Study of the Flux and Transition Time Control Coefficient profiles in a metabolic system *in vitro* and the effect of an external stimulator

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Control of flux and transition time was investigated with a reconstructed rabbit muscle glycolytic system *in vitro* as an experimental model. The results show agreement with the summation property for the Flux Control Coefficients [Kacser & Burns (1973) Symp. Soc. Exp. Biol. 27, 65–104; Heinrich & Rapoport (1974) Eur. J. Biochem. 42, 89–95]. Control of flux is almost exclusively located at the hexokinase- and phosphofructokinase-catalysed steps, whereas control of transition time is distributed more evenly between the enzymes of the system. The summation value of the Transition Time Control Coefficients is near to -1 , suggesting the existence of another Summation Theorem besides that already stated for Flux Control Coefficients. Finally, we study the effect of an external stimulator of the system (fructose 2,6-bisphosphate) on the Control Coefficient profiles. The effect appears to be greater on the Transition Time Control Coefficient distribution than on the Flux Control Coefficients.

INTRODUCTION

Control analysis, as proposed by Kacser & Burns (1973) and Heinrich & Rapoport (1974), aims to describe quantitatively the control in metabolic systems. This approach is mainly based on the definition of two types of coefficients, Control and Elasticity, which express systemic and local sensitivities respectively. Among them our attention here is focused on the first of these, which are defined as:

$$C_P^V = \frac{\partial V}{\partial P} \cdot \frac{P}{V}$$

where V represents a system response of a dependent variable and P any independent variable. Considering the flux (J) through a metabolic system in a given steady state as the response, and the enzyme concentration (e_i) as the independent variable, the above-cited authors defined the Flux Control Coefficient as:

$$C_{e_i}^J = \frac{\partial J}{\partial e_i} \cdot \frac{e_i}{J} \quad (1)$$

and established the Summation Theorem, which applies to any metabolic pathway:

$$\sum_{i=1}^n C_{e_i}^J = 1$$

One important consequence of this Summation Theorem is that in sequential pathways with no special regulatory features (forward inhibition loops, product activation or substrate cycles; see Kacser, 1983) the values of the coefficients are usually between 0 and 1.

The theoretical framework based on these coefficients has been successfully extended to the study of many types of metabolic systems (Kacser, 1983; Fell & Sauro,

1985; Hofmeyr *et al.*, 1986; Sauro *et al.*, 1987; Torres *et al.*, 1988a; for a review see Westerhoff *et al.*, 1984) as well as to the experimental study into the regulation of fluxes and metabolite concentrations (Rapoport *et al.*, 1976; Flint *et al.*, 1980, 1981; Groen *et al.*, 1982a,b; Salter *et al.*, 1986; Torres *et al.*, 1988b; for a review see Meléndez-Hevia *et al.*, 1987).

But there is an aspect of the metabolic regulation that still remains largely unexplored even by the control analysis approach (Hess, 1973; Heinrich & Rapoport, 1975; Reich & Sel'kov, 1981; Easterby, 1981), namely the time dimension of these processes.

Easterby (1981, 1984, 1986) has developed a theoretical approach to the transition time in coupled enzyme assays and has established criteria for optimum design of such systems. He has also dealt with the effect of feedback and with the transition from one state to another.

Heinrich & Rapoport (1975) have considered the control of individual transition time, τ_{s_j} , associated with a given free intermediate S_j , defined somewhat differently from Easterby (1981), in terms of the standard formulation of control analysis. In the now accepted nomenclature (Burns *et al.*, 1985), this is:

$$C_{e_i}^{\tau_{s_j}} = \frac{\partial \tau_{s_j}}{\partial e_i} \cdot \frac{e_i}{\tau_{s_j}} = \frac{\partial(\ln \tau_{s_j})}{\partial(\ln e_i)}$$

where e_i refers to the activity of enzyme E_i and τ_{s_j} is the transition time with respect to the free pool of the intermediate metabolite S_j . These authors applied this approach to the calculation of τ_{s_j} in some simplified systems (Heinrich & Rapoport, 1975) and they also established that:

$$\sum_{i=1}^n C_{e_i}^{\tau_{s_j}} = -1 \quad (2)$$

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which is the Summation Theorem of a given individual free-pool transition time for all the enzymes in the metabolic system.

