Eur. J. Biochem. *158*, 107–115 (1986)

Theoretical analysis of the flux control properties of a substrate cycle

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(Received December 23, 1985/March 2, 1986) – EJB 85 1395

Substrate cycles are able to increase the flux control of a non-equilibrium reaction in a wide range of situations related to the effects of the metabolites involved in the cycle on the reaction producing them. No limit exists for that amplification if appropriate conditions are attained if those effects are positive. In all cases, the ratio between the rate of the reverse reaction and the net flux through the pathway plays an important role in defining the final amplification.

The theory of metabolic control developed by Heinrich and Rapoport [1, 2] and by Kacser and Burns [3] has greatly facilitated the quantitative determination of the control exerted by an enzyme on the flux of a metabolic pathway. Their work has been extended [4–8] and the existing theorems, relating the properties of each step in a metabolic pathway and its role in metabolic regulation, allow us to discuss the flux control distribution among the enzymes of the system. So, by using these theorems, it is possible to discuss theoretically the necessary conditions in the pathway reactions to obtain a determined distribution of the flux control. If this sense, the main purpose of this work is to apply these theorems to the analysis of the control properties of a substrate cycle. The role of such cycles in metabolic regulation has been discussed for a long time although the necessary conditions leading to high flux control for the cycle enzymes has been only partially studied [9–13].

METHODOLOGY

Computation of the flux control coefficients

The flux control coefficients of the enzymes involved in the substrate cycle of Fig. 1 are computed by using the matrix method suggested by Fell and Sauro [6] as functions of the elasticities and rates of the reactions involved in the pathway. Previously published results on the flux control properties of substrate cycles have considered that the ratio between the recycling reaction and the pathway flux (recycling ratio) is an important factor in defining the final flux control [6, 9, 10, 12]. Accordingly, we have computed flux control coefficients as a function of that ratio instead of as a function of absolute rates.

It is important to point out that we do not consider the alteration of flux control as a result of a change in the steady state of the system, since that change varies the set of elasticities of the reactions of the considered pathway. We only take into account the resultant flux control distribution computed by fixing the elasticities for different values of the recycling ratio (α).

Amplification ratio

The influence of the substrate cycle structure on the flux control coefficient of a non-equilibrium reaction (reaction catalyzed by E_2 in Fig. 1) can be quantified by the ratio of the flux control coefficients of E_2 obtained with or without a recycling reaction [6]. The amplification ratio is defined as:

$$\frac{C^J_{2C}}{C^J_{2L}} = \frac{1}{1 - \dfrac{\alpha}{(1+\alpha)} \cdot \dfrac{1 - (\varepsilon^4_P/\varepsilon^3_P) - (\varepsilon^4_S/\varepsilon^1_S)}{1 - (\varepsilon^2_P/\varepsilon^3_P) - (\varepsilon^2_S/\varepsilon^1_S)}} \qquad (1)$$

where C^J_{2C} and C^J_{2L} are the flux control coefficients of E_2 in both situations.

To discuss theoretically the possibility of amplification, Eqn (1) can be rewrittren as:

$$\frac{C^J_{2C}}{C^J_{2L}} = \frac{1}{1 - \dfrac{\alpha}{(1+\alpha)} \cdot K} \qquad (2)$$

where K replaces the ratio of elasticities. The flux control of E_2 is amplified when this ratio is greater than unity.

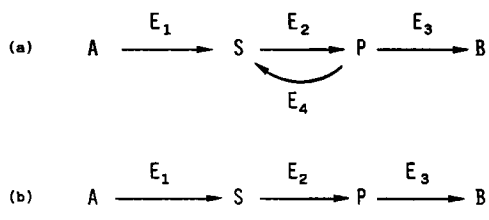

Fig. 1. *Substrate cycle structure.* (a) Substrate cycle: E_i (i = 1, ..., 4) are the enzymes involved in the cycle. S and P are the metabolites of the substrate cycle, while A is a source metabolite and B a sink metabolite. (b) Linear pathway

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Symbols. E_i (i = 1,...) are the enzymes; ε^i_S and ε^i_P are the elasticities of each step with respect to metabolites S and P; C^J_i is the flux control coefficient of enzyme E_i, v_i is the rate of enzyme E_i.

THEORETICAL RESULTS

THE SUBSTRATE CYCLE AS A MECHANISM TO INCREASE THE FLUX CONTROL OF A NON-EQUILIBRIUM REACTION

Theoretical analysis of the amplification ratio: maximum amplification ratio

Amplification can exist as a result of two factors: a set of elasticity coefficents depending on the 'local' properties of the reactions involved in the cycle (K in Eqn 2) and a recycling ratio (α) relating the rate of the recycling reaction and the net flux. Considering that, in the linear pathway of Fig. 1, net flux goes from left to right due to the fact that E_2 catalyzes a non-equilibrium reaction, to evaluate the influence of v_4 on the control properties of E_2 we will also consider a left to right net flux in the substrate cycle, that is $v_2 > v_4$ ($\alpha > 0$).

The actual value of K defines an equation that allows us to compute the resultant amplification as a function of the recycling ratio (Eqn 2). The qualitative behaviour of that function varies according to whether the value of K is over or below unity, as shown in Fig. 2. If K is greater than unity, Eqn (2) has a discontinuity at $\alpha = 1/(K-1)$ giving high amplification when the recycling ratio approaches that boundary from the left [$\alpha < 1/(K-1)$]. When α reaches higher values exceeding that limit, the amplification becomes negative due to the fact that coefficient C^J_{2C} has a negative value in this situation. On the other hand, if K is positive and less than unity, amplification increases with α and tends to a maximum value defined by the mathematical limit of Eqn (2) when α tends to infinity:

$$\lim_{\alpha \to \infty} \frac{1}{1 - \frac{\alpha}{(1+\alpha)} \cdot K} = \frac{1}{(1-K)} \qquad 0 < K < 1 . \qquad (3)$$

In the special case in which $K = 1$, the amplification ratio is equal to $(1 + \alpha)$. If $K < 0$ no amplification is possible.

There is no limit for the amplification that can result in cycles having $K > 1$ if they have the appropriate conditions, although the range of recycling values compatible with that effect can be very reduced for high values of K. Cycles having $0 < K < 1$ will not exceed the amplification ratio corresponding to $K = 1$ what ever the value of α.

