Metabolic Control Analysis – Theory and Experimental Application

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ABSTRACT

This paper discusses the theory, concept and experimental application of metabolic control analysis. This technique has provided a means by which a quantitative description of control structure by an enzyme, substrate and cofactor over a metabolic pathway can be produced. Application of metabolic control analysis results in numerical values (flux control coefficients) showing how much a particular enzyme or step has over a metabolic pathway flux. The value of flux control coefficient for an enzyme is within the range 0 (no control) and 1 (maximum control). We describe two different approaches that have been used to measure flux control coefficients in metabolic pathways, i.e. (1) top-down control analysis (TDCA) and (2) bottom-up control analysis (BUCA) together with the experimental work that has been carried out recently in the study of control structure over carbon flux through lipid biosynthesis pathway in oil palm callus cultures. Using the TDCA of metabolic control analysis, we calculated the control over the lipid biosynthesis pathway exerted by fatty acid synthesis and the Kennedy pathway during complex lipids assembly. BUCA was also applied to evaluate the importance of fatty acid synthase (FAS) in controlling carbon flux from acetate to total lipids in oil palm callus cultures. These data represent the first use of metabolic control analysis to gain quantitative information about control structure over lipid biosynthesis in oil palm and should form the basis of more detailed work which will be invaluable for the rational development of improved, genetically modified oil crops.

INTRODUCTION

A study of any metabolic map will reveal a high degree of complexity in even the simplest of metabolic pathways. Each step, whether an enzyme, a carrier or any other reaction, has a finite affinity for its substrate and is a potential target for inhibition or activation by any one of a large number of effectors. Therefore, at least to some extent, each reaction will affect the flux through that pathway.

In classical terms, the flux through a pathway is described qualitatively in terms of the kinetics of the individual enzymes. In such a qualitative analysis, each enzyme in a chosen metabolic pathway would be isolated and

Keywords: top-down control analysis, metabolic control analysis, flux control coefficients, lipid biosynthesis pathway, oil palm.
purified and its kinetics determined in vitro by rate-substrate concentration measurements, using a suitable assay technique. An enzyme can be declared rate-limiting for a particular pathway if it has the lowest Vmax, or, if at steady-state, it has a ratio of product concentration to substrate concentration far from its equilibrium constant, that is, its mass-action ratio is far from the value of its equilibrium constant. In the pathway below, substrate S1 is converted by enzymes E1 – E4 into product P via the intermediates M1 – M3.

**Figure 1a**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>E1</th>
<th>M1</th>
<th>E2</th>
<th>M2</th>
<th>E3</th>
<th>M3</th>
<th>E4</th>
<th>Product</th>
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<tr>
<td>(Suppose enzyme E2 has been found to be rate-limiting in an in vitro set of experiments and a means exists for it to overexpress in the in vivo organism. If the amount of E2 is, say, doubled by genetic manipulation, will the enzyme still be rate-limiting? As an alternative, take the case where an enzyme, say E3, has a very high Vmax and a specific inhibitor for it is available. If the activity of E3 is now reduced to a fraction of its original value, then at some point, it must become rate-limiting. Both of these somewhat hypothetical examples illustrate the two extremes of control: (i) the activity or amount of the rate-limiting enzyme increased to the extent that it cannot exert any significant control over the pathway flux, and (ii) the activity or amount of an enzyme catalyzing a near-equilibrium reaction reduced to such an extent that it becomes rate-limiting. These two cases show that it is possible for a shift in control to occur within a pathway in response to some effector acting upon it, where the effector may be a product, acting as a feedback inhibitor or activator, or a change in the expression of the enzyme in response to a change in the physiological or developmental state of the organism. Given the number of enzymes in any typical metabolic pathway and the complexity of their interactions, for enzyme-enzyme and enzyme-substrate/product, the likelihood of one single step during rate-limiting is remote (Quant, 1993) especially during changing physiological or developmental conditions. It follows, therefore, that within the two extremes discussed earlier there should be a range of control for each enzyme between no control and complete control (rate-controlling). Rather more likely is that all the enzymes in the pathway exert some control over the flux through the pathway, that is, control is shared. For example, a particular physiological condition may favour high control by certain enzymes while a different physiological condition may show a shift in the distribution of the control.</td>
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control coefficients \( C_i \) of individual enzymes within a metabolic pathway are estimated by titration of the pathway flux as a result of changing the enzyme activity or concentration using a specific inhibitor/activator or some other means such as genetic manipulation (Kacser and Burns, 1979). If an enzyme exerts strong flux control, then changing its activity by a small amount (say, 10%-20%) will give a significant (or proportional) change to the overall flux through the pathway. This is represented by a high flux control coefficient which is expressed as the fractional change in the pathway flux \( (\delta J/J) \) caused by a small fractional change in the enzyme activity or concentration \( (\delta E/E) \). For a linear pathway, the value of flux control coefficient for an enzyme is within the range \( >0 \) to \( <1 \) [i.e. whose variation produces an almost proportional response in the pathway flux] is more important than one with a lower coefficient, say \( C = 0.1 \) (where the variation in its activity only gives a tenth of the effect on the pathway flux). A full control structure of a pathway can be obtained eventually by multiple applications of BUCA on different target enzymes. This will yield information on the flux control coefficients of all the constituent enzymes.

