ELSEVIER

Biochimica et Biophysica Acta 1289 (1996) 175–186

BBA Biochimica et Biophysica Acta

Why and when channelling can decrease pool size at constant net flux in a simple dynamic channel

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Received 9 August 1995; accepted 22 September 1995

Abstract

Cornish-Bowden and Cárdenas (Cornish-Bowden, A. and Cárdenas M.L. (1993) Eur. J. Biochem. 213, 87–92) have suggested that simulation results previously published by us (Mendes, P., Kell, D.B. and Westerhoff, H.V. (1992) Eur. J. Biochem. 204, 255–266) which had demonstrated that large reductions of intermediate pool sizes could be accompanied by increasing channel flux in a model metabolic pathway, were an artefact of changes in the pathway's *overall* flux of the order of 0.0075%, or of inappropriate alterations of enzyme activities. They also asserted to prove that "channelling of an intermediate cannot affect its free concentration at constant net flux". We consider the co-response of the intermediate metabolite concentration ('pool') and the channel flux to changes in kinetic (or thermodynamic) parameters. Both by analytical proofs and by numerical examples we show that this co-response can be positive, negative or null, depending on the parameter change. In particular, we prove that there is always a number of ways of changing parameters such that the intermediate metabolite concentration decreases with increasing channel flux, whether the total flux varies or is constant. We also show that increased stability of the (dynamic) enzyme-intermediate-enzyme complex, as well as a single parameter change that similarly displays no cross-over effects, can lead to decreased intermediate metabolite concentration and increased channel flux at constant total flux. In general, a non-zero co-response of the intermediate metabolite concentration ('pool') and the channel flux to changes in kinetic (or other) parameters is the rule rather than the exception. More specifically: (i) The algebraic analysis ('general proof') given in Cornish-Bowden and Cárdenas (1993) contains the constraint that the elasticities of various steps to the modulation parameters which were used to vary the channel flux at constant net flux were unity. This is an unfortunate and unnecessary constraint which, when lifted, means that the concentration of the pool in the general case can indeed change at constant net flux. A 'simplified proof' given in Cornish-Bowden and Cárdenas (1993) also fails, due in addition to the consequent failure to include mass conservation relations for some of the enzymes. (ii) In the systems studied by Cornish-Bowden and Cárdenas (1993), flux is properly to be considered as a variable (since it varies during the transition to the steady state), and not a parameter, and as such *cannot per se* affect the magnitude of other variables in the steady state. (iii) By relaxing the constraint referred to in (i), above, and by making *dual* modulations (i.e., of more than one parameter at once) which are different from those carried out in Cornish-Bowden and Cárdenas (1993) we find many instances in which channelling (described by a parameter p) does significantly affect the concentration of the pool intermediate C at constant total flux. (iv) In the same pathways, but in which the flux is held constant by setting it via a zero-order flux-generating reaction, the addition of a channel is also able significantly to modulate the size of the pool at constant total flux. Our results show that the effectiveness of channelling in decreasing a pool, even at constant flux, is very much a reality.

1. Introduction

Enzyme–enzyme interactions have long been identified both in vitro and in vivo. These interactions can take many forms. One that has been much in focus lately is that in which a common intermediate of two enzymes, catalysing consecutive reactions of a pathway, diffuses from one active centre to the other without becoming free to mix with a nominal substrate 'pool'. This phenomenon, known as metabolic channelling, has been particularly clearly identified and characterized in long-lived enzyme complexes (with low dissociation constants). One case of this so-called 'static' channelling, in which the enzyme-en-

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SSDI 0304-4165(95)00152-2

zyme complex exists in the absence of the common intermediate, and which has been studied by X-ray crystallography in remarkable detail, is the bacterial tryptophan synthase bienzyme (EC 4.2.1.20). In this case, indole, the common intermediate of the two reactions, travels from the site of its production to the site where it is utilised as a substrate via a 25–30 Å long tunnel [1–3]. It has also been proposed (e.g. [4–9]) that *dynamic* complexes of consecutive enzymes (with higher dissociation constants) are also capable of promoting metabolic channelling. Because such complexes are essentially absent when the common intermediate is not bound, enzymes dissociate easily, and (the widespread) experimental evidence for such dynamic channelling has been gathered by methods other than structural ones (reviewed in [7,10–12]).