Thus, qualitatively, we can consider two kinds of substrate cycles: those which provide an unlimited amplification of the forward flux control coefficient and those for which the effect on this coefficient is limited to $(1 + \alpha)$.

To analyze the amplification, it is important to point out that K results from a combination of the elasticities of the system reactions to S and P. Thus, according to the meaning of K, the same value can result from different sets of elasticities having different metabolic significance. Therefore, an identical amplification ratio can be obtained in cycles with different properties if their elasticities lead to the same value of K.

Optimal conditions to obtain high amplification

From Eqn (1) it is possible to derive the necessary conditions to attain a positive value of K depending on the properties of the reactions involved in the pathways of Fig. 1. Elasticities are determined by the kinetic features of the reactions and can be related to the distance to equilibrium and to substrate saturation of the reaction [14]. Generally, the elasticities of a reaction will be positive to its substrates and negative to its products, except when we consider special

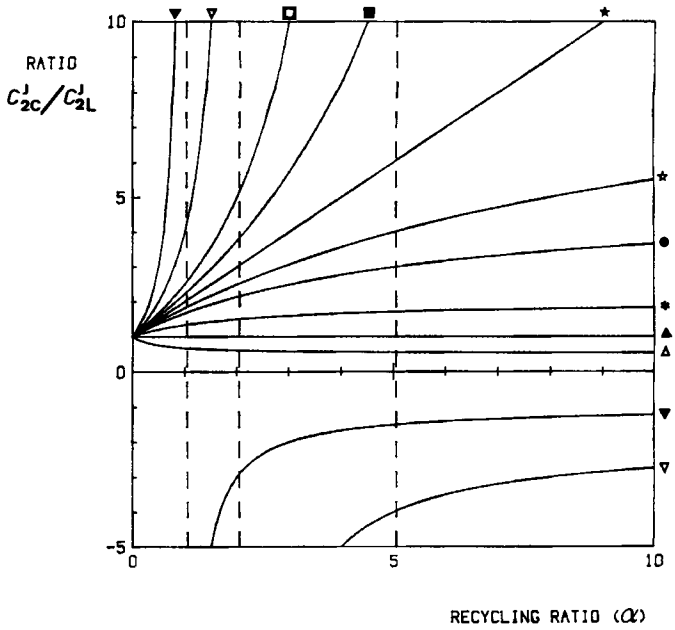

Fig. 2. *The amplification ratio as a function of* K *and* α. We have computed the resultant amplification ratio by using Eqn (6) as a function of the recycling ratio (α) for different values of coefficient K. The dashed lines indicate the limiting value of α compatible with a positive amplification ratio when $K > 1$. Values of K: (△) −1; (▲) 0; (*) 0.5; (•) 0.8; (☆) 0.9; (★) 1; (■) 1.1; (□) 1.2; (▽) 1.5; (▼) 2

situations such as substrate inhibition or product activation. Thus, normally, ε^1_S will be negative and ε^2_S, ε^3_P and ε^4_P will be positive while the sign of ε^4_S and ε^2_P will depend on the kinetics features we consider. According to this, we can write the following equation for K:

$$K = \frac{1 + |\varepsilon^4_P/\varepsilon^3_P| - \varepsilon^4_S/\varepsilon^1_S}{1 - |\varepsilon^2_S/\varepsilon^1_S| - \varepsilon^2_P/\varepsilon^3_P} \qquad (4)$$

from which the necessary conditions to obtain amplification ($K > 0$) can be discussed.

To define these conditions we will consider that, in the linear pathway, $C^J_{2L} > 0$, that is:

$$1 > \varepsilon^2_P/\varepsilon^3_P - |\varepsilon^2_S/\varepsilon^1_S| . \qquad (5)$$

With that restriction, the conditions to reach amplification are:

$$\begin{aligned} &1 > |\varepsilon^4_P/\varepsilon^3_P| + |\varepsilon^4_S/\varepsilon^1_S| \quad \text{if} \quad \varepsilon^4_S \le 0 \\ &1 > |\varepsilon^4_P/\varepsilon^3_P| - |\varepsilon^4_S/\varepsilon^1_S| \quad \text{if} \quad \varepsilon^4_S > 0 . \end{aligned} \qquad (6)$$

The necessary conditions to attain the high amplification ratios related to values of $K > 0$ are more restricted and can not be attained in the absence of positive activation of the cycle reactions by their products (Table 1). When these effects are present, they result in a wide range of possibilities leading to high amplification related to the effects of the concentrations of S and P (S and P) on the enzymes involved in the cycle.

Amplification related to the internal effects of S *and* P

In the absence of positive activation of the cycle reactions, amplification of the flux control coefficient of the forward reaction is limited to $(1 + \alpha)$. The necessary conditions to

attain that maximum have been extensively discussed and appear to be related to the saturation of the cycle reactions [6, 9, 10, 12] (case A in Table. 1).

Theoretical analysis of Eqn (1) indicates that the amplification can exceed the boundary of $(1 + \alpha)$ if the metabolites involved in the substrate cycle have a positive effect on the reactions producing them. Although such positive effects are not a common feature in enzyme reactions, they have been described in some substrate cycles [15–17]; thus it is of interest to analyze the resultant amplification in the other three cases defined in Table 1. These cases have not been considered in previous analysis of substrate cycles and will now be studied here in order to analyze the optimal conditions leading to a high amplification effect. A theoretical example will be discussed for each case.

Table. 1. *Necessary conditions for exceeding an amplification ratio of* $(1 + \alpha)$

Case	Sign of		Necessary conditions
	ε_S^4	ε_P^2	
A	−	−	impossible
B	−	+	$1 > \lvert\varepsilon_P^2/\varepsilon_P^3\rvert - \lvert\varepsilon_S^2/\varepsilon_S^1\rvert > \lvert\varepsilon_P^4/\varepsilon_P^3\rvert + \lvert\varepsilon_S^4/\varepsilon_S^1\rvert$
C	+	−	$\lvert\varepsilon_P^4/\varepsilon_P^3\rvert + \lvert\varepsilon_S^2/\varepsilon_S^1\rvert + \lvert\varepsilon_P^2/\varepsilon_P^3\rvert < \lvert\varepsilon_S^4/\varepsilon_S^1\rvert$
D	+	+	$1 > \lvert\varepsilon_P^2/\varepsilon_P^3\rvert - \lvert\varepsilon_S^2/\varepsilon_S^1\rvert > \lvert\varepsilon_P^4/\varepsilon_P^3\rvert - \lvert\varepsilon_S^4/\varepsilon_S^1\rvert$

Case (B) $\varepsilon_S^4 \leq 0; \varepsilon_P^2 > 0$

In this case, amplification is limited to cycles in which the elasticity to P is greater for E_3 than for E_4 and in which the elasticity of E_4 to S does not surpass that corresponding to E_1 for the same metabolite. In this sense, substrate saturation of the reverse reaction favours the amplification but it is not a necessary condition if its elasticity to P is weaker than the elasticity of E_3 to the same metabolite.

