Metabolic control analysis of biochemical pathways based on a thermokinetic description of reaction rates

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Metabolic control analysis is a powerful technique for the evaluation of flux control within biochemical pathways. Its foundation is the elasticity coefficients and the flux control coefficients (FCCs). On the basis of a thermokinetic description of reaction rates it is here shown that the elasticity coefficients can be calculated directly from the pool levels of metabolites at steady state. The only requirement is that one thermodynamic parameter be known, namely the reaction affinity at the intercept of the tangent in the inflection point of the curve of reaction rate against reaction affinity. This parameter can often be determined from experiments *in vitro*. The methodology is applicable only to the analysis of simple two-step pathways, but in many cases larger pathways can be lumped into two overall conversions. In cases where this cannot be done it is necessary to apply an extension of the thermokinetic description of reaction rates to include the influence of effectors. Here the reaction rate is written as a linear function of the logarithm of the metabolite concentrations. With this type of rate function it is shown that the approach of Delgado and Liao [Biochem. J. (1992) **282**, 919–927] can be much more widely applied, although it was originally based on linearized kinetics. The methodology of determining elasticity coefficients directly from pool levels is illustrated with an analysis of the first two steps of the biosynthetic pathway of penicillin. The results compare well with previous findings based on a kinetic analysis.

INTRODUCTION

Metabolic engineering has been defined as the purposeful modification of intermediary metabolism by recombinant DNA techniques [1–3]. It offers the possibility to design in a systematic fashion new and better strains with improved productivity of both traditional metabolites and novel compounds. It is a multidisciplinary field applying information and techniques from biochemistry, genetics, molecular biology, cell physiology and chemical engineering. For production of metabolites such as amino acids and antibiotics, the key issues in process improvement are productivity, titre and yield of product from the carbon source, and one of the most important aspects in design of new strains is therefore control of cellular fluxes. For this purpose metabolic flux analysis (MFA) combined with metabolic control analysis (MCA) is very useful. With MFA it is possible to quantify the pathway fluxes *in vivo* from measurements of fluxes in and out of the cell [4,5], and it becomes possible to identify possible rigid branch points and alternative pathways to the same metabolite. The influence of alternative pathways on the flux distribution can be examined, and the maximum theoretical yield calculated. However, MFA does not give any information about the regulation of pathway fluxes. For this purpose MCA, which was introduced independently by Kacser and Burns [6] and by Heinrich and Rapoport [7], is useful. With MCA the control structures are quantified through a mathematical formulation based on the so-called elasticity coefficients and control coefficients. Especially useful are the flux control coefficients (FCCs), which quantify the influence of the individual reaction rates (or enzyme activities) on the overall flux through the pathway. For a linear pathway with L enzymic steps the FCCs are given by:

$$C_i^r = \partial \ln(r)/\partial \ln(v_i); \quad i = 1, \ldots, L \tag{1}$$

where r is the steady-state flux through the pathway and v_i is the rate of the ith enzymic reaction. As a consequence of the normalization the FCCs sum to 1, which is normally referred to as the summation theorem. The FCCs are related to the elasticity coefficients through the connectivity theorems:

$$\sum_{i=1}^{L} \epsilon_j^i C_i^r = 0; \quad j = 2, \ldots, L \tag{2}$$

where the elasticity coefficients are given by:

$$\epsilon_j^i = \partial \ln(v_i)/\partial \ln(X_j); \quad i = 1, \ldots, L \text{ and } j = 2, \ldots, L \tag{3}$$

and X_j is the concentration of the jth metabolite (X_1 is the concentration of the substrate and X_{L+1} is the concentration of the product).

