Occurrence of paradoxical or sustained control by an enzyme when overexpressed: necessary conditions and experimental evidence with regard to hepatic glucokinase

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It is widely assumed that the control coefficient of an enzyme on pathway flux decreases as the concentration of enzyme increases. However, it has been shown [Kholodenko and Brown (1996) *Biochem. J.* **314**, 753–760] that enzymes with sigmoidal kinetics can maintain or even gain control with an increase in enzyme activity or concentration. This has been described as ‘paradoxical control’. Here we formulate the general requirements for allosteric enzyme kinetics to display this behaviour. We show that a necessary condition is that the Hill coefficient of the enzyme should increase with an increase in substrate concentration or decrease with an increase in product concentration. We also describe the necessary and sufficient requirements for the occurrence of paradoxical control in terms of the flux control coefficients and the derivatives of the elasticities. The derived expression shows that the higher the control coefficient of an

allosteric enzyme, the more likely it is that the pathway will display this behaviour. Control of pathway flux is generally shared between a large number of enzymes and therefore the likelihood of observing sustained or increased control is low, even if the kinetic parameters are in the most favourable range to generate the phenomenon. We show that hepatic glucokinase, which has a very high flux control coefficient and displays sigmoidal behaviour within the hepatocyte *in situ* as a result of interaction with a regulatory protein, displays sustained or increased control over an extended range of enzyme concentrations when the regulatory protein is overexpressed.

Key words: allosteric enzymes, co-operativity, glucokinase, metabolic control, regulatory protein.

INTRODUCTION

Metabolic control analysis (‘MCA’) has made it possible to describe quantitatively how systemic variables (e.g. pathway fluxes) are controlled by individual enzymes and transporters ([1,2]; reviewed in [3]). The flux control coefficient of an enzyme has been defined as the fractional change in pathway flux divided by the fractional change in the enzyme activity responsible for the flux change. Because in many cases the sum of the flux control coefficients of the enzymes involved in a metabolic pathway is equal to 1, the relative importance of different enzymes in the control of flux in a pathway can be revealed by the control coefficients. However, although an enzyme that displays a high control coefficient can have a strong potential to influence the flux, the validity of the control coefficient as a predictive tool is limited. Control coefficients are defined for small (infinitesimal) changes in enzyme activity and cannot describe the effects of large changes. To predict the effects of large changes from control coefficients, Small and Kacser [4,5] derived simple relationships between the control coefficients and the flux changes expected for large finite changes in enzyme concentrations. These derivations were based on the assumption that the relation between pathway flux and enzyme concentration or activity approximates a hyperbola. A consequence of this derivation is the widely held assumption that, within a metabolic pathway,

inhibition of an enzyme causes the control exerted by that enzyme over pathway flux to increase, whereas activation causes its control to decrease [6,7]. Accordingly, some methods devised to measure the control coefficients experimentally are based on this assumption [3]. Kholodenko and Brown [8] have demonstrated that this assumption does not always hold true. If one of the pathway enzymes displays allosteric kinetics, the pathway can show increased control with activation and decreased control with inhibition of an enzyme or group of enzymes. This theoretical phenomenon has been described as ‘paradoxical control behaviour’ [8]. It has also been shown [8] that this type of control will not occur if the kinetics of the allosteric enzyme is described by the Hill equation. Its occurrence requires more complex enzyme kinetics, such as those described by the Monod–Wyman–Changeux (MWC) or the Koshland–Némethy–Filmer (KNF) models. Although the observation that control does not decrease when the enzyme concentration is increased can give the metabolic system unusual regulatory properties with a number of important implications, until now no detailed study to identify the necessary and sufficient conditions for this behaviour has been reported. Here we identify the properties of allosteric enzymes that are needed to generate this type of behaviour and to analyse potential enzyme candidates. Our analysis shows that liver glucokinase is a strong candidate for sustained or increased control. We confirm this hypothesis from experimental data of

Abbreviations used: MWC model, Monod–Wyman–Changeux model; KNF model, Koshland–Némethy–Filmer model; GKRP, regulatory protein of glucokinase.

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glycogen synthesis flux (from 7.5 mM glucose) in hepatocytes with various degrees of expression of glucokinase and its regulatory protein.