The positive effect of P on E_2 allows the boundary of $(1 + \alpha)$ to be exceeded in the amplification ratio. In that case, the critical parameter in achieving high amplification is the difference between $|\varepsilon_P^2/\varepsilon_P^3|$ and $|\varepsilon_S^2/\varepsilon_S^1|$.

Although the saturation of the forward reaction permits a wide range of elasticity sets compatible with amplification, it is not a necessary condition to achieving the effect if the conditions of Table 1 B, are fulfilled.

To examine some of these possibilities, we have computed the resultant amplification ratio with the conditions shown in Fig. 3. Amplification over $(1 + \alpha)$ occurs as a result of the activation of forward reaction by its product and, in the chosen conditions, it is related to the lowest values of $|\varepsilon_S^2/\varepsilon_S^1|$ and $|\varepsilon_S^4/\varepsilon_S^1|$ considered in the example.

In Fig. 3d, no amplification is possible because the value of $|\varepsilon_S^4/\varepsilon_S^1|$ surpasses the maximum value compatible in Eqn (6)

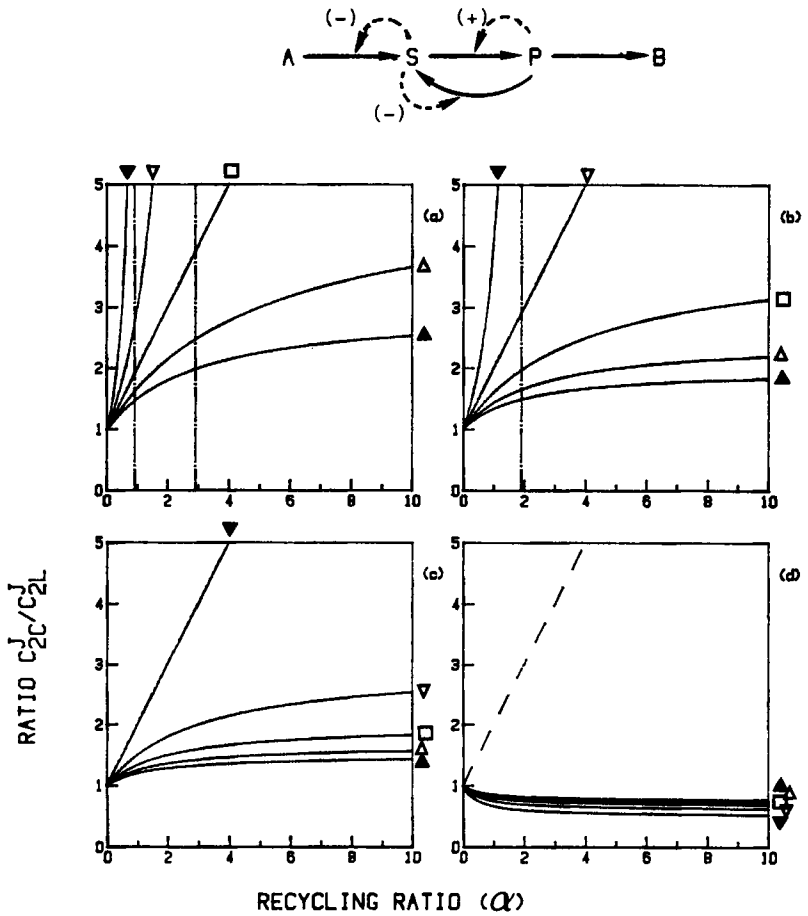

Fig. 3. *Amplification with* $\varepsilon_S^4 \leq 0$ *and* $\varepsilon_P^2 > 0$. The amplification ratio was computed with: $\varepsilon_P^2/\varepsilon_P^3 = 1.2$; $\varepsilon_P^4/\varepsilon_P^3 = 0.5$. Variation of $\varepsilon_S^4/\varepsilon_S^1$: (a) 0.1; (b) 0.2; (c) 0.3; (d) 0.7. Variation of $\varepsilon_S^2/\varepsilon_S^1$: (▼) −0.4; (▽) −0.5; (□) −0.6; (△) −0.7; (▲) −0.8. The dashed line corresponds to an amplification ratio equal to $(1 + \alpha)$. Vertical lines mark the discontinuity in the amplification ratio when $\alpha = 1/(K - 1)$ and $K > 1$