A disadvantage of using BUCA is that specific ways of manipulating each enzyme or step are necessary but are not always possible. In addition, when working with a pathway newly under investigation, it may be difficult to decide which steps are likely to show strong flux control and, therefore, are to be examined initially. Likewise, the total number of feasible experiments which need to be done will also be unclear.

Top-down Control Analysis (TDCA)

The TDCA approach aims to simplify experimental procedure by measuring group flux control coefficients \( *C_i \) instead of the individual components (Brown et al., 1990; Quant, 1993; Brand, 1996). It involves conceptually dividing the whole pathway into two or three blocks connected by a system intermediate. For a linear metabolic pathway, the system is divided into two blocks of reactions, e.g. Block A and Block B (Figure 1b). Thus, Block A comprises all the reactions involved in the production of \( \chi \) (the system intermediate) and Block B, the group of enzymes which convert \( \chi \) to complex metabolites or end product. Thus, \( J_{\chi} \) is the system flux from the initial substrate to \( \chi \), while \( J_{\chi} \) is the system flux from \( \chi \) to the product (Figure 1b). This single pool of a chosen unique intermediate allows interaction between the two parts of the system, such that the blocks of reactions do not interact except via the intermediate. Thus, TDCA on such a linear system would be able to indicate how important each of the blocks of reactions is at controlling the system fluxes to \( J_{\chi} \) or from \( J_{\chi} \) by allowing calculation of group flux control coefficients for Block A \( *C_{JA} \) and Block B \( *C_{JB} \), respectively.

TDCA on a branched system (Figure 1c) will describe precisely how important each of the blocks of reactions (Block A; Block B; Block C) is at controlling the level of the intermediate \( \chi \) and flux through the same or the other two blocks \( (J_{\chi}, J_{\chi}) \) to product 1 or product 2, \( (J_B \) and \( J_C \)) by allowing calculation of different group flux control coefficients \( *C_{JA}; *C_{JA}; *C_{JA}; *C_{JB}; *C_{JB}; *C_{JC}; *C_{JC}; *C_{JC} \). This approach has been described in detail by Brown et al. (1990), Quant (1993) and Brand (1996). Thus, TDCA can give quantitative measurements of flux control without the necessity of guessing which enzyme step will turn out to be important or of having specific enzyme inhibitors available. TDCA is also more widely applicable for studying metabolic control (Brown et al., 1990; Quant, 1993; Brand, 1996) because it can provide an immediate overview of the control structure of the whole pathway, thus giving information on the control exercised by large sections of a complex pathway.