That channelling exists in real metabolic systems does not in itself indicate the magnitude of any effects that might follow from the addition of a metabolic channel to a pathway exhibiting otherwise purely pool-type behaviour. Many authors have suggested possible advantages, or at least consequences, of metabolic channelling for cellular metabolism (e.g., [11–18]), of which perhaps the most common, in addition to possible effects on the flux through various metabolic branches, is that channelling could lead to a decrease in the steady-state concentration of the intermediate metabolite (pool). In contrast with these suggestions, Cornish-Bowden [19] published simulations in which the addition of a channel to a model pathway did not decrease the concentration of the intermediate metabolite (and actually slightly increased it). Although his simulations were restricted to very specific thermodynamic and kinetic parameters, he generalised his conclusions, arguing that "channelling has no effect on the free concentration of a channelled intermediate in a pathway" [19]. We subsequently showed [20], by performing simulations on the same model studied in [19], that this was an incorrect overgeneralisation: in particular, if the reaction catalysed by the enzymes forming the channel had a K_{eq} higher than 1, and if the enzyme removing the product of the channel reaction was kinetically competent, channelling in the stated model could decrease the steady-state concentration of the pool by factors of as much as 1000, independently of the mechanism of the terminal reaction and under conditions of essentially (approximately) constant overall flux. If the channel was a 'static' channel, the decrease in the pool could be to arbitrarily low levels, and this conclusion also held for a system in which other reactions could consume the pool intermediate. Easterby [59] had also showed that channelling could decrease the size of a pool, particularly in a static channel. Thus, we concluded that, given appropriate parameters, the addition of a channel to an otherwise unchannelled pathway is perfectly capable of bringing metabolite concentrations to low levels.

However, Cornish-Bowden and Cárdenas [21] then claimed, inter alia, that the simulation results previously published by us [20] resulted from variations of overall pathway flux or 'cross-over effects' and that channelling would not decrease the pool size at constant net flux.

In this paper we prove analytically that there are always parameter changes that lead to increased channelling flux and decreased intermediate metabolite concentrations even at constant net flux. We also show that increased channelling flux caused by increased stability of the ternary enzyme-substrate-enzyme complex can be accompanied by decreased concentrations of the (free) intermediate metabolite. In general, whether the intermediate metabolite concentration decreases or increases depends on the way increased channelling flux is effected. Flux optimization procedures could therefore employ the possibility of increasing the proportion of flux through channelling complexes and decreasing intermediate metabolite concentrations (cf. [22]).

[21] also contained a number of statements concerning the cross-over theorem [23] which is anyway well known to fail (e.g., [24,25]). In sum, however, the incorrect arguments raised in [21], and related to the purported inability of channelling to modulate a pool at constant net flux, are gross overgeneralisations which seem to stem (i) from an unnecessary restriction in the coupling of pathway parameters, and (ii) from the confusion of a parameter and a variable. In answer to the question "Channelling can affect concentrations of metabolic intermediates at constant net flux: artefact or reality?", it is clear that the answer is reality. Depending on how channelling flux is increased the concentrations of pool metabolites can decrease, increase or remain at the original values.

2. Model and methods

The model used here to study the effects of channelling on a linear pathway is depicted in Fig. 1a and has been described previously [19–21]. It represents a pathway of four consecutive enzyme-catalysed reactions in which the two middle enzymes can associate and channel their intermediate metabolite, whilst a proportion of the intermediate may be released to the 'bulk' solution, thus forming a pool. Because we are specifically interested in studying the effects of channelling per se, the elementary reactions of the two interacting enzymes were detailed explicitly, whilst the other two were represented by single steps with Michaelis-Menten-type kinetics. Similarly, since we wish to describe the effects of the channel per se on the 'naked' (channel-free) pathway, we also delineate the equivalent case in which the channelling steps (7 and 8 in Fig. 1a) are absent (Fig. 1b); in such a circumstance, the kinetics of enzymes 2 and 3 (steps 2–5) may be accounted for by the reversible Michaelis-Menten rate law (and one can compute the kinetic constants from the rate constants). We would stress that this model is the same as the one studied *numerically* in [19]. However, the model represented *graphically* in Scheme 3 of that paper, as well as the