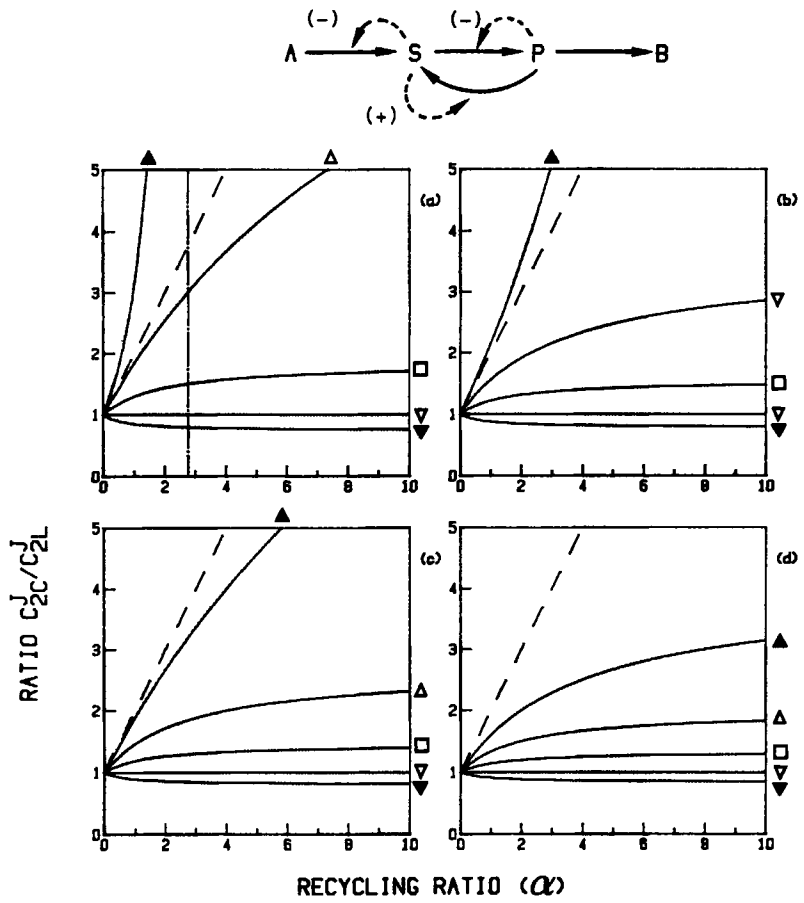

Fig. 4. *Amplification with* $\varepsilon^4_S > 0$ *and* $\varepsilon^2_P \leq 0$ *(Case C).* The amplification ratio was computed with: $\varepsilon^2_P/\varepsilon^3_P = 0$; $\varepsilon^4_P/\varepsilon^3_P = 1.5$. Variation of $\varepsilon^2_S/\varepsilon^1_S$: (a) −0.1; (b) −0.4; (c) −0.6; (d) −1.0. Variation of $\varepsilon^4_S/\varepsilon^1_S$: (▼) −0.1; (▽) −0.5; (□) −1.0; (△) −1.5; (▲) −2.0. The dashed and vertical lines have the same meaning as in Fig. 3

with a value of $|\varepsilon^4_P/\varepsilon^3_P| = 0.5$. The amplification ratio becomes negative when the recycling ratio surpasses the value $1/(K-1)$ (vertical lines in Fig. 3) as a consequence of the resultant negative value of C^J_{2C}. Thus, high positive amplification is restricted to a range of values of the recycling ratio below this boundary.

Case (C) $\varepsilon^4_S > 0; \varepsilon^2_P \leq 0$

The positive effect of S on E_4 permits the amplification when the elasticity to P is greater for E_4 than for E_3 (i. e. non-saturated reverse reaction). As in the preceding case, amplification can occur over the limit $(1 + \alpha)$ even if neither the forward nor the reverse reaction of the cycle are saturated by their substrates.

According to the condition shown in Table 1 C, the critical parameter to obtain high amplification is $|\varepsilon^4_S/\varepsilon^1_S|$. Depending on the value of the other elasticity ratios defining the system, high amplification can be reached if there is a sufficient activation of E_4 by its product. In some circumstances, i.e. if cycle reactions are substrate-saturated and the negative effect of P on E_2 is low, high amplification can be attained with a low positive effect of S on E_4.

As an example, we have computed the amplification ratio in a cycle having a non-saturated reverse reaction activated by its product (Fig. 4.). This activation favours the possibility of attaining amplification over $(1 + \alpha)$ according to the conditions of Table 1C. That effect, in the conditions of Fig. 4, is related to low values of $|\varepsilon^2_S/\varepsilon^1_S|$, but could be reached with higher values of that ratio if $|\varepsilon^4_S/\varepsilon^1_S|$ increases. No amplification occurs when the elasticities do not fulfil Eqn (6) (▼ in Fig. 4).

Case (D) $\varepsilon^4_S > 0; \varepsilon^2_P > 0$

In this case, if the amplification condition expressed in Eqn (6) is fulfilled, the critical parameter to attain high amplification is $|\varepsilon^2_P/\varepsilon^3_P| - |\varepsilon^2_S/\varepsilon^1_S|$. No substrate saturation of the cycle reactions are required to achieve high amplification but if both reactions are saturated by their substrates the resultant amplification is always greater than $(1 + \alpha)$ whenever the ratio $|\varepsilon^2_P/\varepsilon^3_P|$ is less than unity.

As an example of the amplification provided by the simultaneous activation of E_2 and E_4 by their products, we show in Fig. 5 the computed amplification ratio in a substrate cycle where these effects are present. Amplification increases when $|\varepsilon^4_S/\varepsilon^1_S|$ and $|\varepsilon^2_S/\varepsilon^1_S|$ increase and exceed the limited of $(1 + \alpha)$ for the highest values of these ratios considered in the example. According to Eqn (6), no amplification occurs if $|\varepsilon^4_S/\varepsilon^1_S|$ is less than 0.5 due to the value of $|\varepsilon^4_P/\varepsilon^3_P|$ (Fig. 5a).

DISTRIBUTION OF THE FLUX CONTROL AMONG THE ENZYMES OF THE CYCLE

The amplifiation defined in Eqn (1) quantifies the effect of the recycling reaction on the flux control of the forward