Here we present an experimental study based on a reconstructed rabbit muscle glycolytic pathway with an experimental system *in vitro*. The aim of this work was to study the flux control and transition time control of the system developed under the usual conditions of enzyme assay. We also study the effect on control distribution of an external effector of the glycolytic pathway, fructose 2,6-bisphosphate.

THEORY

In this work we start from the concept of transition time, τ , defined as follows.

Consider a pathway bounded by two metabolites, X_s and X_p , at independently held constant external concentrations. This pathway contains any number of enzymes and pools between them, as shown in Scheme 1.

The mechanism of each enzyme is unspecified, allowing any degree of reversibility and any degree of saturation. The constancy of the concentrations of X_s and X_p might be thought of as based on the same assumptions as apply to an 'ordinary' enzyme assay generating an initial rate; this could include that the concentration of X_p is zero, so that the last step is virtually irreversible, but this is not a necessary feature of the system. Alternatively, the concentrations of X_s and X_p can be maintained by some other mechanism outside the system under consideration.

Provided that there is an electrochemical potential difference between X_s and X_p , there will be a net flux and, at steady state, a constant rate of production of X_p with time (see Fig. 1). In such state the system flux J is the amount of X_p removed from the system ($X_{p,out}$), per unit of time, i.e. in steady state $J = d(X_{p,out})/dt$. On the other hand, at steady state all intermediate pool concentrations, both free and bound, have time-invariant values. Here we deal only with the case that the concentrations of enzyme-bound intermediates are negligible, which is the usual condition in enzyme assays *in vitro*. Under this assumption the transition time can be considered as the summation of all individual transition times for every free pool, these having the physical meaning of the average time one specified molecule spends in that pool. Its value is given by the ratio of concentration of the whole pool, $\sigma = \sum s_j$, over the flux through it:

$$\tau = \sum_{j=1}^m \tau_{s_j} = \frac{\sum s_j}{J} = \frac{\sigma}{J}$$

From this expression is directly derived the physical meaning of transition time as the mean time that, in a metabolic system at steady state, one specified molecule spends inside the system. Fig. 1 serves also to show the graphical interpretation of this magnitude. In the usual conditions of assay *in vitro* intercepts of the flux asymptote with the time axis give us the transition time directly.

Obviously the transition time so defined is a dependent variable of the metabolic system as well as the flux J , or

Scheme 1.

the concentration of a metabolite S_j . Therefore we can define its corresponding Control Coefficient:

$$C_{e_i}^{\tau} = \frac{\partial \tau}{\partial e_i} \cdot \frac{e_i}{\tau}$$

which is used in our treatment below.

EXPERIMENTAL SYSTEM

We have used as experimental system a reconstructed metabolic pathway of commercial enzymes from rabbit muscle that converts glucose into glycerol 3-phosphate through the glycolytic pathway. The composition of the reaction mixture is given in Table 1. Steady state was verified by observing a constant flux and ensured in each experiment by the following experimental conditions. (1) The flux produced with 5 mM-glucose ensured that glucose was not a limiting factor. (2) ATP concentration was buffered by phosphocreatine and creatine kinase. Flux of the pathway was assayed by recording NADH decay in the glycerol-3-phosphate dehydrogenase reaction. The kinetics of the commercial enzymes were studied in the same conditions as those for the system flux experiments; their activities were assayed (Bergmeyer, 1974) and found to be the same as those indicated by the manufacturer.