The FCCs can be determined experimentally [8,9], but this is very laborious, because it requires independent variation of the activity *in vivo* of all the enzymes within the pathway. MCA is therefore often based on information of the kinetics of the individual reactions from which the elasticity coefficients and thereafter the FCCs can be calculated by using the summation and connectivity theorems [10,11]. In the literature there are several examples of this approach, e.g. for the analysis of the glycolysis in *Saccharomyces cerevisiae* [12,13] and for the analysis of the penicillin biosynthetic pathway in *Penicillium chrysogenum* [14,15]. However, the lack of information about kinetics *in vivo* and the daunting task of obtaining this for the individual enzymic reactions impede a more widespread use of this approach. Delgado and Liao [16,17] introduced another approach to determining the FCCs. By assuming linearized kinetics, they derived a set of equations that enable the direct determination of the FCCs from measurements of the metabolite pools during transients. Besides determination of the FCCs, this ingenious approach can also be used to determine the so-called metabolite concentration control coefficients [18]. The approach was used to determine the FCCs for a reconstituted partial glycolytic pathway, and the calculated FCCs were found to correspond quite well to those determined experimentally by enzyme titration [19].

Abbreviations used: ACVS, ACV synthase; FCCs, flux control coefficients; IPNS, isopenicillin N synthetase; LLD-ACV, δ-(L-aminoadipyl)-L-cysteinyl-D-valine; MCA, metabolic control analysis; MFA, metabolic flux analysis.

It remains to be shown, however, that their approach can be applied more widely.

This paper decribes a new approach to determining the elasticity coefficients. On the basis of non-equilibrium thermodynamics, the kinetics of enzymic reactions can be described as linear functions of the change in Gibbs free energy of the reaction, a so-called thermokinetic description. For a simple two-step pathway this leads to a direct relation between the elasticity coefficients and the change in Gibbs free energy for the reactions. For more complex pathways, it is necessary either to use the top-down approach [20] to evaluate the FCCs for different segments of the pathway or to use the approach of Delgado and Liao [17,18], which is shown to be applicable also when a thermokinetic description of reaction rates is applied.

THERMOKINETIC DESCRIPTION OF ENZYMIC REACTIONS

Classic thermodynamics considers only equilibrium states; it therefore provides little insight into the mechanisms of transformation occurring in cellular pathways. Thus the second law of thermodynamics, together with the Gibbs chemical equilibrium principle, gives information on whether a reaction or conversion can proceed, i.e. whether it is feasible, in a certain direction, but it gives no information of the rate of reaction or conversion. In his pioneering work Onsager [21], however, proposed that thermodynamics could be extended to describe non-equilibrium systems, and he derived linear flow-force relations, so-called phenomenological equations, where the flow (or reaction rate) v is specified as a linear function of the thermodynamic driving forces A_i, i.e. for the ith flow:

$$v_i = \sum_{i=1}^{L} L_{ij} A_j \tag{4}$$

For a chemical reaction the thermodynamic driving force is given by the reaction affinity (equal to minus the change in free energy of the reaction). L_{ji} are phenomenological coefficients, which are constrained by the requirement of the overall dissipation function to be non-negative [22]. Furthermore according to Onsager's reciprocal relations the matrix of coefficients is symmetrical, i.e. $L_{ij} = L_{ji}$ [21,22].

The linear force relations in eqn. (4) are only valid close to equilibrium, and because many cellular processes operate far from equilibrium this prevents a wider application of the concept. From an empirical analysis of different cellular processes a linear relation is, however, often found between the flow and the driving force of the process, even if the process operates far from equilibrium. The best-known example is the linear relation between the rate of respiration and specific growth rate found for many microbial systems (see, for example, [23]), but other examples have been described, e.g. for the individual processes of the oxidative phosphorylation [24,25]. Rottenberg [26] and van der Meer et al. [27] showed that this might be a consequence of the special kinetics of enzymic reactions (see also the discussion in [28]). To illustrate this, consider the simple, reversible, enzyme-catalysed conversion of a substrate into a product. The net forward reaction rate v of this reaction is given by:

$$v = \frac{v_{S,max} c_S/K_S - v_{P,max} c_P/K_P}{1 + c_S/K_S + c_P/K_P} \tag{5}$$