Essential Language of Control Analysis - Definition of their Mathematical Forms

The underlying concepts of MCA which underpin the ideas of this technique are contained in four essential expressions (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). Let us consider Figure 1a as the specimen pathway for explaining the following:

Flux control coefficients. For a pathway flux consisting of several steps from the initial substrate to final product, the importance of each step in the pathway \( (E_1, E_2, E_3, etc.) \) in controlling the pathway flux \( J \) in a steady-state is expressed by its flux control coefficient \( C_{E_i} \). Mathematically, the flux control coefficient, \( C \), for enzyme, \( E_i \), over a pathway flux, \( J \), can be written as;

\[
C_{E_i} = \frac{(\delta J/J)(E_i \delta E_i)}{(\delta E_i/E_i)}
\]

and similarly for \( C_{E_2}, C_{E_3}, etc. \). Thus, \( C_{E_i} \) can be defined as the fractional change in the pathway flux \( (\delta J/J) \) in response to a small fractional change in the enzyme under study \( (\delta E_i/E_i) \) when the intermediates in the pathway are allowed to relax to a new steady-state.

The summation theorem. The flux
control coefficients sum to one (Kacser and Burns, 1973) although there are exceptions to this rule (Kacser et al., 1990; van Dam et al., 1993; Brand et al., 1994). The summation theorem for n enzymes or steps is thus:

\[ C_{E1}' + C_{E2}' + C_{E3}' + \ldots + C_{En}' = 1 \]

Therefore, in a linear pathway consisting of enzymes with normal kinetics \( (i.e. \text{where substrates stimulate and products inhibit the reaction rate}) \), the individual flux control coefficients will be between zero (no control) and one (complete control).

However, negative flux control coefficients may be possible in a branched pathway where stimulation of an enzyme in one branch may decrease the flux through a competing branch (Fell, 1997). In such systems, summation of the flux control coefficients can also exceed 1.

**Elasticity coefficients.** Elasticity coefficients describe the effect of an effector (inhibitor, activator or pathway metabolite) on the flux through an individual enzyme. Elasticity coefficients can be defined as the fractional change in the flux through an enzyme, \( \frac{\delta\nu}{\nu} \), for a small fractional change in the concentration of the effector, \( \frac{\delta\chi}{\chi} \). Thus,

\[ \varepsilon = \left( \frac{\delta\nu}{\nu} \right) \left( \frac{\delta\chi}{\chi} \right) \]

**Connectivity theorem.** The connectivity theorem relates the flux control coefficients and elasticity coefficients as:

\[ (C_{E1}') \frac{\delta\nu_{E1}}{\nu_{E1}} + (C_{E2}') \frac{\delta\nu_{E2}}{\nu_{E2}} + \ldots + (C_{En}') \frac{\delta\nu_{En}}{\nu_{En}} = 0 \]

**TOP-DOWN CONTROL ANALYSIS (TDCA) – A SPECIFIC EXAMPLE**

This section describes the experimental application of TDCA to the lipid biosynthesis pathway in oil palm callus cultures as a specific example. For a detailed account of these experiments, see Ramli et al. (1998; 2001a). This study represents the first use of TDCA to examine the overall lipid biosynthesis pathway in oil palm callus cultures.
biosynthetic pathway. Lipid biosynthesis in the so-called C18:3 plants (Browse and Sommerville, 1991), such as oil palm, involves the co-operation of two different subcellular compartmentations – the plastid and the endoplasmic reticulum (Figure 2a). Fatty acids are produced by de novo synthesis in the plastid and then exported mainly to the cytosol. This gives rise to the eukaryotic lipids which have C18 fatty acids at the sn-2 position while the small amount of complex lipid formation in the plastid gives rise to prokaryotic lipids with C16 fatty acids at the sn-2 position (Browse and Sommerville, 1991). These fatty acids are activated to acyl-CoAs in the cytosol which are then transported into the endoplasmic reticulum where synthesis of the storage lipid, triacylglycerol, takes place predominantly via the glycerol 3-phosphate or Kennedy pathway (Kennedy, 1961). For the purpose of TDCA, we need to simplify conceptually the whole lipid biosynthetic pathway to a simple linear pathway (Figure 2b) comprising two blocks of reactions (Block A – fatty acid synthesis and Block B – complex lipid assembly).

The fatty acid synthesis (Block A) and the triacylglycerol assembly (Block B) pathways were manipulated independently and indirectly via changes in the level of the system intermediate, acyl-CoA. These changes in the steady-state levels of the intermediate were achieved directly by manipulation with exogenous oleate, and they were monitored by incorporation of radiolabel from labelled substrate, [1-14C]acetate, which was used as the precursor for fatty acid synthesis. In these experiments (Ramli et al., 1998; 2001a), the fluxes through the two blocks of reactions were measured independently: J_A was measured by [1-14C]acetate labelling and TAG assembly (J_B) was followed directly by using [U-14C]glycerol (Figure 2b).