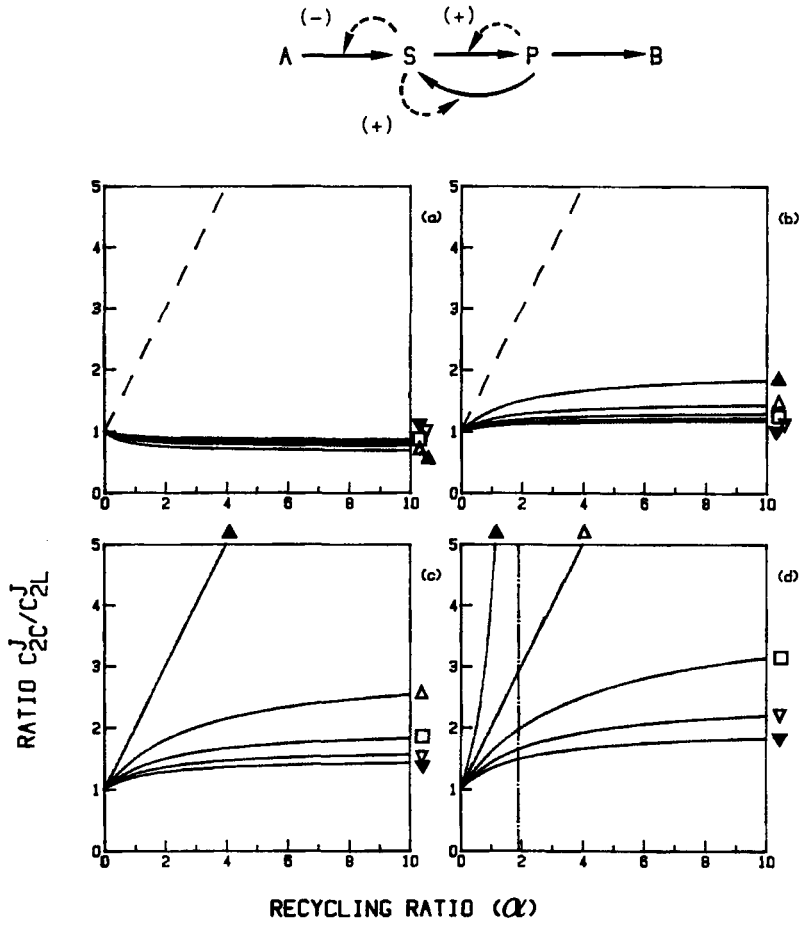

Fig. 5. *Amplification with* $\varepsilon^4_S > 0$ *and* $\varepsilon^2_P > 0$ *(Case D).* The amplification ratio was computed with: $\varepsilon^2_P/\varepsilon^3_P = 1.2$; $\varepsilon^4_P/\varepsilon^3_P = 1.5$. Variation of $\varepsilon^4_S/\varepsilon^1_S$: (a) −0.5; (b) −0.6; (c) −0.7; (d) −0.8. Variation of $\varepsilon^2_S/\varepsilon^1_S$: (▼) −0.4; (▽) −0.5; (□) −0.6; (△) −0.7; (▲) −0.8. The dashed and vertical lines have the same meaning as in Fig. 3

reaction but does not quantify the resultant modifications on the other enzymes of the system. So, the different conditions leading to amplificaton can result in a different pattern of flux control distribution that must be evaluated in order to discuss its metabolic significance.

As an example of this situation, we have computed the distribution of the flux control among the four enymes involved in the substrate cycle of Fig. 1a in the situations defined by the signs of ε^4_S and ε^2_P (Table 1). In each case, we have chosen a set of elasticities leading to $K = 1$, thus, an amplification ratio equal to $(1 + \alpha)$ (Fig. 6).

As a general result, the introduction of a recycling reaction increases all the flux control coefficients by means of the effect of the recycling ratio value on them. Although the relative effect of the cycle on the flux control coefficient of E_2 is the same in all cases, the distribution of the flux control among the four enzymes is rather different as a result of the particular set of elasticities leading to $K = 1$ in each situation. So, enzymes E_1 and E_3 can play quite a different role in the flux control distribution due to the resultant structure in each case. For instance, to reach an amplification of $(1 + \alpha)$ when ε^4_S and ε^2_P are not positive, involves zero values for all the elasticity ratios appearing in Eqn (1). As a result, E_1 and E_3 have flux control coefficients equal to zero while $C^J_2 = (1 + \alpha)$ and $C^J_4 = -\alpha$. In the other cases, C^J_1 and C^J_3 are different from zero and can attain high values depending on the recycling ratio. In some situations, their values can be greater than the ones corresponding to C^J_2 and C^J_4, resulting in a higher amplification of the flux control of the first and third enzymes with regard to the initial condition ($\alpha = 0$) (Fig. 6c).

Thus, the forward and reverse reactions are not necessarily dominant in the final pattern of flux control distribution resulting from the existence of a recycling reaction opposing a non-equilibrium reaction. In fact, the necessary conditions for having low values for the control coefficients of E_1 and E_3 appear to be very restricted and limited to cases A and D. Therefore, in the resultant flux control distribution the four enzymes could play a significant role that must be analyzed in each case.

On the other hand, cycles having a value of K above unity have a discontinuity at $\alpha = 1/(K - 1)$ for the amplification ratio (Fig. 2). Such a discontinuity appears also in the flux control coefficients in such a way that the distribution of the flux control changes radically from one side to another of this boundary (results not shown). Over that boundary the flux control coefficient of E_2 becomes negative while the corresponding coefficient of E_4 becomes positive. So, the recycling ratio becomes a critical parameter in defining the final flux control distribution. Such situations could be involved in the change of the sense of the net flux as a response to external effectors, although it would be necessary to study their dynamic properties in order to evaluate such a capacity.

