quad \text{(B-3)}$$

Since $v_s$ belongs to the kernel of the (reduced) stoichiometric matrix $N$ (defined in Appendix A), one can write (Reder, 1988):

$$Zv_s = 0 \quad \text{(B-4)}$$

Thus:

$$ZA^+ \frac{dx}{dt} = v_s \quad \text{(B-5)}$$

Eqn. (B-5) can now be integrated between an initial instant $t_0 = 0$ and any arbitrary time point $t$ to give:

$$ZA^+ \Delta x = v_s t \quad \text{(B-6)}$$

Eqn. (B-6) is the generalized matrix version of eqn. (4). It can be expressed in terms of the normalized Flux Control Coefficients if the assumptions stated in the Appendix A about $Z$ hold. Using eqn. (A-18), the above equation becomes:

$$CJ^{-1}A^+ \Delta x = t \quad \text{(B-7)}$$

If one considers a single row of the steady-state flux-control matrix, eqn. (B-7) can be written as:

$$[a_1, a_2, \ldots, a_n] \Delta x = t \quad \text{(B-8)}$$

where

$$[a_1, a_2, \ldots, a_n] = C^x J^{\text{sp}} A^+ \quad \text{(B-9)}$$

or

$$[a_1, a_2, \ldots, a_n] AJ = C^x \quad \text{(B-10)}$$

Here $C^x$ is a row vector whose elements are $C^x_{ij}$. Eqn. (B-8) is the matrix version of eqns. (4), (20a), (20b) and (20c) in the main paper. Also, from eqns. (B-9) and (B-10) one can see that the coefficients in eqn. (B-8) are related to the stoichiometric coefficients, the steady-state fluxes and the Flux Control Coefficients. Thus eqns. (5), (22a), (22b) and (22c) can be readily seen.

**REFERENCES**


**APPENDIX C**

In this Appendix we show how the mass balance or other stoichiometric constraints vanish from the constraints among the transient metabolite concentrations after post-multiplying by the (full) stoichiometric matrix \(A\). Assuming \(A\) has dimension \(n \times r\), where \(r\) is the total number of reactions in the pathway and \(n\) is the total number of metabolites (internal plus external), we follow the same approach as in Appendix A and decompose \(A\) as:

\[
A = LA_R
\]  
(C-1)

where \(A_R\) is an \(n_o \times r\) matrix formed by the first \(n_o\) rows of \(A\) that constitute a basis for its row space, and \(L\) is an \(n \times n_o\) matrix that has the form:

\[
L = \begin{bmatrix} I_{n_0} \\ I_{-n_o} \end{bmatrix}
\]  
(C-2)

where \(I_{n_0}\) is \((n-n_o) \times n_o\) and \(I_{-n_o}\) is the \(n_o \times n_o\) identity matrix.

With these definitions, one can easily show that all the structural conservation relationships among all metabolites can be expressed by:

\[
[I_{n_0} - I_{-n_o}] \Delta x = 0
\]  
(C-3)

where the \(n \times 1\) concentration change vector \(\Delta x\) includes all external and internal metabolites.

All constraints among the transient metabolite concentrations will be a linear combination of eqns. (B-6) and (C-3). Then one can write:

\[
B \Delta x = v_n
\]  
(C-4)

where

\[
B = ZA^* + \beta[I_{n_0} - I_{-n_o}]
\]  
(C-5)

and \(\beta\) is an arbitrary constant.

To determine the Flux Control Coefficients and according to eqn. (B-10) one has to post-multiply the coefficients in the constraint [eqn. (C-5)] by the full stoichiometric matrix \(A\):

\[
BA = ZA^*A + \beta[I_{n_0} - I_{-n_o}]A
\]  
(C-6)

By eqn. (C-1):

\[
BA = Z + \beta[I_{n_0} - I_{-n_o}]L A_R
\]  
(C-7)

Using the partitioned form of \(L\) [eqn. (C-2)]:

\[
BA = Z + \beta[I_{n_0} - I_{-n_o}][I_{n_o} I_{-n_o}]A_R
\]  
(C-8)

Multiplying the partitioned matrices:

\[
BA = Z + \beta[I_{n_0} - I_{-n_o}]A_R = Z
\]  
(C-9)

or

\[
BA = Z + \beta[I_{n_0} - I_{-n_o}]A_R = Z
\]  
(C-10)

and the structural or stoichiometric conservation equations vanish when one post-multiplies by \(A\).

**APPENDIX D**

As shown in eqn. (B-10), the Flux Control Coefficients are given by:

\[
[C_{i_1}^{\alpha_k} C_{i_2}^{\alpha_k} C_{i_3}^{\alpha_k} ... C_{i_r}^{\alpha_k}] = [a_1^k a_2^k a_3^k ... a_r^k]A J
\]  
(D-1)

where the \(k\) denotes the branch on which the Flux Control Coefficients are based.

In linear (unbranched) pathways there are no special relationships among the Flux Control coefficients. On the other hand, in branched pathways some relationships can be extracted and their number and form will depend on the topology of the reaction network. For the configuration depicted in Fig. 6, the stoichiometric matrix \(A\) is given by eqn. (24). In this case eqn. (D-1) can be explicitly written as:

\[
[C_{i_1}^{\alpha_k} C_{i_2}^{\alpha_k} C_{i_3}^{\alpha_k} C_{i_4}^{\alpha_k}] = [(a_1^k - a_2^k)J_1 (a_2^k - a_3^k)J_2 (a_3^k - a_4^k)J_3
\]

\[
(a_4^k - a_1^k)J_4 (a_1^k - a_2^k)J_2] (A_1^k - A_2^k)J_3]
\]  
(D-2)

where \(J_1 = J_2 = J_3 = J_4 = J_0\) and \(J_5 = J_c\).

It can be shown that the following relation among the Flux Control Coefficients holds (e.g. Fell & Sauro, 1985):

\[
l_j(C_{i_j}^{\alpha_k} + C_{i_j}^{\alpha_k}) = l_{jn} C_{i_j}^{\alpha_k}
\]  
(D-3)

Substituting \(C_{i_1}^{\alpha_k}\), \(C_{i_2}^{\alpha_k}\) and \(C_{i_4}^{\alpha_k}\) using eqn. (D-2) we obtain:

\[
a_1^k = a_2^k
\]  
(D-4)

Thus the coefficients of \([x_{i_1}(t) - x_{i_1}(0)]\) and \([x_{i_2}(t) - x_{i_2}(0)]\) in eqn. (20a) must be the same. Since we must delete variables so that the mass-balance constraint fails, deletion of \([x_{i_1}(t) - x_{i_1}(0)]\) or \([x_{i_2}(t) - x_{i_2}(0)]\) leads to \(a_1^k = a_2^k = 0\). Following the same argument, one can show that deletion of \(X_1\) and \(X_4\) (\(a_1^k = a_4^k = 0\)) leads to the calculation of \(C_{i_1}^{\alpha_k}\), and that deletion of \(X_1\) and \(X_4\) (\(a_1^k = a_4^k = 0\)) leads to the calculation of \(C_{i_4}^{\alpha_k}\).

**REFERENCE**