RESULTS AND DISCUSSION

Control behaviour dependence on elasticities

The control exerted by a reaction step on the steady-state flux can be described quantitatively by the control coefficient [1–3]. This coefficient represents the relative change in the flux per relative change in the rate $[(v_i/J)(dJ/dv_i)]$ and is symbolized by $C_{v_i}^J$. The effect that a relative change in the rate of a step has on the control exerted by that step can be calculated by the following quantity (referred to as the ‘control response ratio’ by Kholodenko and Brown [8]):

$$B^i = v_i \frac{dC_{v_i}^J/dp_i}{dv_i/dp_i} \quad (1)$$

where p_i is a parameter affecting the rate v_i only, and a rate change (dv_i) is brought about by a change in p_i . The metabolite concentrations are assumed constant when a change dv_i is imposed, and they are allowed to evolve to a new steady state at which $dC_{v_i}^J$ is determined. Let us for the moment consider two sequential enzymes, E_1 and E_2 , linked by a single metabolite (X) as depicted in Scheme 1. The concentrations of the initial substrate S and the final product P are assumed to be constant, whereas the concentration of the linking metabolite X depends on the kinetic parameters of the enzymes. A sustained or increased control coefficient with an increase in the rate of an enzyme catalysed reaction will appear when $B^i \geq 0$. Kholodenko and Brown [8] derived the general equation for the control response ratio B^i . Here we consider the simple case when p_i is the maximal catalytic activity or enzyme concentration. In this case, the reaction rate v_i depends linearly on p_i and therefore the enzyme elasticities $\epsilon_X^1 = (X/v_1)(dv_1/dX)$ and $\epsilon_X^2 = (X/v_2)(dv_2/dX)$ do not depend on p_i . The flux control coefficients of enzymes in Scheme 1 ($C_{v_1}^J$ and $C_{v_2}^J$) are functions of the elasticities, which depend on the metabolite concentration X. This concentration is a function of the rates v_1 and v_2 . Therefore B^1 and B^2 defined in eqn (1) can be expressed as:

$$B^1 = XC_{v_1}^J \left[\frac{dC_{v_1}^J}{d\epsilon_X^1} \frac{d\epsilon_X^1}{dX} + \frac{dC_{v_1}^J}{d\epsilon_X^2} \frac{d\epsilon_X^2}{dX} \right] \quad (2)$$

$$B^2 = XC_{v_2}^J \left[\frac{dC_{v_2}^J}{d\epsilon_X^1} \frac{d\epsilon_X^1}{dX} + \frac{dC_{v_2}^J}{d\epsilon_X^2} \frac{d\epsilon_X^2}{dX} \right] \quad (3)$$

The flux and concentration control coefficients in terms of the elasticities are:

$$C_{v_1}^J = \frac{\epsilon_X^2}{\epsilon_X^2 - \epsilon_X^1} \quad C_{v_2}^J = \frac{-\epsilon_X^1}{\epsilon_X^2 - \epsilon_X^1} \quad (4)$$

$$C_{v_1}^X = \frac{1}{\epsilon_X^2 - \epsilon_X^1} \quad C_{v_2}^X = \frac{-1}{\epsilon_X^2 - \epsilon_X^1} \quad (5)$$

Scheme 1 Two-enzyme pathway

Note that $C_{v_1}^J + C_{v_2}^J = 1$ and $C_{v_1}^X + C_{v_2}^X = 0$ (see [1,2]).

The derivatives of the flux control coefficients with respect to the elasticities are:

$$\frac{dC_{v_1}^J}{d\epsilon_X^1} = \frac{\epsilon_X^2}{(\epsilon_X^2 - \epsilon_X^1)^2} \quad \frac{dC_{v_1}^J}{d\epsilon_X^2} = \frac{-\epsilon_X^1}{(\epsilon_X^2 - \epsilon_X^1)^2} \quad (6)$$

$$\frac{dC_{v_2}^J}{d\epsilon_X^1} = \frac{-\epsilon_X^2}{(\epsilon_X^2 - \epsilon_X^1)^2} \quad \frac{dC_{v_2}^J}{d\epsilon_X^2} = \frac{\epsilon_X^1}{(\epsilon_X^2 - \epsilon_X^1)^2} \quad (7)$$

Substituting the control coefficients and their derivatives in the expressions for B^1 and B^2 we find that the condition for sustained or increased control is the same for both rates:

$$B^1 = B^2 = \frac{-X\epsilon_X^1\epsilon_X^2}{(\epsilon_X^2 - \epsilon_X^1)^3} \left(\frac{1}{\epsilon_X^2} \frac{d\epsilon_X^2}{dX} - \frac{1}{\epsilon_X^1} \frac{d\epsilon_X^1}{dX} \right) \geq 0 \quad (8)$$

We shall assume that the product elasticity ϵ_X^1 is negative and the substrate elasticity ϵ_X^2 is positive (i.e. ‘normal’ kinetics). This means that the substrate activates, and the product inhibits, the corresponding rates (substrate inhibition and product activation are not considered). Under these assumptions, the necessary and sufficient condition given in equation (8) simplifies to [8]:

$$\frac{1}{\epsilon_X^2} \frac{d\epsilon_X^2}{dX} - \frac{1}{\epsilon_X^1} \frac{d\epsilon_X^1}{dX} \geq 0 \quad (9)$$

Because $\epsilon_X^1 < 0$ and $\epsilon_X^2 > 0$, it is a necessary condition for the appearance of a paradoxical behaviour that $d\epsilon_X^1/dX \geq 0$ or $d\epsilon_X^2/dX \geq 0$. Next we show how these conditions can be expressed in terms of the Hill coefficients of the enzymes involved.