MATERIALS AND METHODS

Chemicals

ATP, NADH, phosphocreatine, Hepes and all the enzymes given in Table 1, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucose monohydrate and other reagents were obtained from E. Merck, Darmstadt, Germany.

Titration experiments

All titration experiments were carried out in the cuvette of a Hitachi 100-60 spectrophotometer. Fluxes and transition times were measured by recording NADH decay at 340 nm and 37 °C. The total volume of incubation mixture was 2 ml containing the final concentration of enzymes, substrates and cofactors shown in Table 1. Reactions were started by addition of hexokinase to the reaction mixture. In titration assays diluted enzymes were added to the mixture at convenient concentrations according to the experiment. Flux and transition time values were normalized with respect to the value in basal conditions (without any titrating enzyme added, only the basal activities being present). The sums of enzyme activities (basal and added) were used to obtain the corresponding e_i values.

Modulation of flux and transition time

Flux was recorded and measured in the absorbance range from 0.8 to 0.3 A , where NADH decay was always linear. Flux modulations were carried out as described elsewhere (Torres *et al.*, 1986).

The method used to assay transition time (τ) is illustrated in Fig. 1. The flux of end product X_p was assayed by means of a continuous recording of NADH concentration, giving curves like the one represented in the Figure. It is not necessary to record the complete progress of X_p concentration versus time from starting time. It is only necessary to know precisely the start of

Table 1. Composition of the experimental system

Enzyme	Activity (μmol of product of reaction/min)
Hexokinase (EC 2.7.1.2)	0.028
Phosphoglucose isomerase (EC 5.3.1.9)	0.105
Phosphofructokinase (EC 2.7.1.11)	0.030
Fructose-bisphosphate aldolase (EC 4.1.2.13)	0.133
Triose-phosphate isomerase (EC 5.3.1.1)	0.670
Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8)	0.390
Creatine kinase (EC 2.7.3.2)	1.00
Substrates, salts, cofactors etc.	Concentration (mM)
Glucose	5
ATP	0.5
NADH	0.56
Fructose 2,6-bisphosphate	0.002 or 0.01
Phosphocreatine	10
Hepes	50
K ⁺	100
Mg ²⁺	7
NH ₄ ⁺	3
EDTA	1
Dithiothreitol	2.5

the reaction, as well as the value of the flux extrapolated to the steady state in the progress curve X_p concentration versus t in order to assay τ correctly. Because of the conditions of our system *in vitro*, the fractions of pool bound to enzymes are negligible in relation to the total

pool σ ; thus transition time is given by the intercepts of the asymptote of steady-state flux with the time axis.

Once the value of τ of the system for a specific state was measured, we modulated every one of its enzyme activities by enzyme titration. From these data we constructed graphs like that shown in Fig. 3, from which $C_{e_i}^T$ values were calculated.

Determination of Control Coefficients

Flux Control Coefficients. These were determined essentially as described previously (Torres *et al.*, 1986) from normalized results of titration experiments. The hyperbolic relationship found between flux and enzyme activity is described by:

$$J_N = \frac{Q_1 \cdot e_i}{Q_2 + e_i} \quad (3)$$

where J_N is the normalized flux, e_i is the enzyme concentration of the enzyme E_i and Q_1 and Q_2 are normalized specific constants of the system that include all parameters of the system with exception of e_i . From eqns. (3) and (1), the following expression for the Flux Control Coefficients of e_i is obtained (Torres *et al.*, 1986):

$$C_{e_i}^J = \frac{Q_1 - 1}{Q_1} \quad (4)$$

Q_1 values were calculated by means of double-reciprocal plots of normalized values of flux and e_i obtained by enzyme titration.