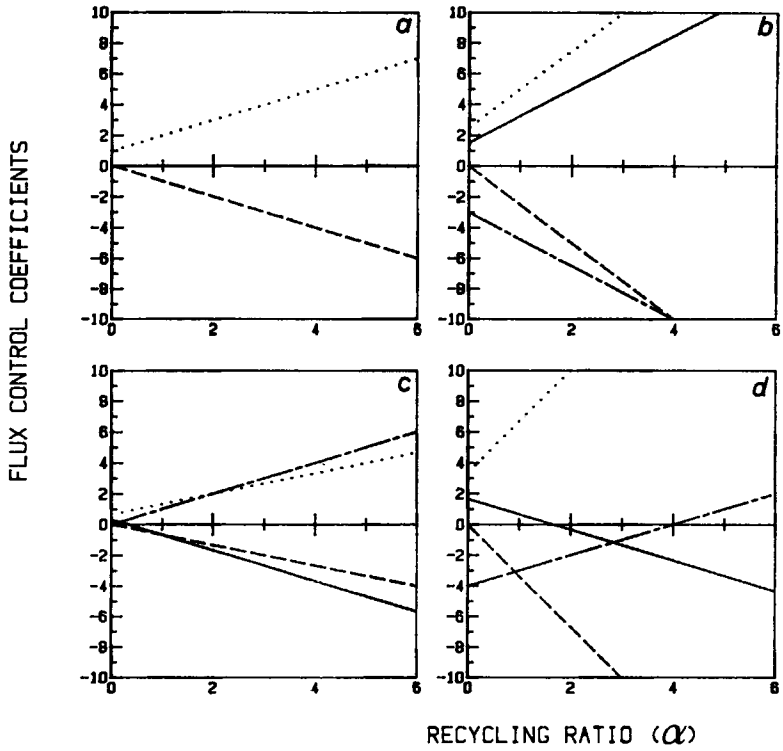

Fig. 6. *Distribution of the flux control among the enzymes of a substrate cycle.* The flux control distribution among the enzymes of the substrate cycle has been computed using the analytical expressions obtained by applying the control theorems (Eqn 3). (a) Case A: $\varepsilon_S^2/\varepsilon_S^1 = 0$; $\varepsilon_S^4/\varepsilon_S^1 = 0$; $\varepsilon_P^2/\varepsilon_P^3 = 0$; $\varepsilon_P^4/\varepsilon_P^3 = 0$. (b) Case B: $\varepsilon_S^2/\varepsilon_S^1 = -0.6$; $\varepsilon_S^4/\varepsilon_S^1 = 0.1$; $\varepsilon_P^2/\varepsilon_P^3 = 1.2$; $\varepsilon_P^4/\varepsilon_P^3 = 0.5$. (c) Case C: $\varepsilon_S^2/\varepsilon_S^1 = -0.5$; $\varepsilon_S^4/\varepsilon_S^1 = -2.0$; $\varepsilon_P^2/\varepsilon_P^3 = 0$; $\varepsilon_P^4/\varepsilon_P^3 = 1.5$. (d) Case D: $\varepsilon_S^2/\varepsilon_S^1 = -0.5$; $\varepsilon_S^4/\varepsilon_S^1 = -0.8$; $\varepsilon_P^2/\varepsilon_P^3 = 1.2$; $\varepsilon_P^4/\varepsilon_P^3 = 1.5$. (——) E_1; (·····) E_2; (–·–·–) E_3; (– – – –) E_4

DEPENDENCE OF THE FLUX CONTROL DISTRIBUTION ON THE STRUCTURE OF THE PATHWAY

There are many optimum possibilities, depending on the kinetic features we consider, leading to high amplification and to a high resultant flux control coefficient for the forward reaction. Nevertheless, the necessary conditions resulting from our theoretical analysis could be insufficient if we take into account the structure of the pathway where the cycle is included. Just as when we discussed the role of an enzyme in flux control, it is not possible to determine whether certain isolated properties will determine a major role in flux control without considering the structure and the metabolic state of the pathway where the cycle is included.

As an example of this problem, we have computed the resultant flux control distribution in a theoretical pathway having two substrate cycles connected by two reactions (those catalyzed by E_3 and E_5 in Fig. 7). Due to the complexity of that system, it is very difficult to derive the analytical expressions relating the elasticities to the flux control coefficients, although it is possible to compute them numerically by solving the set of equations defined by the control theorems. The matrix method suggested by Fell and Sauro [6] is easy and fast to apply by using a computer.

Following this method, we have calculated flux control coefficients by considering the same conditions and by increasing, simultaneously and by the same value, the recycling ratio in both cycles. The resultant distribution is very different depending on the value of ratio $\varepsilon_B^3/\varepsilon_B^5$ and, according to the results of the metabolic control theory, the first cycle has a major role when that ratio has a low absolute value. Control moves to the second cycle when that ratio increases. In both cases, the flux control distribution is different from the resultant pattern when we consider each cycle alone.

The resultant flux control distribution is therefore strongly dependent on the pathway structure in such a way that the derived properties of the cycle, when studied alone, can be opposed by the pathway structure giving a final distribution in which the role of the cycle enzymes in control has changed.

So, the control properties discussed below must be considered as 'optimal' properties that must be evaluated in each case by taking into account the whole structure of the system. The observed importance of certain substrate-cycle enzymes in integrating several regulatory signals suggest the possible existence of some optimal design in the properties of the pathway to concentrate the flux control on substrate cycles. A more detailed study is necessary to define these optimal conditions.

AMPLIFICATION AND STABILITY

The proof of the control theorems has been limited to systems that fulfil a number of conditions [5]: metabolite concentrations must be spatially homogeneous, reaction rates must be proportional to the total concentration of the enzyme and the system must be in an unique and assymptotically stable steady state completely determined by the values of the internal and external parameters (metabolite concentrations, effector concentrations, etc.). Thus, in order to analyze the control properties of a metabolic pathway by using these theorems, it is necessary to be sure that the system under analysis fulfils that set of conditions.

In this sense, a critical point in considering the ability of a substrate cycle in amplifying the flux control of a non-