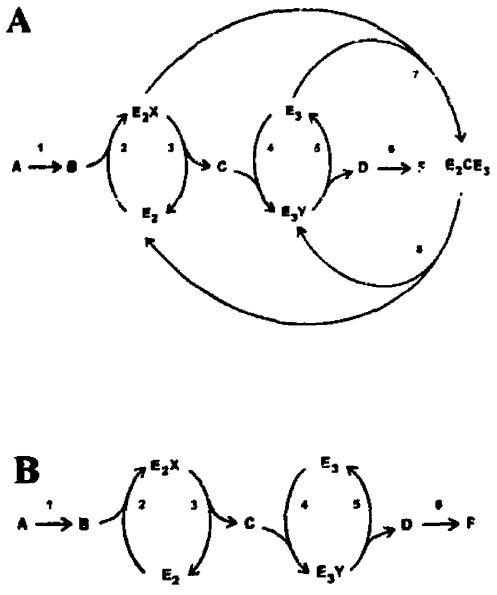

Fig. 1. Model of dynamic channelling in a four-enzyme pathway. (A) the full schematic model (for the corresponding mathematical model see Fig. 2); (B) the same pathway without channelling, in which the interactions between enzymes 2 and 3 that allow for the channelling of C are absent. Arrowheads refer to the positive direction of fluxes; all steps except 6 are treated as kinetically reversible.

model represented in Fig. 1 of [21], is significantly different from this; conservation of enzyme molecules is not included in that model, with the effect, for example, that E_2X is converted to E_2CE_3 without ever binding E_3. Because such incomplete arrow diagrams can be misleading, we think it is appropriate to present the graphical representation of the full model. The dynamic equations of the mathematical elaboration of the model are given in Fig. 2. They are essentially identical to (although somewhat more general than) with the equations employed earlier in [19–21]. The variables of this model are expressed as time derivatives. They include the internal metabolite concentrations [B], [C], and [D] as well as the concentrations of the enzyme forms $[E_2]$, $[E_2X]$, $[E_3]$, $[E_3Y]$, and $[E_2CE_3]$. Because of conservation of the total concentration of enzymes 2 and 3, only three of the latter are independent. Although the fluxes are also variables, they are not independent of the internal metabolite concentrations, and are expressed as functions of the latter; thus they do not appear explicitly in Fig. 2.

The numerical data displayed herein were obtained as previously [20] with the simulation program GEPASI 2 [26,27] on a personal computer equipped with an Intel 80486 processor running MS-DOS and MS-Windows 3.1.

$$\frac{d[B]}{dt} = \frac{\frac{V^f[A]}{K_{ms}} - \frac{V^r[B]}{K_{mp}}}{1 + \frac{[A]}{K_{ms}} + \frac{[B]}{K_{mp}}} - \lambda_2(k_{+2}[B][E_2] - k_{-2}[E_2X])$$

$$\frac{d[E_2]}{dt} = \lambda_3 q(k_{+3}[E_2X] - k_{-3}[E_2][C]) - \lambda_2(k_{+2}[B][E_2] - k_{-2}[E_2X]) + \lambda_8 p(k_{+8}[E_2CE_3] - k_{-8}[E_2][E_3Y])$$

$$\frac{d[E_2X]}{dt} = \lambda_2(k_{+2}[B][E_2] - k_{-2}[E_2X]) - \lambda_3 q(k_{+3}[E_2X] - k_{-3}[E_2][C]) - \lambda_7 p(k_{+7}[E_2X][E_3] - k_{-7}[E_2CE_3])$$

$$\frac{d[C]}{dt} = q(\lambda_3(k_{+3}[E_2X] - k_{-3}[E_2][C]) - \lambda_4(k_{+4}[E_3][C] - k_{-4}[E_3Y]))$$

$$\frac{d[E_3]}{dt} = \lambda_5(k_{+5}[E_3Y] - k_{-5}[E_3][D]) - \lambda_4 q(k_{+4}[E_3][C] - k_{-4}[E_3Y]) - \lambda_7 p(k_{+7}[E_2X][E_3] - k_{-7}[E_2CE_3])$$

$$\frac{d[E_3Y]}{dt} = \lambda_4 q(k_{+4}[E_3][C] - k_{-4}[E_3Y]) - \lambda_5(k_{+5}[E_3Y] - k_{5}[E_3][D]) + \lambda_8 p(k_{+8}[E_2CE_3] - k_{-8}[E_2][E_3Y])$$

$$\frac{d[D]}{dt} = \lambda_5(k_{+5}[E_3Y] - k_{-5}[E_3][D]) - \frac{V[D]}{K_m + [D]}$$

$$\frac{d[E_2CE_3]}{dt} = p(\lambda_7(k_{+7}[E_2X][E_3] - k_{-7}[E_2CE_3]) - \lambda_8(k_{+8}[E_2CE_3] - k_{-8}[E_2][E_3Y]))$$