Transition Time Control Coefficients. The enzyme-titration method was also used to determine the $C_{e_i}^T$. We fitted data of normalized transition time (τ) and e_i parameters to an exponential function such as:

$$\tau = A \cdot e^{B e_i} \quad (5)$$

Fig. 1. Progress curve of the reaction (thick curve) of a metabolic system that converts an initial substrate X_i into a final product X_p , through a chain of enzyme steps, 'empty' of intermediates at starting time ($t = 0$), and evolving asymptotically to a stable steady state

From starting time the flux of X_p production, J_p , increases reaching the steady-state value. The slope of the asymptote to the progress curve (thin line) gives us the steady-state flux (J_p)_{ss}. The intercept of the flux asymptote with the time axis gives us the transition time directly and the intercept with the ordinate axis gives the concentration of the whole pool (σ); see Easterby (1981). No recording of the first moments of the reaction (usually lost during mixture of reagents in conventional spectrophotometers) is necessary, but it is essential to know the starting time of the reaction.

Fig. 2. Flux enzyme titration of the system

The Figure shows the results of flux enzyme titration with hexokinase (●) and phosphofructokinase (▲) in the experimental conditions described in the Materials and methods section with 2 μM -fructose 2,6-bisphosphate (a) and 10 μM -fructose 2,6-bisphosphate (c). Basal flux of the metabolic pathway is expressed as 1 on the ordinate axis, and the point marked $\square$ in every curve corresponds to the flux with the enzyme present in basal conditions. Modulation with hexokinase or phosphofructokinase to the system changes significantly the flux around the basal enzyme concentration value, showing substantial values of their Flux Control Coefficients. Further modulation on any other enzymes of the system does not highly modify the flux (results not shown). The double-reciprocal plots (b) and (d) give us, as the intercepts with the ordinate, the $1/Q_1$ values, which have been used in eqn. (4).

where A and B are empirical constants of the same nature as Q_1 and Q_2 . By using the first derivative of τ with respect to e_i it is possible to obtain:

$$C_{e_i}^{\tau} = B \cdot (e_i)_{\text{basal}} \quad (6)$$

It is worth noting here that eqn. (5) is an empirical equation and not an analytical solution, which we have used to improve the determination of Transition Time Control Coefficients from experimental data. A and B parameters were determined from the linear plot of $\ln \tau_N$ versus e_i , where τ_N represents the normalized transition time.

RESULTS AND DISCUSSION

The results of enzyme titrations of the system in the experimental conditions described in Table 1 are shown in Figs. 2 and 3.

Fig. 2 shows the modulations on the flux. We show only the results of enzyme titration with hexokinase and phosphofructokinase, the only ones that have reasonably high values of Flux Control Coefficients. In Figs. 2(a)

and 2(b) plots are the results obtained when a low concentration of fructose 2,6-bisphosphate was used (2 μM), and in Figs. 2(c) and 2(d) those with saturating concentrations of this effector (10 μM).

Modulations on transition time are shown in Fig. 3, also in conditions of low and high fructose 2,6-bisphosphate concentrations. Only the enzymes with important values of Transition Time Control Coefficients are represented. In Figs. 3(a) and 3(b) the plots correspond to conditions of 2 μM -fructose 2,6-bisphosphate, and in Figs. 3(c) and 3(d) to those of 10 μM of this effector.

In Table 2 are shown the Control Coefficient profiles measured from experiments such as those given in Figs. 2 and 3. This Table also shows the agreement of the Summation Theorem of Flux Control Coefficients and strongly suggests a similar Summation Theorem for the Transition Time Control Coefficients. Finally, Table 3 shows the A and B parameter values from which Transition Time Control Coefficients were calculated.