Control behaviour dependence on the Hill coefficient

The fractional rate (Y) for an enzyme is defined as:

$$Y = \frac{v}{V_{\max}} \quad (10)$$

where v is the reaction rate and $V_{\max}$ is the maximal enzyme rate that can be obtained by changes in the concentrations of substrates or products. We shall designate by X any of these concentrations. The Hill coefficient h is often used to describe cooperative properties of allosteric enzymes. It is defined as follows:

$$h \equiv \delta \frac{d \ln[Y/(1-Y)]}{d \ln X} \quad (11)$$

where $\delta = 1$ if X is a substrate and $\delta = -1$ if X is a product. Because Y is a function of X , h is usually a function of X . An exception is the relationship given by the Hill equation, $Y = X^n/(K + X^n)$, where the Hill coefficient is constant, and one of its particular cases: the hyperbolic equation ($h = 1$). From eqn (11) it can be shown that this quantity is related to the elasticity coefficient, $\epsilon_X^Y \equiv (X/Y)(dY/dX)$, as follows:

$$\epsilon_X^Y = \delta(1-Y)h \quad (12)$$

As $1-Y$ is between 0 and 1, ϵ_X^Y and δh are both of the same sign, satisfying the inequality $|\epsilon_X^Y| \leq |h|$. Differentiation of eqn (12) leads to:

$$\frac{1}{\epsilon_X^Y} \frac{d\epsilon_X^Y}{dX} = \frac{1}{1-Y} \frac{d(1-Y)}{dX} + \frac{1}{h} \frac{dh}{dX} \quad (13)$$

From the definition of Y [eqn (10)] it follows that the elasticity coefficients of the rates are identical with those of the corresponding fractional rates, namely $\epsilon_X^1 = \epsilon_X^{Y_1}$ and $\epsilon_X^2 = \epsilon_X^{Y_2}$. Taking into account these equalities we can substitute eqn (13) into eqn (9) (twice), obtaining the condition for sustained or increased control behaviour in terms of the fractional rates and the Hill coefficients:

$$\left[\frac{1}{1-Y_2} \frac{d(1-Y_2)}{dX} - \frac{1}{1-Y_1} \frac{d(1-Y_1)}{dX} \right] + \left[\frac{1}{h_2} \frac{dh_2}{dX} - \frac{1}{h_1} \frac{dh_1}{dX} \right] \geq 0 \quad (14)$$

Next we examine the signs of the quantities appearing in these inequalities. By definition, $1-Y_1 > 0$ and $1-Y_2 > 0$ (we exclude the limit cases $1-Y_1 = 0$ and $1-Y_2 = 0$ to avoid vanishing denominators). From the assumption of 'normal' kinetics (substrates activate, and products inhibit, reaction rates) an increase in X increases Y_2 and decreases Y_1 ; therefore $d(1-Y_2)/dX < 0$ and $d(1-Y_1)/dX > 0$. Taking these signs into account, we find that the expression enclosed in the first brackets of eqn (14) is negative. As a consequence, it is a necessary (but not sufficient) condition for sustained or increased control that the expression in the second brackets is positive, i.e.

$$\frac{1}{h_2} \frac{dh_2}{dX} - \frac{1}{h_1} \frac{dh_1}{dX} > 0 \quad (15)$$

Another consequence of the assumption of 'normal' kinetics (i.e. $\epsilon_X^1 < 0$ and $\epsilon_X^2 > 0$) is that $h_2 > 0$ and $h_1 > 0$ [see eqn (12)]. Accordingly, the final necessary condition for paradoxical control behaviour, expressed in terms of the Hill coefficients, is:

$$\frac{dh_1}{dX} < 0 \quad \text{or} \quad \frac{dh_2}{dX} > 0 \quad (16)$$

This means that to obtain sustained or increased control behaviour in Scheme 1 it is necessary that either the Hill coefficient of the enzyme of the first step decreases, or the Hill coefficient of the enzyme of the second step increases, with increasing concentration of the intermediate metabolite. Importantly, this condition is valid irrespective of the details of the rate equations involved.