where c_S and c_P are the concentrations of substrate and product respectively, $v_{S,max}$ is the maximum forward reaction rate (at high substrate concentrations and $c_P = 0$), $v_{P,max}$ is the maximum backwards reaction rate (at high product concentrations and $c_S = 0$), and K_S and K_P are the corresponding Michaelis–Menten constants. Using the definition of the change in Gibbs free energy for the conversion of substrate into product, the kinetics can be written as a function of the reaction affinity A [27,28]:

$$\frac{v}{v_{S,max}} = \frac{e^{A/RT} - 1}{[K_S/(c_S + c_P) + 1]\, e^{A/RT} + v_{S,max}/v_{P,max}\,[K_P/(c_S + c_P) + 1]} \tag{6}$$

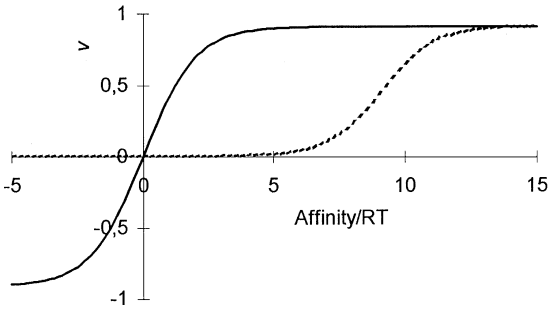

Figure 1 The forward reaction rate v of an enzyme-catalysed reaction as a function of the reaction affinity calculated from eqn. (6)

The sum of the concentrations of substrate and product is assumed to be constant (and equal to 1). The solid line depicts a thermodynamically reversible reaction with $v_{S,max} = v_{P,max} = 10$ and $K_S = K_P = 10$ (corresponding to an equilibrium constant K_{eq} of 1). The broken line depicts a thermodynamically irreversible reaction with $v_{S,max} = K_S = 10$, $v_{P,max} = 0.1$ and $K_P = 100$ (corresponding to an equilibrium constant K_{eq} of 1000). Adapted from [28].

A direct application of eqn. (6) is limited by the fact that the net forward reaction rate is a function of two variables, namely the concentrations of both the substrate and the product (strictly speaking, the chemical potential of these two compounds). If one applies the natural physical constraint that the sum of the substrate and product concentrations should be constant, then the net forward reaction rate is a function of the reaction affinity only [27], and Figure 1 shows the reaction rate as a function of the reaction affinity for two sets of kinetic parameters.

For the reversible reaction (solid line in Figure 1) it is seen that proportionality between the reaction rate and reaction affinity holds for a wide range of net forward reaction rates. Thus for this type of reaction the phenomenological equations (4) can generally be applied far from equilibrium. However, for the irreversible reaction (broken line in Figure 1) proportionality between v and the reaction affinity only holds for very small net forward reaction rates. In the relevant range of reaction rates (between 0.1 and 0.9) an assumption of proportionality would be a very poor approximation to the actual relation between v and reaction affinity. However, around $v = 0.5$, a linear approximation to the relation seems possible, i.e.:

$$v = L^{\#}(A - A^{\#}) \tag{7}$$

where $A^{\#}$ is the reaction affinity at the intercept of the tangent of the curve at the inflection point and $L^{\#}$ is the slope of the tangent at the inflection point. These two parameters are functions of the kinetic parameters [27,28]:

$$L^{\#} = v_S + v_P/4RT \tag{8}$$

$$\frac{A^{\#}}{RT} = \ln\left(\frac{v_S}{v_P}\right) - 2\frac{v_S - v_P}{v_S + v_P} \tag{9}$$

with v_S and v_P given by:

$$v_S = \frac{v_{S,max}}{1 + K_S/(c_S + c_P)}; \quad v_P = \frac{v_{P,max}}{1 + K_P/(c_S + c_P)} \tag{10}$$

Normally the ratio of $v_{S,max}$ to $v_{P,max}$ is constant, and $A^{\#}$ is therefore independent of the enzyme activity. However, it is a function of the reaction conditions through the term $c_S + c_P$. Westerhoff and van Dam [28] also analysed a number of other kinetic expressions, and in all cases found that for a certain range of free energy differences (or reaction affinities) a linear relation could be assumed.