Inspection of Flux and Transition Time Control Coefficients in Table 2 shows some interesting aspects of the

Fig. 3. Transition time enzyme titration of the system

Results for transition time obtained from modulation of the concentrations of several enzymes (●, hexokinase; □, phosphoglucose isomerase; ▲, phosphofructokinase; ■, triose-phosphate isomerase; △, aldolase) are shown in the two conditions of concentration of fructose 2,6-bisphosphate analysed: $2 \mu\text{M}$ (a) and (b), and $10 \mu\text{M}$ (c) and (d). Basal transition time is expressed as 1 on the ordinate axis, and the point marked □ in every curve corresponds to the transition time measured with the enzyme present in basal conditions. Only modulation with these enzymes yields important changes in transition time, showing substantial values of their Transition Time Coefficients (see Table 2). The linear plot of $\ln(\text{normalized transition time})$ versus enzyme activity (e_i) gives us the parameter value of B (slope), from which we can calculate the Transition Time Control Coefficient by means of eqn. (6). For graphical convenience hexokinase and phosphofructokinase enzyme concentrations are multiplied by 10 in all plots, and the aldolase enzyme concentration is multiplied by 5 in (b) and (d) for the same reason.

Table 2. Control Coefficient profiles

The experimental conditions are indicated in Table 1. (a) With $2 \mu\text{M}$ -fructose 2,6-bisphosphate: in these conditions basal flux was $0.025 \pm 0.002 \mu\text{mol}$ of final product/min and τ_{basal} was 11 ± 0.7 min. (b) With $10 \mu\text{M}$ -fructose 2,6-bisphosphate: in these conditions the basal flux was $0.047 \pm 0.0017 \mu\text{mol}$ of final product/min and the τ_{basal} was 7 ± 0.3 min. Results in the Table are means $\pm$ S.D. for three different experiments.

Enzyme	(a) With $2 \mu\text{M}$ -fructose 2,6-bisphosphate		(b) With $10 \mu\text{M}$ -fructose 2,6-bisphosphate	
	$C_{e_i}^J$	$C_{e_i}^r$	$C_{e_i}^J$	$C_{e_i}^r$
Hexokinase	$+0.20 \pm 0.03$	-0.46 ± 0.025	$+0.40 \pm 0.09$	-0.33 ± 0.1
Phosphoglucose isomerase	$+0.01 \pm 0.005$	-0.11 ± 0.09	0.0	-0.25 ± 0.045
Phosphofructokinase	$+0.65 \pm 0.1$	-0.71 ± 0.15	$+0.50 \pm 0.042$	-0.60 ± 0.085
Aldolase	$+0.05 \pm 0.003$	$+0.05 \pm 0.007$	0.0	$+0.28 \pm 0.025$
Triose-phosphate isomerase	$+0.06 \pm 0.006$	$+0.22 \pm 0.08$	0.0	-0.06 ± 0.007
Glycerol-3-phosphate dehydrogenase	$+0.02 \pm 0.001$	-0.08 ± 0.004	$+0.05 \pm 0.008$	-0.07 ± 0.009
$\sum_{i=1}^n C_{e_i}^{J,r}$	$+0.99 \pm 0.09$	-1.09 ± 0.15	$+0.95 \pm 0.15$	-1.03 ± 0.15

Table 3. Values of *A* and *B* parameters

Values of the *A* and *B* parameters of eqn. (5) are given. Only enzymes having high values of these parameters are shown. Transition Time Control Coefficients were calculated by using eqn. (6), which was derived from eqn. (5), where *A* and *B* are constant parameters. As stated in the text, this equation was proposed in order to improve the estimation of the coefficients from experimental data. Results in the Table are means $\pm$ s.d. for three different experiments.

Enzyme	(a) With 2 μ M-fructose 2,6-bisphosphate			(b) With 10 μ M-fructose 2,6-bisphosphate		
	C_{ei}^*	<i>A</i>	<i>B</i>	C_{ei}^*	<i>A</i>	<i>B</i>
Hexokinase	-0.46 ± 0.025	1.58 ± 0.3	-8.21 ± 0.4	-0.33 ± 0.1	1.39 ± 0.5	-5.89 ± 0.25
Phosphoglucose isomerase	-0.11 ± 0.09	1.11 ± 0.25	-0.52 ± 0.1	-0.25 ± 0.045	1.29 ± 0.33	-4.16 ± 0.66
Phosphofructokinase	-0.71 ± 0.15	2.03 ± 0.7	-11.83 ± 0.9	-0.6 ± 0.085	1.82 ± 0.45	-2.85 ± 0.37
Aldolase	—	—	—	$+0.28 \pm 0.025$	0.75 ± 0.22	-0.28 ± 0.05
Triose-phosphate isomerase	$+0.22 \pm 0.08$	0.8 ± 0.2	-0.22 ± 0.08	—	—	—