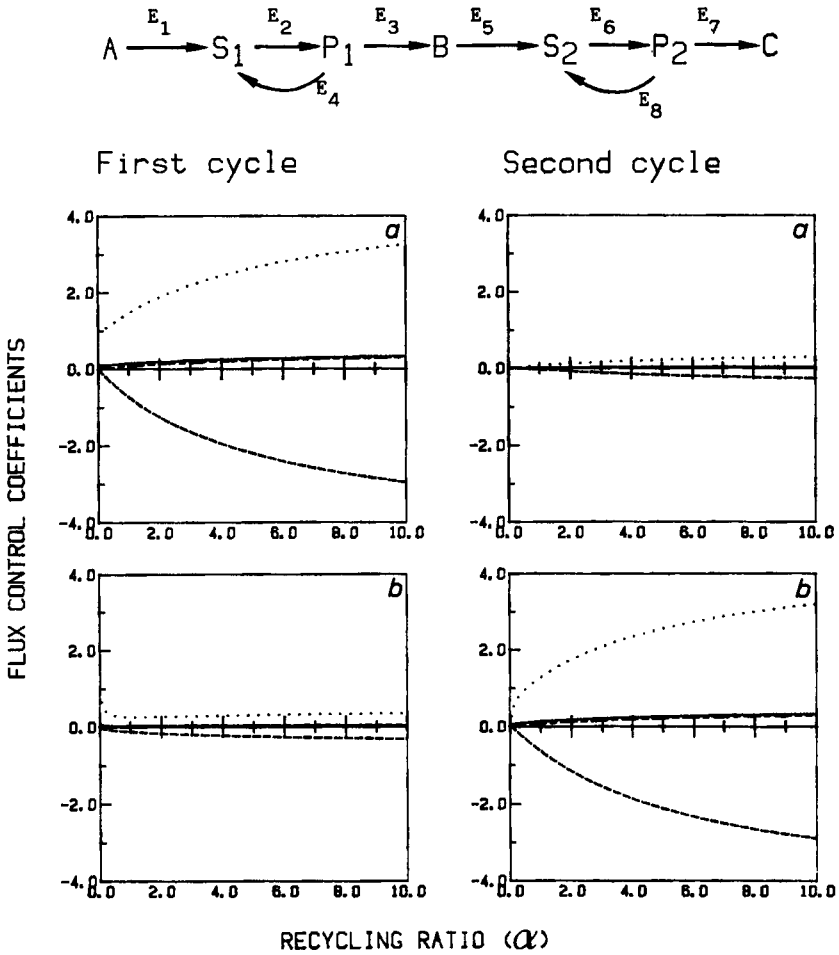

Fig. 7. *Flux control distribution in a pathway with two substrate cycles.* The flux control distribution of this system has been computed numerically with the following set of elasticities: $\varepsilon^3_B/\varepsilon^5_B$: (a) -0.1; (b) -10. $\varepsilon^2_{S_1}/\varepsilon^1_{S_1} = \varepsilon^6_{S_2}/\varepsilon^5_{S_2} = -0.1$; $\varepsilon^4_{P_1}/\varepsilon^3_{P_1} = \varepsilon^8_{P_2}/\varepsilon^7_{P_2} = 0.1$; $\varepsilon^2_{P_1}/\varepsilon^3_{P_1} = \varepsilon^6_{P_2}/\varepsilon^7_{P_2} = 0$; $\varepsilon^4_{S_1}/\varepsilon^1_{S_1} = \varepsilon^8_{S_2}/\varepsilon^5_{S_2} = 0$. (———) E_1 (first cycle), E_5 (second cycle); (·····) E_2 (first cycle), E_6 (second cycle); (–·–·–) E_3 (first cycle), E_7 (second cycle); (– – – –) E_4 (first cycle), E_8 (second cycle)

equilibrium reaction is to show the stability of the system under the special conditions leading a high amplification ratio.

The dynamic behaviour of the substrate cycle of Fig. 1 is defined by a system of two differential equations:

$$\begin{cases} \dfrac{dS}{dt} = v_1 - v_2 + v_4 = f(S,P) \\ \dfrac{dP}{dt} = v_2 - v_3 + v_4 = g(S,P) \end{cases} \tag{7}$$

whose steady-state condition is:

$$\begin{aligned} f(S,P) &= 0 \\ g(S,P) &= 0. \end{aligned} \tag{8}$$

The stability analysis of the steady state can be performed by linearizing Eqn (7) around the steady state (represented by σ) using a Taylor series and neglecting the non-linear terms [18, 19] resulting in the variational system:

$$\begin{cases} \dfrac{dx_1}{dt} = \left[\dfrac{\delta f}{\delta S}\right]_\sigma \cdot x_1 + \left[\dfrac{\delta f}{\delta P}\right]_\sigma \cdot x_2 \\ \dfrac{dx_2}{dt} = \left[\dfrac{\delta g}{\delta S}\right]_\sigma \cdot x_1 + \left[\dfrac{\delta g}{\delta P}\right]_\sigma \cdot x_2 \end{cases} \tag{9}$$

where x_1 ($i = 1, 2$) are the variation of S and P with respect to their steady-state values (S_σ, P_σ).

Using matrix notation, the variational equations can be rewritten as:

$$\dot{x} = Jc \cdot x. \tag{10}$$

The stability of the steady state of the substrate cycle can be studied by analyzing the stability properties of the linearized system [18, 19] that depend on the value of the trace (tr) and the determinant (det) of matrix Jc. So, if tr $(Jc) < 0$ and det $(Jc) > 0$, the system is stable. Otherwise, the system is unstable except when tr $(Jc) = 0$ and det $(Jc) > 0$ which corresponds to a neutrally stable steady state resulting in an oscillatory behaviour around the steady-state values.

Derivatives of functions $f(S,P)$ and $g(S,P)$ at steady state (S_σ, P_σ) can be evaluated as a function of the elasticity coefficients by using the results of Eqn (11):

$$\begin{aligned} \frac{\delta v_i}{\delta S} &= \varepsilon^i_S \cdot \frac{v_{i\sigma}}{S_\sigma} \\ \frac{\delta v_i}{\delta P} &= \varepsilon^i_P \cdot \frac{v_{i\sigma}}{P_\sigma} \end{aligned} \qquad i = 1, 2, 3, 4 \tag{11}$$

where $v_{i\sigma}$ ($i = 1, \ldots, 4$) is the steady-state rate of each reaction.