The basic assumption for the above analysis is that the sum of concentrations for the substrate and the product is constant. For many pathway reactions this assumption is not reasonable, but often there are other physical constraints on the concentrations. Thus one of the concentrations might be kept constant by external conditions or very tight intracellular regulation, or its concentration might vastly exceed the Michaelis–Menten constant for the enzyme acting on it. Rottenberg [26] showed that also for these cases the relation between reaction rate and driving force becomes nearly linear. It therefore seems that eqn. (7) is often a very good approximation to the rate of enzyme-catalysed reactions.

METABOLIC CONTROL ANALYSIS

MCA of two-step pathways based on thermokinetics

We now consider the simple two-step pathway:

$$g_{11}X_1 + g_{12}X_2 = 0; \quad g_{22}X_2 + g_{23}X_3 = 0 \tag{11}$$

where g_i are stoichiometric coefficients, positive for products, negative for reactants, and zero for non-participating compounds (e.g. $g_{13} = 0$ and $g_{21} = 0$). This pathway can either consist of two enzymic reactions or be a result of lumping several enzymic reactions into two overall reactions. The reaction affinity for the ith reaction is given by:

$$A_i = -\Delta G_i^{\circ'} - \boldsymbol{RT}\ln\left(\prod_{k=1}^{3} X_k^{g_{ik}}\right); \quad i = 1, 2 \tag{12}$$

where $G_i^{\circ'}$ is the change in standard free energy of the ith reaction.

We now assume that the rate of each of the two reactions can be described as a function of the reaction affinity according to eqn. (7). With this thermokinetic description of the reaction kinetics the elasticity coefficients for the two reactions with respect to the intermediate X_2 are given by:

$$\epsilon_2^i = \frac{1}{v_i}\frac{\partial v_i}{\partial \ln(X_2)} = \frac{1}{v_i}\frac{\partial v_i}{\partial A_i}\frac{\partial A_i}{\partial \ln(X_2)}; \quad i = 1, 2 \tag{13}$$

where the two partial derivatives can be evaluated from eqns. (7) and (12):

$$\epsilon_2^i = -\frac{g_{i2}RT}{A_i - A_i^{\#}}; \quad i = 1,2 \tag{14}$$

Thus if $A_1^{\#}$ and $A_2^{\#}$ are known, the elasticity coefficients for the two reactions can be calculated directly from the reaction affinity. For reversible reactions $A_i^{\#}$ is zero, and eqn. (14) therefore allows the calculation of the elasticity coefficients directly from measurements of the metabolite levels at steady state. Westerhoff et al. [29] derived a similar relation for the elasticity coefficient for enzyme reactions at equilibrium, but used the mass action ratio rather than the reaction affinity. To be able to control the flux through the pathway, at least one step is normally irreversible, i.e. it operates far from equilibrium. In these cases it is necessary to know $A_i^{\#}$, which can be determined from a plot of reaction rate against reaction affinity. Because $A_i^{\#}$ is independent of the activity of the enzyme *in vivo* and depends only on the Michaelis–Menten constants, on the ratio of the maximal forward and maximal backward reaction rates and on $c_S + c_P$, it is in principle possible to determine $A_i^{\#}$ from experiments *in vitro*. When corresponding data on reaction rate and reaction affinity are evaluated it is nevertheless important to ensure that the enzyme activity is constant, because otherwise $L^{\#}$ varies and linearity between v and $A^{\#}$ does not hold. If the enzyme activity is changing, it is in principle possible to normalize the reaction rate with the enzyme activity because $L^{\#}$ is proportional to the enzyme activity (or to v_{max}).

Determination of $A^{\#}$ from experiments *in vitro* have the same limitations as other experiments *in vivo*, namely that the conditions *in vivo* might not be correctly simulated, e.g. if factors such as cytoskeleton organization influence the kinetics.