Next we shall sketch an explanation for the meaning of the necessary condition [eqn (16)]. As shown previously by Acerenza and Mizraji [9], the Hill coefficient h for a binding system at equilibrium satisfies $h-1 = C_X^A$, where $C_X^A = (X/A)(dA/dX)$ represents the relative change in the global affinity A of the binding system relative to the change in the ligand concentration (X). Co-operativity, i.e. $h \neq 1$, means that the global affinity changes with ligand concentration. In addition, it has been shown [9] that this result also applies to what Monod called K -enzyme systems [10], in which the fractional saturation is replaced by the fractional rate defined in eqn (10) and X is the reactant concentration. For instance, in this case, condition $dh_2/dX > 0$ in eqn (16) is equivalent to $dC_X^{A2}/dX > 0$. Therefore in Scheme 1 the necessary condition for obtaining an increase in the flux control coefficient C_{vi}^J when the corresponding rate v_i is increased (i.e. to obtain paradoxical control behaviour) is that C_X^A of a component enzyme changes with X or, in other words, that the relative change in its global affinity relative to the change in reactant concentration must change with reactant concentration.

Next we shall give examples of rate equations of allosteric enzymes that could form part of the diagram in Scheme 1; we discuss whether they fulfil the necessary condition in eqn (16).

Figure 1 Dependence of the Hill coefficient on the concentration of the substrate in an allosteric enzyme

(a) The curve was generated with an MWC tetramer [eqn (17)] with the parameters taking the following values: $L = 10000$, $K_R = 1$. (b) The curve was generated with a KNF dimer [eqn (18)] with positive co-operativity ($K_1 = 10 > K_2 = 1$) (solid line), and negative co-operativity ($K_1 = 3 < K_2 = 15$) (broken line).

Let us consider the second step of the Scheme, where X is the substrate. If in the range of concentrations of X in which the system functions the rate law could be reasonably adjusted to a Hill equation, $Y = X^h/(K + X^h)$, the Hill coefficient h_2 [eqn (11)] is constant and its derivative dh_2/dX with respect to substrate concentration is zero. In this case, provided that the first step in Scheme 1 has Michaelis–Menten kinetics, eqn (16) is not satisfied and paradoxical behaviour cannot be observed. Equally, the same applies to the particular case of hyperbolic kinetics ($h_2 = 1$). Other models used to describe the kinetics of co-operative enzymes have a variable Hill coefficient with substrate concentration. For instance, in Figure 1(a) we show the plot of h_2 against X for an exclusive-binding MWC tetramer with the following rate equation:

$$v = V_{\max} \frac{X/K_R}{(1 + X/K_R)[L/(1 + X/K_R)^4 + 1]} \quad (17)$$

where K_R and L are kinetic parameters. In this Figure we see that the necessary condition, $dh_2/dX > 0$, is valid between $X = 0$ and the value of X that makes h_2 maximum (the interval in which h_2 increases with X). In addition, the smaller X is, the greater is the magnitude of dh_2/dX . It can readily be shown that dh_2/dX calculated from eqn (17) depends on two quantities only: X/K_R and L . In Figure 2 we show (area in white) the region of values of these quantities for which $dh_2/dX > 0$. In general, in this Figure we see that the necessary condition is satisfied in the region of low values of saturation (X/K_R relatively small). For low values of L a positive dh_2/dX requires a value of X/K_R below approx. 0.3, whereas for high values of L , the greater L is, the wider is the range of values of X/K_R for which the necessary condition [eqn (16)] is fulfilled.

Figure 2 Dependence of the derivative of the Hill coefficient with respect to the substrate on the kinetic parameters of the allosteric enzyme

The kinetics of the allosteric enzyme is described by an MWC model [eqn (17)]. The dependence of the derivative of the Hill coefficient of the allosteric enzyme with respect to its substrate on L and X/K_R is shown. The white area corresponds to the region in which this derivative is positive and the black area to the region in which it is negative. Contour lines (1 and 2) represent curves where the derivative of the Hill coefficient with respect to its substrate takes positive constant values (1 and 2) respectively.

The MWC model used previously [eqn (17)] cannot describe enzymes that show negative co-operativity. For this purpose the KNF model has been widely used. In Figure 1(b) we show the plot h_2 against X for a KNF dimer (with identical subunits), whose rate equation is:

$$v = V_{\max} \frac{(X/K_1)(1 + X/K_2)}{X^2/(K_1K_2) + 2X/K_1 + 1} \quad (18)$$

where K_1 and K_2 represent the microscopic dissociation constants for the singly occupied and doubly occupied dimer respectively. The analysis of the case of positive co-operativity, $K_1 > K_2$ (solid line), is similar to that discussed for the MWC model, obeying condition eqn (16) in the interval between $X = 0$ and the value of X that makes h_2 maximum. However, the case of negative co-operativity, $K_1 < K_2$ (broken line), is quite different. In this situation the necessary condition $dh_2/dX > 0$ is satisfied for concentrations greater than the value of X that minimizes h_2 .