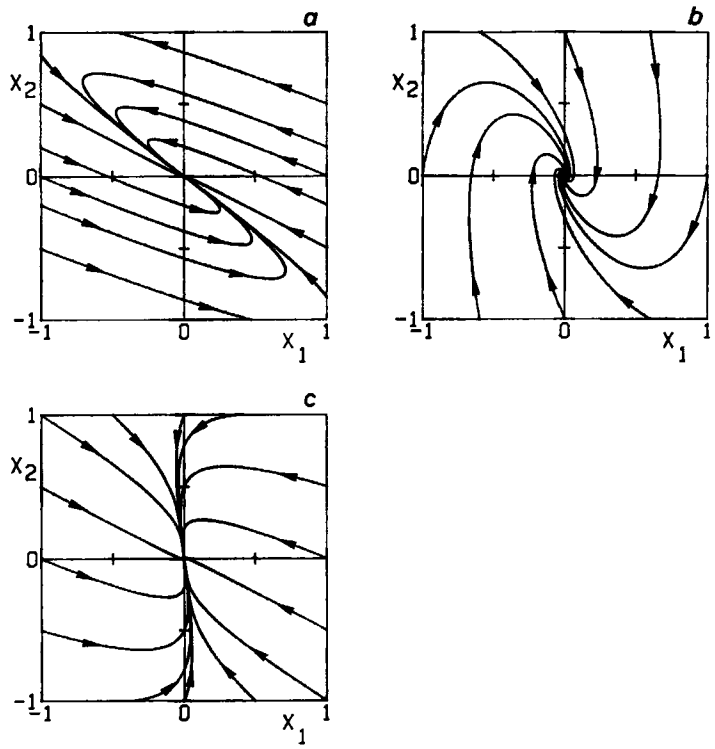

Fig. 8. *Phase plane analysis of the steady state of a substrate cycle.* Elasticities are chosen, in each case, to be compatible with high amplification of the flux control coefficient of the forward reaction. X_1 and X_2 are the deviations of the steady-state values of the concentrations of S and P respectively. (a) Case B (Fig. 3a, ▽) $\varepsilon_S^1 = -0.2$; $\varepsilon_S^2 = 0.1$; $\varepsilon_S^4 = -0.02$; $\varepsilon_P^2 = 0.6$; $\varepsilon_P^3 = 0.5$; $\varepsilon_P^4 = 0.25$; $\alpha = 0.5$; $P_\sigma/S_\sigma = 2$. (b) Case C (Fig. 4a, ▲) $\varepsilon_S^1 = -0.25$; $\varepsilon_S^2 = 0.025$; $\varepsilon_S^4 = 0.5$; $\varepsilon_P^2 = 0$; $\varepsilon_P^3 = 0.5$; $\varepsilon_P^4 = 0.75$; $\alpha = 0.5$; $P_\sigma/S_\sigma = 5$. (c) Case D (Fig. 5d, ▲) $\varepsilon_S^1 = -0.5$; $\varepsilon_S^2 = 0.4$; $\varepsilon_S^4 = 0.4$; $\varepsilon_P^2 = 0.3$; $\varepsilon_P^3 = 0.25$; $\varepsilon_P^4 = 0.375$; $\alpha = 3$; $P_\sigma/S_\sigma = 1$

By using these relationships, the linearized system can be rewritten as:

$$\dot{x} = [\boldsymbol{E} \cdot (v_\sigma \cdot \boldsymbol{D})]' \cdot (x_\sigma)^{-1} \cdot x \qquad (12)$$

where v_σ and x_σ are diagonal matrices of steady-state values of rates and metabolite concentrations, and:

$$E = \begin{pmatrix} \varepsilon_S^1 & \varepsilon_S^2 & 0 & \varepsilon_S^4 \\ 0 & \varepsilon_P^2 & \varepsilon_P^3 & \varepsilon_P^4 \end{pmatrix} \qquad D = \begin{pmatrix} 1 & 0 \\ -1 & 1 \\ 0 & -1 \\ 1 & -1 \end{pmatrix}$$

So, we can evaluate $\mathrm{tr}(Jc)$ and $\det(Jc)$ as a function of system elasticities, rates and metabolite concentrations at steady state.

A phase plane analysis can be performed by using this aproximation by perturbing the system of (Eqn 12) around its steady state. Using this technique it is possible to show that necessary conditions to attain high amplification (Table 1) are compatible with stable steady-state dynamics. In Fig. 8 we show the phase plane portrait for cases B, C and D in Table 1, choosing a set of conditions compatible with a high amplification ratio. In all the cases, stability depends on the ratio P_σ/S_σ, which is determined by the kinetic properties of the enzymes and the steady-state value of net flux.

So, in case B, the system is unstable if the ratio P_σ/S_σ is low (less that 0.7 in the conditions of Fig. 8a) while in the other cases instability appears to be associated with high values of that ratio.

In the example of Fig. 8, the cycles are stable in the range of recycling ratio values compatible with high amplification. A value of α over the boundary compatible with that effect can lead to an unstable steady state which, in special conditions, determines an oscillatory behaviour.

Thus, as a result of this analysis, stability has been shown to be compatible with a set of conditions leading to high amplification allowing to apply the control theorems to the study of such cases.

DISCUSSION

In agreement with earlier results in this field [6, 9, 10, 12, 13], our theoretical analysis has shown that substrate cycles are able to increase the flux control of a non-equilibrium reaction in quite different situations. The necessary conditions to reach such a capacity depend on the 'local' properties of the reactions involved in the cyclic structure and, particularly, on the internal effects of the metabolites of the cycle on the reactions producing them. In opposition to the results published about the control properties of substrate cycles [6, 9, 10, 12, 13], this amplification is not limited to $(1 + \alpha)$ if these effects are positive.

An example of such a situation appears in the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in which phosphofructokinase is activated by fructose 1,6-bisphosphate [15–17]. Such a positive effect defines a substrate cycle similar to case B when it works in a glycolytic sense and a cycle similar to case C when it works in a gluconeogenic sense. In both situations, such an activation can provide a high amplification of the flux control of the forward reaction (phosphofructokinase or fructose 1,6-bisphosphatase) that could be of in-

terest in the control of the pathway flux by external effectors. The possibility of attaining that situation without saturation of the cycle reactions can be important for the dynamic response to these effectors, although a more detailed dynamic analysis is required to evaluate its metabolic relevance (this study is in progress in our laboratory).

The recycling ratio is an important factor in defining amplification, which, as a general rule, increases with the recycling. Moreover, when the elasticities of the system permit the limit of $(1 + \alpha)$ in the amplification ratio to be exceeded, the recycling plays a critical role in defining the distribution of the flux control. So, an identical set of elasticities can lead to positive or negative values of the flux control coefficient of the forward reaction depending on the value of the recycling ratio, resulting in a very different pattern of flux control distribution (results not shown). Such a property can play a role in the change of the sense of the pathway flux due to the change of the sign of the control coefficients.

The role of substrate cycles in metabolic regulation must be discussed in relation to the redistribution of the flux control among the enzymes of the system as a result of the new metabolic structure. Although the ability of substrate cycles to increase the flux control of a non-equilibrium reaction is well demonstrated from the theoretical analysis described in this paper, it is not easy to extrapolate from that analysis its role in the control of the flux of a more complex pathway. So, depending on the structure and properties of the whole system, the control will be more or less distributed among the different steps of the pathway affecting the role of the substrate cycle in the final distribution. The observed importance of certain substrate cycle enzymes in integrating several regulatory signals [15, 20] suggests the possible existence of some optimal design in the properties of the pathway to concentrate the flux control on substrate cycles. The dynamic properties of such structures will favour the response of the pathway flux to effectors acting over its enzymes. The variation of the metabolic state, which involves changes in elasticities and, thus, in the distribution of the flux control, would change the role of each cycle and could switch the control from one substrate cycle to another in pathways where these structures are present [13].

This work has been supported by a grant of *Comissió Interdepartemental de Recerca i Innovació Tecnològica* (CIRIT) *de la Generalitat de Catalunya* (AR-211) and a grant of the University of Barcelona [*Ajut a la Investigació per a Professors de la Universitat de Barcelona* (1984)].

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