values of these coefficients. The first one that we wish to note refers to how an external effector of the pathway (namely fructose 2,6-bisphosphate acting on phosphofructokinase) affects the distribution of Flux and Transition Time Control Coefficients in different ways. The distribution of Flux Control Coefficients shows important changes in the case of phosphofructokinase and hexokinase, the enzymes that share the major part of control. It can also be seen that the effects on the flux can be quite different from the effects on the transition time for the modulation of the same enzyme with different effector concentrations. All this set of results shows a different behaviour in both kinds of control (flux and time), these being different aspects of the whole response of a metabolic system.

The Transition Time Control Coefficient profile (see Table 2) shows important changes in almost all the coefficients. (a) Phosphoglucose isomerase and aldolase increase the absolute value of their Transition Time Control Coefficients. But, whereas phosphoglucose isomerase always has a negative influence on the transition time of the system, aldolase, which has a significant control on neither flux nor time at the low concentration of fructose 2,6-bisphosphate, and still does not control flux at the saturating concentration of that effector, shows, however, a positive Transition Time Control Coefficient ($+0.28$) in these latter conditions. It is worth noting here that this behaviour of aldolase in controlling flux and time is not entirely new. Results obtained in our laboratory with a rat muscle glycolytic system (Riol-Cimas & Meléndez-Hevia, 1988) have shown that an increase in aldolase activity increases also the time response of the system. (b) Hexokinase, phosphofructokinase and triose-phosphate isomerase decline in time control with a high concentration of effector, the last enzyme showing the biggest decline in absolute values. Moreover, this enzyme changes its Transition Time Control Coefficient values from positive to negative. It means that at low concentrations of fructose 2,6-bisphosphate an increase in the concentration of this enzyme enhances the transition time of the system, but the opposite happens at saturating concentrations of the effector.

The second aspect refers to the satisfaction of the C_{ei}^* Summation Theorem above considered. Because the system used *in vitro* in our experiments is a 'closed' system, with no diverting branches or feedforward inhibition or substrate inhibition, none of the Flux Control Coefficients will be negative (Kacser, 1983). Thus all the Flux Control Coefficients must be positive (or zero) and their sum must add up to 1 (Kacser & Burns, 1973; Heinrich & Rapoport, 1974). On the other hand, although the Transition Time Control Coefficients have positive and negative values, their sum tends to -1 , suggesting the existence of an equivalent Summation Theorem for Transition Time Control Coefficients. However, it is worth noting here that this suggested relationship is different from that presented by Heinrich & Rapoport (1975) (eqn. 2) because of the different definition of the Transition Time Control Coefficients. In the case of those authors the dependent variable considered, τ_{s_j} , is the individual transition time, associated with a given free intermediate S_j , whereas in the present paper τ is the total transition time related to the total mass of free intermediate pools, $\sigma = \sum s_j$.

This aspect, together with the above-mentioned rela-

tive independence of the two kinds of control, fluxes and time, is not discussed in the present paper.

Finally, the results on the effects of an external stimulator of glycolysis on the Control Coefficient profiles of the pathway suggest that other effectors of the pathway (i.e. P_i , citrate or ADP) could also provoke bigger changes on the transition time response than on the flux response. Presumably the evolutionary selection of these effectors has occurred, not only on the basis of their action on the flux, but also on the other responses of the system, such as the transition time, relevant to the metabolic role of the tissue. We consider that further investigation of these aspects will be an important area in the future research in this field.

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