It should be noted that similar reasoning could be applied if we consider the first enzyme to be allosteric. In this case we can obtain sustained or increased control if we consider the first enzyme to be allosterically inhibited by its product. For simplicity we shall consider the case in which only the second enzyme is allosteric.

Finally, we shall consider the whole of Scheme 1. We shall assume that the enzyme in the first step follows a Michaelis-Menten equation of the form:

$$v_1 = \frac{V_f S/K_s - V_r X/K_x}{1 + S/K_s + X/K_x} \quad (19)$$

and that the enzyme in the second step (v_2) follows an MWC model with a rate law, as in eqn (17). The steady state of the system can be obtained by equating the difference between v_1 and v_2 to zero and solving for the concentration of the variable

Figure 3 Dependences of control response ratio and the derivative of the Hill coefficient with respect to the linking metabolite on the kinetic parameters of the allosteric enzyme

The kinetics of the allosteric enzyme (second) is described by an MWC model [eqn (17)]; the first enzyme is described by the Michaelis-Menten equation [eqn (19)]. The parameter values were $V_f = 200$, $S = 1$, $K_s = 1$, $K_x = 0.005$, $V_r = 0$, $V_{\max} = 10^{2.5}$. The dependences of control response ratio and the derivative of the Hill coefficient of the allosteric enzyme with respect to its linking metabolite on L and K_R are shown. The white area corresponds to the region in which control response ratio and the derivative of the Hill coefficient with respect to the linking metabolite are both positive (paradoxical control behaviour) and the black area corresponds to the region in which both magnitudes are negative. The grey area corresponds to the region in which the derivative of the Hill coefficient with respect to the linking metabolite is positive and the control response ratio is negative.

metabolite X . Differentiation and normalization of v_1 and v_2 render the expressions for the elasticities ϵ_X^1 and ϵ_X^2 respectively. Subsequently, the elasticities are differentiated, obtaining $d\epsilon_X^1/dX$ and $d\epsilon_X^2/dX$. The steady-state values of the elasticities and their derivatives can be introduced in eqn (9) to obtain the condition for sustained or increased control. Note that in our conditions $B^1 = B^2 \equiv B$ [eqn (8)]. dh_2/dX can be calculated in a similar way from eqns (10) and (11) and the steady-state values of X . In Figure 3 we show the region of values of K_R and L (corresponding to the co-operative enzyme), where B and dh_2/dX are positive (white), B is negative and dh_2/dX is positive (grey) and B and dh_2/dX are negative (black). Here we see that to obtain sustained or increased control (white region), relatively large values of both K_R and L are required (although if K_R is sufficiently high the necessary condition $dh_2/dX > 0$ is also fulfilled for low values of L). In Figure 4 we consider an equivalent case of K_x and K_R (corresponding to the first and second steps respectively) for a high value of L . The results show that an increase in K_R brings the system nearer to the region of sustained or increased control, whereas an increase in K_x diverges from this behaviour. Therefore the Michaelis constants of X for the first and second steps have opposite effects on the control behaviour.

Dependence of control behaviour on flux control distribution between the enzymes of the pathway

Here we shall study how the condition of sustained or increased control behaviour depends on the distribution of flux control between the enzymes of the pathway. We start from eqns (2) and (3). The derivatives of the flux control coefficients with respect to

Figure 4 Dependences of control response ratio and the derivative of the Hill coefficient with respect to the linking metabolite on the kinetic parameters of the first and second enzymes

The rate equations used were as in Figure 3. The parameter values were $V_f = 200$, $S = 1$, $K_S = 1$, $V_r = 0$, $V_{\max} = 10^{2.5}$, $L = 10000$. The dependences of control response ratio and the derivative of the Hill coefficient of the allosteric enzyme with respect to its linking metabolite on K_X and K_R are shown. The white area corresponds to the region in which control response ratio and the derivative of the Hill coefficient with respect to the linking metabolite are both positive (paradoxical control behaviour) and the black area corresponds to the region in which both magnitudes are negative. The grey area corresponds to the region in which the derivative of the Hill coefficient with respect to the linking metabolite is positive and the control response ratio is negative.

the elasticities [eqns (6) and (7)] can be expressed in terms of the control coefficients [eqns (4) and (5)] as follows [11]:

$$\frac{dC_{v_1}^J}{d\epsilon_X^1} = C_{v_1}^J C_{v_1}^X \quad \frac{dC_{v_1}^J}{d\epsilon_X^2} = C_{v_2}^J C_{v_1}^X \quad (20)$$

$$\frac{dC_{v_2}^J}{d\epsilon_X^1} = C_{v_1}^J C_{v_2}^X \quad \frac{dC_{v_2}^J}{d\epsilon_X^2} = C_{v_2}^J C_{v_2}^X \quad (21)$$