The group or overall flux control coefficients for the two groups of enzymes over lipid biosynthesis pathway can be calculated from the group elasticities (*ε^{BlkA}_X and *ε^{BlkB}_X) of the two subsystems to acyl-CoA (X) (Equation 1b), which, in turn, are calculated from the flux data (Equation 1a). The overall flux control coefficients of the groups of enzymes involved in fatty acid biosynthesis and in the conversion of acyl-CoA to complex lipids (Figure 2a) are C^{JTL}_{BlkA} = 0.64 and C^{JTL}_{BlkB} = 0.36, respectively. These results showed that as much as 60% of the control of lipid biosynthesis pathway can reside in the enzymes of fatty acid biosynthesis and therefore posed several questions, such as, if ACCase really has strong flux control as reported earlier in leaves (Page et al., 1994). The results also agree with a previous study (Ramli and Sambanthamurthi, 1996) which suggested that KAS II is important in controlling fatty acid biosynthesis in the oil palm mesocarp. The results are also in agreement with other studies where it has been suggested that the fatty acid supply is more important than the activity of lipid assembly enzymes for TAG accumulation in oil crops (Bao and Ohlrogge, 1999; Bafor et al., 1990). It is important to note that about 40% of the overall control is exerted by the steps comprising Block B. This would suggest that increasing the expression of genes for the enzyme steps in Block B would not necessarily increase the product yield and could explain the disappointing results obtained in many transgenic crops (Ohlrogge and Jaworski, 1997).

**CONTROL OF FATTY ACID PRODUCTION IN OIL PALM CALLI – A SPECIFIC EXAMPLE OF BOTTOM-UP CONTROL ANALYSIS (BUCA) APPLICATION**

This section describes the application of BUCA to measure flux control coefficients. Figure 3 simplifies the experiment used to estimate the flux control coefficient for fatty acid synthase (FAS) over lipid biosynthesis in oil palm callus cultures (see Ramli, 1999 for experimental details). For flux manipulation in control analysis, we require a small but measurable inhibition of the target enzyme’s activity (Fell, Equation 1. Determination of *ε_X*, *ε_{BlkB}*, *C^{JTL}_{BlkB} and *C^{JTL}_{BlkA}.

(a) Calculation of *ε^{BlkA}_X, *ε^{BlkB}_X*

\[
*\epsilon^{BlkA}_X = \frac{\delta J_A}{J_A} \left( \frac{X}{\delta X} \right) = \frac{\delta J_A}{\delta X} \left( \frac{X}{X} \right)
\]

\[
*\epsilon^{BlkB}_X = \frac{\delta J_B}{J_B} \left( \frac{X}{\delta X} \right) = \frac{\delta J_B}{\delta X} \left( \frac{X}{X} \right)
\]

where *ε^{BlkA}_X and ε^{BlkB}_X are group elasticity coefficients for Block A and Block B with respect to the system intermediate, cytosolic acyl-CoA (X). (\(\frac{\delta J_A}{J_A}\)) and (\(\frac{\delta J_B}{J_B}\)) are the fractional changes in the steady-state fluxes through Block A and Block B, respectively, in response to a small fractional change in the level of A (\(\frac{\delta X}{X}\)).

(b) Calculation of *C^{JTL}_{BlkB} and *C^{JTL}_{BlkA}*

\[
*\epsilon^{JTL}_{BlkA} = \frac{\epsilon^{BlkA}_X}{\epsilon^{BlkB}_X} - \epsilon^{BlkA}_X
\]

\[
*\epsilon^{JTL}_{BlkB} = \frac{\epsilon^{BlkB}_X}{\epsilon^{BlkA}_X} - \epsilon^{BlkB}_X
\]
(a) A simplified plant lipid biosynthesis pathway.

(b) The complex system (a) can be ‘reduced’ to a simplified version (into a linear pathway) for TDCA.