With the elasticity coefficients determined from eqn. (14), the FCCs can be determined from the summation and connectivity theorems as mentioned in the Introduction section. Thus the thermokinetic description allows a complete evaluation of the MCA coefficients from measurements of the metabolite pool levels at steady state.

Generalization to more complex pathways

Eqn. (14) can also be applied to pathways with more than two reactions, but a strict requirement is that there are no regulation loops that span more than one reaction, e.g. if the last metabolite causes feedback inhibition of the first reaction in a pathway with more than two steps. This is because the thermokinetic description allows quantification of the influence of the substrate and product only on the reaction rate, and does not include any influence of effectors. In many cases pathways only consist of one or two irreversible reactions, with the other reactions being close to equilibrium. This allows the lumping together of several reaction steps, and hereby one can end up with a pathway structure such as that shown in eqn. (11). In this case the reaction affinities are those for the overall conversions. Because $A_i^{\#} = 0$ for reactions operating close to equilibrium, the value of this parameter for the lumped set of reactions is still given by the corresponding value for the irreversible reaction. When equilibrium reactions are lumped together with one irreversible reaction, it is not possible to quantify the FCCs exactly for the individual steps, but because reactions close to equilibrium have small FCCs by definition, the value of the overall FCC for the lumped set of reaction, will correspond quite well to that of the irreversible reaction. Alternatively one can apply the top-down approach [20], where the centring is done around each metabolite and in each case the group (or block) FCCs are determined.

In cases where it is not possible to lump the individual pathway reactions into two overall reactions and where regulation loops extend over more than one reaction, the approach described above cannot be applied directly. However, if we rewrite the thermokinetic expression of eqn. (7) as:

$$v_i = a_i \sum_{j=1}^{L+1} k_{ij}\ln(X_j) + b_i \tag{15}$$

it can be shown (see the Appendix) that the approach of Delgado and Liao [17] can be applied to quantify the FCCs. As mentioned in the Introduction, this approach permits a direct determination of the FCCs from measurements of the metabolite pools during a transient. In eqn. (15) a_i, b_i, and k_{ij} are kinetic parameters. For substrates and products the k_{ij} are identical with the stoichiometric coefficients, whereas for effectors they are empirical parameters. For a compound that does not influence the kinetics, i.e. either substrates, products or effectors, k_{ij} is zero. For the simple case where there are no effectors, a_i becomes equal to $L_i^{\#}$ and b_i becomes equal to $-L_i^{\#}A_i^{\#}$, whereas in the general case

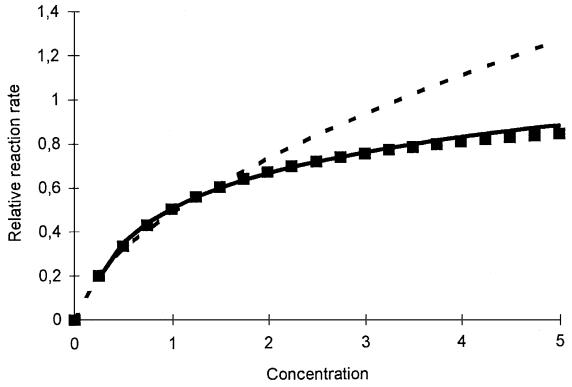

Figure 2 Comparison of different types of kinetics

The data points are Michaelis–Menten kinetics with $v_{max} = 1$ and $K_m = 1$. The solid line is the best fit of the kinetics given by eqn. (15) to the Michaelis–Menten kinetics and the broken line is the best fit of power-law kinetics. In both cases the best fit was found from linear regression around the K_m value, i.e. for concentrations between 0.25 and 1.75.

they should be considered as empirical parameters. On the basis of the discussion in the previous section, eqn. (15) will always be a better approximation to reaction kinetics than the linearized kinetics assumed by Delgado and Liao [17], and a wider application of their approach is therefore justifiable. Recently it was demonstrated that the approach of Delgado and Liao is extremely sensitive to errors in measurements of the pathway metabolites [30], and a practical application of the approach still needs to be demonstrated.