Substituting these expressions into eqns (2) and (3) we obtain:

$$B^1 = X(C_{v_1}^X)^2 \left(C_{v_1}^J \frac{d\epsilon_X^1}{dX} + C_{v_2}^J \frac{d\epsilon_X^2}{dX} \right) \quad (22)$$

$$B^2 = X(C_{v_2}^X)^2 \left(C_{v_1}^J \frac{d\epsilon_X^1}{dX} + C_{v_2}^J \frac{d\epsilon_X^2}{dX} \right) \quad (23)$$

These expressions show that the condition for sustained or increased control in terms of the control distribution is:

$$C_{v_1}^J \frac{d\epsilon_X^1}{dX} + C_{v_2}^J \frac{d\epsilon_X^2}{dX} \geq 0 \quad (24)$$

This inequality shows that the sign of B depends on the signs and magnitudes of $C_{v_1}^J$, $C_{v_2}^J$, $d\epsilon_X^1/dX$ and $d\epsilon_X^2/dX$. If we assume 'normal' kinetics ($\epsilon_X^1 < 0$ and $\epsilon_X^2 > 0$), $C_{v_1}^J$ and $C_{v_2}^J$ are positive [eqn (4)]. If, in addition, $d\epsilon_X^1/dX < 0$, as is the true for the rate equations normally used, it is a necessary condition for the fulfilment of eqn (24) that $d\epsilon_X^2/dX > 0$. However, to ensure that the condition in eqn (24) is satisfied $C_{v_2}^J$ must be sufficiently high (given that $C_{v_1}^J + C_{v_2}^J = 1$). In summary, to obtain sustained or increased control behaviour in the second step, this must have a substrate elasticity that increases with substrate concentration

and, in addition, the fraction of control exerted by this step must be adequately high.

These results have been extended to the case in which the enzyme that displays an unusual dependence on the elasticity with respect to the substrate concentration is the third in a pathway with three steps (substrates X_1 and X_2). The condition for sustained or increased control in terms of the control distribution obtained in this case is:

$$B^3 = C_{v_1}^J \left[\left(X_1 \frac{d\epsilon_{X_1}^1}{dX_1} \right) [C_{v_3}^{X_1}]^2 \right] + C_{v_2}^J \left[\left(X_1 \frac{d\epsilon_{X_1}^2}{dX_1} \right) [C_{v_3}^{X_1}]^2 \right. \\ \left. + \left(X_2 \frac{d\epsilon_{X_2}^2}{dX_2} \right) [C_{v_3}^{X_2}]^2 + \left(X_2 \frac{d\epsilon_{X_1}^2}{dX_2} + X_1 \frac{d\epsilon_{X_2}^2}{dX_1} \right) [C_{v_3}^{X_1} C_{v_3}^{X_2}] \right] \\ \left. + C_{v_3}^J \left[\left(X_2 \frac{d\epsilon_{X_2}^3}{dX_2} \right) [C_{v_3}^{X_2}]^2 \right] \geq 0 \quad (25)$$

Assuming 'normal' kinetics, as previously with two enzymes, we can see that the only term that can be positive in this expression is $C_{v_3}^J [(X_2 (d\epsilon_{X_2}^3/dX_2)) [C_{v_3}^{X_2}]^2]$. To obtain sustained or increased control a high value of $C_{v_3}^J$ is therefore necessary, in addition to a positive value of $d\epsilon_{X_2}^3/dX_2$, to compensate for the other negative terms in eqn (25) and to achieve a zero or positive value of B^3 .

Example of the influence of a kinetic parameter of the non-allosteric enzyme on the control behaviour