Notes: (a) A simplified plant lipid biosynthesis pathway showing fatty acid formation in the plastid. The acyl-ACPs produced by this process are either used directly for plastidial lipid production or hydrolysed and exported to the cytosolic compartment as acyl-CoAs. The latter are used by the acyltransferases of the Kennedy pathway on the endoplasmic reticulum.

Abbrev.: ACP, acyl carrier protein; ACCase, acetyl-CoA carboxylase; KAS, β-ketoacyl ACP synthase; NEFA, nonesterified fatty acids.

(b) The complex system (a) can be ‘reduced’ to a simplified linear pathway. The pathway reactions in lipid biosynthesis have been grouped together to a conceptually simplified system as described in the text. Carbon flux from the substrate pool, acetate, enters Block A to produce fatty acids. The fatty acids are then exported to the cytosol as acyl-CoAs, esters to serve as substrates for the Kennedy pathway enzymes during triacylglycerol synthesis on the endoplasmic reticulum. Therefore, the two blocks of reactions are connected via a single pool of cytosolic acyl-CoA (as the system intermediate). For the purpose of measuring the kinetics for each block (i.e. \( J_A \) or \( J_B \)) independently, \([U-^{13}C]\)acetate is used as the carbon precursor for fatty acid synthesis in the plastid while complex lipid assembly was followed conveniently with \([U-^{13}C]\)glycerol which has proven to be selective for endoplasmic reticulum activities.

Figure 2. TDCA of lipid biosynthesis pathway.
Thus, FAS activity was assayed in the presence of a range of concentrations of a specific inhibitor (diflufenican/DFF). In this study, we investigated the effect of changing the FAS activity ($\gamma_{FAS}$) on the carbon flux from [1-14C]acetate ($J_{TL}$) to lipid biosynthesis. The flux control coefficient for FAS was calculated from the ratio of the tangents, at zero inhibitor concentration, to the curve for the total pathway flux and for the FAS activity versus inhibitor concentration using the SIMIFIT package (Bardsley and Prasad, 1997) and by the definition (Figure 3d):

$$C_{TL}^{FAS} = \frac{\Delta J_{TL}}{\Delta \gamma_{FAS}}$$

where $C_{FAS}$ is the flux control coefficient of FAS over the total lipid biosynthesis pathway flux ($J_{TL}$) and $\gamma_{FAS}$ is the activity of FAS. Calculation of the flux control coefficient gave a value of 0.51. The results (Ramli, 1999) suggest that in oil palm callus cultures, approximately 50% of the control over lipid biosynthesis pathway is exerted by the FAS reactions. Therefore, the results are in agreement with previous observations (Ramli and Sambanthamurthi, 1996) and also from temperature manipulation studies (Ramli et al., 1998; 2001b) that KAS II (which is a component of FAS) may play a vital role in the lipid biosynthesis pathway.

\[ \frac{J_{TL}}{\gamma_{FAS}} \]

**CONCLUSION**

Although our knowledge of molecular and basic biochemistry of plant lipid biosynthesis is now quite extensive, there is still a major gap in the understanding of the regulation and control of such metabolism. This paucity of knowledge on control sites is important because gene manipulation is an expensive exercise. Thus, the manipulation of a single gene, even one calculated on current knowledge to be important, often has little or no effect on the overall flux. Moreover, although coordinate changes can be induced by manipulating one gene, antisensing often has no action unless very powerful, and there are com-

(a) Manipulation of enzyme activity (e.g. FAS activity using DFF as a specific inhibitor).

(b) Measurement of the effect of DFF on enzyme activity and total pathway flux.

(c) Plot of inhibition curves (FAS activity and total pathway flux versus inhibitor concentrations).

(d) Calculation of flux control coefficient.
pensatory mechanisms for plants to deal with many gene expression changes. The study has shown that metabolic control analysis can serve as an effective means for studying the control of pathway fluxes, quantitatively. The data arising from these studies also confirmed other findings from qualitative analysis (e.g. Ramli et al., 1998). Thus, the work has provided a useful new way of looking at metabolic control in oil crops. The experimental results presented in this article represent the first use of flux control analysis to examine the lipid biosynthesis pathway and, thus, provide a foundation for further, much needed, experiments.

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