The kinetic expression, eqn. (15), is analogous to the power-law kinetic presentation on which biochemical systems analysis is based (reviewed in [31]). However, as illustrated in Figure 2, the kinetics of eqn. (15) gives a better representation of Michaelis–Menten-type kinetics over the entire range of substrate concentrations, and it is therefore to be preferred over the power-law kinetic presentation.

EXAMPLE: MCA OF THE PENICILLIN BIOSYNTHETIC PATHWAY

To illustrate the methodology described above for calculating the elasticity coefficients and the FCCs we consider the penicillin biosynthetic pathway, of which all the pathway metabolites and enzyme activitities have been measured during fed-batch cultivations [5,14,32]. The penicillin biosynthetic pathway has now been almost completely elucidated [33]. The pathway consists of three enzymic steps, of which the first two are identical in all biosynthetic pathways for β-lactam antibiotics. On the basis of a kinetic model for this pathway, it has been found that control is mainly exerted by the first two steps of the pathway [15], and in the following we therefore consider only these steps:

(1) formation of the tripeptide δ-(L-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) from the amino acids L-aminoadipic acid, L-cysteine and L-valine, a reaction catalysed by ACV synthetase (ACVS), which is a large multifunctional enyme; and

(2) conversion of LLD-ACV into isopenicillin N, a reaction catalysed by isopenicillin N synthetase (IPNS), which is an iron-dependent oxidase.

The changes in free energy for both reactions have been calculated by P. N. Pissarra and J. Nielsen (unpublished work), and both reactions were found to be very exogenic, with changes in free energy of the order of -130 kJ/mol for the ACVS-catalysed reactions and -480 kJ/mol for the IPNS-catalysed reaction. Thus both reactions are operating far from equilibrium.

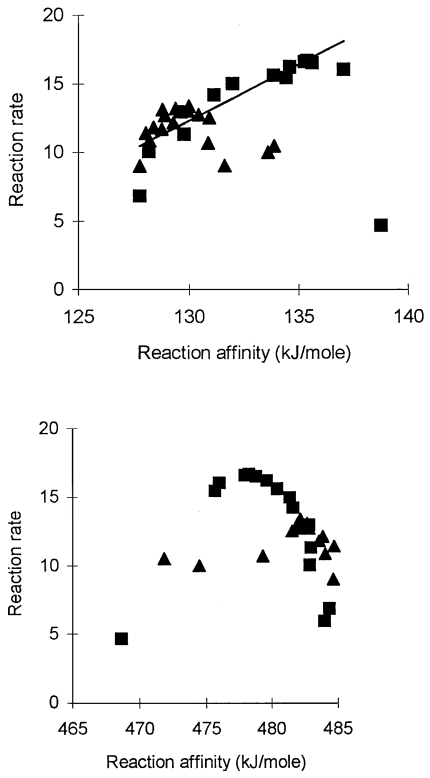

Figure 3 Reaction rate (mol/h per g dry weight) as a function of reaction affinity (kJ/mol) for the two first steps of the penicillin biosynthetic pathway

The data were obtained from two different fed-batch cultivations during which the reaction rate of the two reactions varied slowly. Symbols: ▲, data for the fed-batch cultivation FB023; ■, data for the fed-batch cultivation FB028. The reaction affinities were calculated as described by P. N. Pissarra and J. Nielsen (unpublished work) with data from [5,32]. Upper panel, reaction rate as a function of reaction affinity for the ACVS-catalysed reaction; lower panel, reaction rate as a function of reaction affinity for the IPNS-catalysed reaction.