For a two-enzyme pathway in which the first enzyme follows Michaelis–Menten kinetics [eqn (19)] and the second enzyme is allosteric, displaying sigmoidal rate dependence as described by eqn (17), we shall examine how the condition of increased control behaviour depends on the kinetic parameter K_X of the first enzyme. The range of values of K_X analysed was between 10^{-5} and 10^5 . The values of the other kinetic parameters were $V_f = 200$, $S = 1$, $K_S = 1$, $V_r = 0$, $L = 10000$ and $K_R = 1$. We find that increased control is observed only for values of K_X lower than 0.25. Figure 5(a) shows three examples of how the flux control coefficients of the second enzyme (E_2) change with an increase in its maximal catalytic activity ($V_{\max}$) for three different values of K_X . The curve for $K_X = 0.005$ (solid line) corresponds to a typical profile of increased control behaviour. The curve for $K_X = 50$ (dotted line) corresponds to the region far away from increased control behaviour. In this case there is an abrupt decrease in the value of $C_{v_2}^J$. $K_X = 0.5$ (broken line) corresponds to intermediate behaviour: the control coefficient decreases to an intermediate value and remains practically constant over a wide range of values of $V_{\max}$, beyond which it decreases. Figure 5(b) confirms that, for $K_X = 0.005$, eqn (9) is fulfilled in exactly the same range of $V_{\max}$ values as that in which an increase in the control coefficient is observed (compare with Figure 5a). For $K_X = 50$ or $K_X = 0.5$ we see that eqn (9) does not take a positive value and accordingly the control coefficient never increases. This is in agreement with the control behaviour observed in the flux control coefficient for these K_X values (compare with Figure 5a). Figure 5(c) shows the derivative of the Hill coefficient of the second enzyme with respect to the linking metabolite (dh_2/dX) for the three values of K_X considered above. The necessary (but not sufficient) condition for increased control behaviour ($dh_2/dX > 0$) is accomplished for $K_X = 0.005$ in a range of values of $V_{\max}$ wider than the region in which we see this behaviour. For the curves corresponding to systems in which $K_X = 50$ or $K_X = 0.5$ it should be noted that there is also a wide range of $V_{\max}$ values in which dh_2/dX is positive but increased control is not observed because the sufficient condition $[(1/\epsilon_X^2)(d\epsilon_X^2/dX) - (1/\epsilon_X^1)(d\epsilon_X^1/dX)]$ is not met.

Figure 5 Effect of changes in a kinetic parameter of the first enzyme (K_X) on the occurrence of paradoxical control behaviour

The rate equations used were as in Figure 3. The parameter values were $V_i = 200$, $S = 1$, $K_S = 1$, $V_r = 0$, $L = 10000$, $K_R = 1$. The values for K_X were as follows: solid line, $K_X = 0.005$; broken line, $K_X = 0.5$; dotted line, $K_X = 50$. The plots show the following: (a) control coefficient of the second enzyme, (b) eqn 9 $[(1/\epsilon_X^2)(d\epsilon_X^2/dX) - (1/\epsilon_X)(d\epsilon_X^1/dX)]$, (c) the derivative of the Hill coefficient of the second enzyme with respect to the linking metabolite (dh_2/dX) for a range of activities of the second enzyme (V_{max}) from $10^{-2.5}$ to 10^{10} .

From this study we conclude that the kinetic parameters of the non-allosteric enzyme can counteract the tendency of an allosteric enzyme to produce increased control behaviour, even if the kinetic parameters of the allosteric enzyme are in the most favourable region to produce this effect.

Experimental case

Good candidates to test for sustained or increased control would be allosteric enzymes with a high control coefficient of pathway flux. Hepatic glucokinase (hexokinase IV) is a very good example with which to test this model because it has a high flux control coefficient on glycogen synthesis [12,13] and because it displays sigmoidal kinetics not only in solution [14] but also in the hepatocyte *in situ* [15]. The sigmoidal kinetics *in situ* is the result of interaction with a 68 kDa regulatory protein (GKRP), which is a competitive inhibitor of glucokinase [16]. Glucose is both the substrate for the enzyme and the regulator that causes the dissociation of glucokinase from GKRP [17]. The sigmoidal kinetics of glucokinase *in situ* is the combined effect of the sigmoidal effect of glucose in dissociating the enzyme from GKRP and the sigmoidal kinetics of the free enzyme [13] and is dependent on the concentration of GKRP [18]. Here we

Figure 6 Glycogen synthesis flux dependency versus glucokinase overexpression at different levels of GKRP

Curves were fitted with the Taylor expansion in logarithmic space given by $\log J = a \log J_0 + c(\log GK - \log GK_0) + (b/2)(\log GK - \log GK_0)^2$. (a) Representative experiment of three different culture experiments done at a fixed GKRP concentration of 0.7 unit/mg of protein. (b) Representative experiment of three different culture experiments done at a fixed GKRP concentration of 1.4 units/mg of protein. (c) Curves corresponding to each concentration of GKRP (in units/mg of protein) tested. Each curve corresponds to the average of the curves corresponding to three different culture experiments done at the same concentration of GKRP (at each concentration of GKRP only the common part of the different curves is represented). Rates of glycogen synthesis are expressed as nanomol of glucose incorporated into glycogen per 3 h per mg of cell protein. Glucokinase activity is expressed as m-units/mg of cell protein.