The change in free energy for the first reaction was calculated from the data from fed-batch cultivations. When the rate of the ACVS-catalysed reaction, which varied during the fed-batch cultivations, is plotted as a function of the calculated reaction affinity, a linear relation between the two is found (Figure 3, upper panel). The activity of ACVS was found to be approximately constant duing fed-batch cultivations [14]; the linear plot therefore allows a determination of the parameters in the thermokinetic description of eqn. (7). Thus $L^{\#}$ is estimated as 0.82×10^{-6} mol²/h per g dry weight per kJ and $A^{\#}$ as 115 kJ/mol.

For the IPNS-catalysed reaction there is no similar linear relation between the reaction rate and reaction affinity (Figure 3, lower panel); in fact the reaction rate decreases for increasing reaction affinities. This is explained by a decrease in the activity of this enzyme throughout the fed-batch cultivations [14]. The thermokinetics of eqn. (7) can therefore not be applied directly. However, if, as discussed previously, the reaction rate is normalized with respect to the measured enzyme activity, a linear relation between relative rate and reaction affinity is obtained (Figure 4) and $A^{\#}$ is determined as 465 kJ/mol.

With the parameters of the thermokinetic description determined, we can calculate the elasticity coefficients and the FCCs at different times of the fed-batch cultivation. Strictly speaking, application of the summation and connectivity theorems requires a steady state, which is not fulfilled during the fed-batch

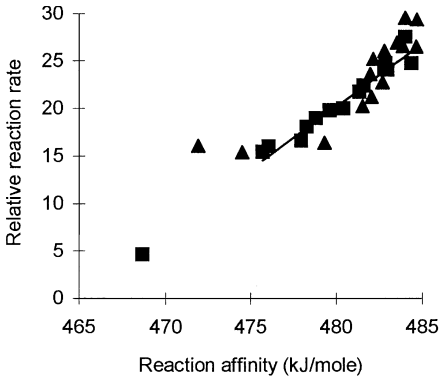

Figure 4 Relative reaction rate (reaction rate divided by enzyme activity) as a function of reaction affinity (kJ/mol) for the IPNS-catalysed reaction

The reaction rate was normalized with respect to the measured enzyme activity reported by Nielsen and Jørgensen [14].

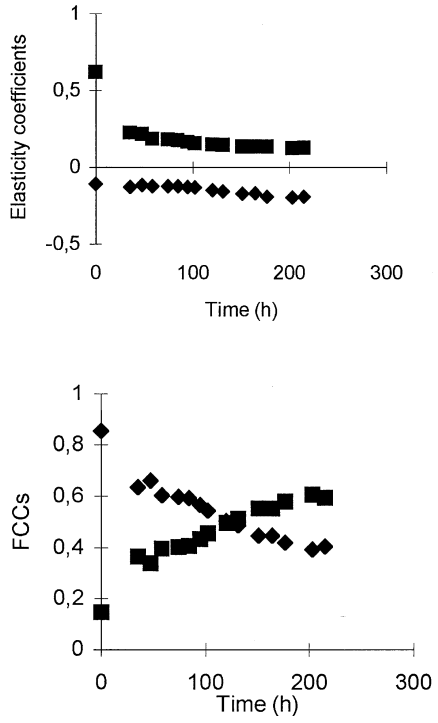

Figure 5 MCA based on the thermokinetic description of the first two steps in the penicillin biosynthetic pathway

The elasticity coefficients and FCCs were calculated at different times of cultivation during a fed-batch cultivation (FB028 of [32]). Upper panel: elasticity coefficients for ACVS (◆) and IPNS (■). The elasticity coefficients were calculated from the change in free energy at the given time and from eqn. (14) with $A^{\#}_{ACVS} = 115$ kJ/mol and $A^{\#}_{IPNS} = 465$ kJ/mol. Lower panel: FCCs for ACVS (◆) and IPNS (■). The FCCs were calculated from the elasticity coefficients from the summation and connectivity theorems.