analyse data from the experiments of de la Iglesia et al. [18] for the double-logarithmic plot of glycogen synthesis flux at 7.5 mM glucose plotted against total glucokinase activity at different levels of expression of GKRP (Figure 6). In these experiments glucokinase and GKRP were overexpressed by up to 5-fold and up to 2-fold respectively above endogenous levels. The double-logarithmic plots were fitted with the Taylor expansion in logarithmic space given by $\log J = a \log J_0 + c(\log GK - \log GK_0) + (b/2)(\log GK - \log GK_0)^2$. Figures 6(a) and 6(b) show representative experiments done at fixed levels of GKRP representing the endogenous level of GKRP [Figure 6(a), 0.7 unit/mg] and 2-fold overexpression [Figure 6(b), 1.4 units/mg]. Figure 6(c) shows the average of the curves corresponding to each GKRP concentration for the four concentrations of GKRP studied. The sign of b is an estimate of the sign of the 'control response ratio (B')' given in eqn (1). In our study, for the curves in Figure 6(c), b is negative for the two lower levels of GKRP studied ($b = -1.3$ for 0.7 unit/mg of protein; $b =$

– 1.7 for 0.98 unit/mg of protein), which means that the control coefficient of glucokinase decreases when the enzyme is overexpressed. At a GGRP concentration of 1.16 units/mg of protein, b is practically zero ($b = -0.1$), which means that the control coefficient value is sustained when glucokinase concentration or activity increases. Finally, at a GGRP concentration of 1.4 units/mg of protein, b is positive ($b = +1.0$), which means that the control exerted by glucokinase increases when the enzyme is overexpressed. The finding that control is either sustained or even increased at certain levels of GGRP confirms that the regulation of glucokinase by GGRP in hepatocytes confers on the hepatocyte the ability to maintain a high control by glucokinase on glycogen synthesis flux (at 7.5 mM glucose) over a wide range of glucokinase concentrations.

Final comments

An easy test to determine whether an allosteric enzyme might display sustained or increased control is from a Hill plot {i.e. $\partial \ln[Y/(1-Y)]$ against $\ln X$ }, where the slope is the Hill coefficient [eqn (11)]. The occurrence of this behaviour could be expected only in the range of concentrations of X in which this plot is not a straight line (where the slope changes with X). If X is a substrate (product) we must look for a region where the slope increases (decreases) with X [eqn (16)]. As we have seen, this requirement for the shape of the Hill plot is a necessary but not sufficient condition to obtain increased control when the enzyme is studied *in situ*.

It has been demonstrated that muscle phosphofructokinase, which has allosteric kinetics, has a low flux control coefficient [19]. It is thought that most allosteric enzymes would similarly be expected to display low control on pathway flux (reviewed in [3]). If the allosteric enzyme catalyses the step v_2 , and v_1 represents the other enzyme in the pathway, we would expect that $C_{v_1}^J > C_{v_2}^J$. In this case, if an allosteric enzyme is embedded in the pathway and has a low control on the flux, then sustained or increased control is, in principle, difficult to observe [see eqn (24)].

From control analysis we can conclude that one of the advantages of regulation of glucokinase by GGRP is that the high control of glycogen synthesis flux displayed by glucokinase is maintained over a wide range of enzyme concentrations. It is noteworthy that the free activity of glucokinase in the cytoplasm shows very large changes, both as a result of rapid translocation of the enzyme from the nucleus to the cytoplasm in response to a high concentration of either glucose or precursors of fructose 1-phosphate and also as a result of changes in the total cellular concentration of both glucokinase and GGRP. During starvation the total cell content of both glucokinase and GGRP decrease, however, the decrease in glucokinase is much greater than for GGRP [20]. In this situation the ratio of GGRP to glucokinase increases and is therefore similar to the those in experiments shown in Figure 6 at higher levels of GGRP. During refeeding, glucokinase increases more rapidly than GGRP and this would therefore be equivalent to overexpression of glucokinase at the highest level of GGRP when the control coefficient increases. This would be advantageous in a situation where glucose needs to be efficiently stored as glycogen.

Finally it should be noted that the existence of sustained or increased control of a step in a metabolic pathway could be useful in biotechnology. Very often, an enzyme controlling the flux in a wild-type organism loses its control when it is overexpressed, resulting in a disappointing increase in the flux.

However, activation of an enzyme that displays sustained or increased control properties would have similar effects on the flux over a wide range of enzyme activities. Here we have studied, in a linear pathway, the necessary conditions required to obtain this type of behaviour. In addition, it has been shown by Kholodenko and Brown [8] that paradoxical control behaviour can also occur in branched pathways. In this situation, the control exerted by an enzyme over the flux through the pathway branch in which that enzyme is located, increases on activation of the enzyme. These theoretical results, together with the experimental evidence reported here, suggest that overexpression of an enzyme that exhibits sustained or increased control after stimulation could be, at least in some particular situations, an effective way of increasing the flux of a metabolic pathway.

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