experiments, but, as discussed in [15], pseudo-steady state can be assumed for the biosynthetic pathway. The results of the calculations for one fed-batch cultivation are shown in Figure 5 (similar results were found for another fed-batch cultivation). It is observed that there is a shift in flux control from the first reaction to the second during the cultivation. The picture is exactly the same as that found on the basis of kinetic analysis of the pathway [14,15]. This shift in flux control has been ascribed to accumulation of the tripeptide LLD-ACV, which was hypothesized to inhibit the ACVS-catalysed reaction [14]. Recent analysis of the purified enzyme from *P. chrysogenum* confirms this hypothesis (H. B. Aa. Theilgaard, K. N. Kristiansen, C. M. Henriksen and J. Nielsen, unpublished work). The fact that the thermokinetic description allows quantification of the decreasing reaction rate when LLD-ACV accumulates (the concentration of the three precursor amino acids was approximately constant) indicates that the inhibition of the ACVS-catalysed reaction by LLD-ACV is a result of mass action, i.e. the equilibrium of the desorption of LLD-ACV from ACVS is shifted towards the enzyme–product complex and therefore leaves less enzyme available for catalysis.

CONCLUSION

On the basis of a thermokinetic description of enzymic reaction, it is shown that the elasticity coefficients of MCA can be calculated directly from the reaction affinity (or the change in free energy). The only requirement is a knowledge of a single thermodynamic parameter, which may be determined from experiments either *in vitro* or *in vivo*. The advantage of this method is that it allows the MCA parameters to be determined from steady-state measurements of the pool levels. However, the method relies on an assumption of a linear relation between reaction rate and reaction affinity, which need to be evaluated in each case. If the assumption is not checked, the calculated elasticity coefficients should be considered as only preliminary estimates that need to be checked by another method, e.g. from knowledge of the enzyme kinetics.

The method applies only to two-step pathways, but with the top-down approach the elasticity coefficients and the FCCs can also be calculated for more complex pathways. Alternatively, an extension of the thermokinetic description, where the reaction rate is specified as a linear function of the logarithm of the metabolite concentration, can be applied; the FCCs can then be determined from metabolite measurements during transients as described by Delgado and Liao [17].

The method is straightforward to apply and, as illustrated for the analysis of the first two reactions in the penicillin biosynthetic pathway, it allows quantification of the elasticity coefficients and the FCCs without a detailed kinetic model containing a large number of parameters.

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APPENDIX

The approach of Delgado and Liao ([17] in the main paper) is based on the following equation:

$$\sum_{i=1}^{L} C_i \Delta v_i = 0 \tag{A1}$$

where $v_i = v_i - v_{ss}$ is the change in rate of the ith reaction during the transient compared with the steady-state reaction rate v_{ss}. This equation is easily derived from the connectivity theorem if linearized kinetics is assumed ([16] in the main paper). In the following it will be shown that eqn. (A1) also holds when the kinetics is given by eqn. (15) (in the main paper). First we find the elasticity coefficient:

$$\epsilon_j^i = b_i k_{ij} / v_{ss} \tag{A2}$$

where v_{ss} is the steady-state flux through the pathway. When eqn. (A2) is inserted in the connectivity theorem we get:

$$\sum_{i=1}^{L} C_i b_i k_{ij} = 0 \tag{A3}$$

On multiplication with $\Delta \ln(X_j) = \ln[X_j(t_2)] - \ln[X_j(t_1)]$ we obtain:

$$\sum_{i=1}^{L} C_i b_i k_{ij} \Delta \ln(X_j) = 0 \tag{A4}$$

If we add for all j we get:

$$\sum_{j=2}^{L} \sum_{i=1}^{L} C_i b_i k_{ij} \Delta \ln(X_j) = \sum_{i=1}^{L} C_i b_i \sum_{j=2}^{L} k_{ij} \Delta \ln(X_j) = 0 \tag{A5}$$

which is seen to reduce to eqn. (A1), since from eqn. (15) (in the main paper) we have:

$$\Delta v_i = b_i \sum_{j=2}^{L} k_{ij} \Delta \ln(X_j) \tag{A6}$$

Received 20 May 1996/19 August 1996; accepted 4 September 1996