Fig. 2. The full mathematical model. This model describes the concentrations of the internal metabolites and enzyme complexes (the variables of the model) as a function of kinetic and rate constants and of the concentration of the precursor A. The parameters λi are used to modulate the rate of the steps i. The parameter q multiplies the four constants associated with steps 3 and 4, while p multiplies the four constants associated with steps 7 and 8. In some simulations (see text) p is varied to change the proportion of flux through the channel and q is adjusted so that the net flux remains constant (to a specific accuracy). In other simulations one or both of p and q are kept constant and equal to 1. Finally, in some simulations, p and q modify a more restricted set of rate constants (see text).

Some results were checked with the program SCAMP [28,29] on the same computer.

3. Results

As previously [20], and as followed in [21], we use metabolic control analysis (MCA, [30,31], recently reviewed in [32,33]) and enzyme kinetics, together with steady-state simulations, to study the effects of metabolic channelling. Although MCA has traditionally been used for systems with independent conversions of metabolite pools, it has also been applied to specific problems of channelled systems [34–38]. Recently, it has been shown that MCA is quite capable of dealing with such systems in general if one describes the action of channelling enzymes by explicitly considering the enzyme states (with the inherent conservation of moieties) and their elementary reaction steps [39]. The first comment to make, however, as has indeed been widely stressed in the pertinent literature, is that in the MCA (and indeed generally) it is crucial to distinguish between parameters and variables [30,36,40–44]. Parameters are properties of the experimental system set either by the experimenter (e.g., temperature, pH) or by nature (kinetic and rate constants) that remain constant during an experiment (including during the transition to a steady state), whilst the latter (such as the concentrations of intermediary metabolites or the flux) only attain a constant value (apart from their characteristic fluctuations [41]) as steady state is achieved; the steady-state magnitude of any variable is determined by those of all the parameters [30]. Changes in the magnitude of any variable cannot affect the steady-state magnitude of any other variable (except for systems which are inherently multistable and in which the perturbation had pushed the system away from the original basin of attraction (see [44,46]), but these are not considered by MCA [47] and are beyond the scope of the present discussion).

Likewise, changes in the magnitude of a variable flux, or flux ratio, cannot by themselves affect the steady-state concentration of a metabolite. By contrast, changes in parameter values affect flux ratios and concentrations. In MCA, this is quantified by a response coefficient [48]. Also, and most often, both the concentrations and fluxes change when a parameter is changed. The changes in concentration and in a flux may then be compared by taking their ratio. For small modulations this leads to the co-response coefficient defined by Hofmeyr et al. [49]. Importantly, the magnitude and even the sign of a co-response depends on the parameter that is modulated.

This point is relevant here because the question raised initially was: "does increased channelling reduce the concentration of the channelled metabolite?". In view of the above, the question is ill-phrased. Instead one should ask for the 'co-response' of the intermediate metabolite concentration with the extent of channelling, i.e., how that concentration varies with channelling. However, the question is then incomplete, since it should specify which parameter is modulated so as to affect the extent of channelling. Accordingly we note that the title of the present paper must also be read with caution. The question to be addressed cannot be why and when the phenomenom of channelling of flux can decrease pool size. It must be the extent to which channelling in the transitive sense, i.e., the guiding by a certain parameter change of flux through the channel, can cause the concentration of a metabolite to change.

In this paper we shall examine the co-response of the intermediate metabolite concentration and the extent of channelling for a number of different ways of modulating the parameters of the system. We shall do this both analytically and by numerical simulations. At each point we shall treat the parameter change considered in [21] as one of the cases. One question addressed is whether indeed, the direction in which the metabolite concentration varies when channelling varies is different for different parameter changes. The pertinent question, whether there is always at least one way to increase the extent of channelling such that the concentration of the intermediate metabolite decreases at constant total flux, is addressed analytically, and answered in the affirmative.

3.1. Algebraic analyses

3.1.1. Proof that there are always ways to increase the extent of channelling such that the concentration of the intermediate metabolite decreases; the case of a linear pathway

In this subsection we shall prove analytically that for any case described by Fig. 1a, there are ways of varying channelling at constant total flux J, such that [C] decreases whilst the flux through the channel increases.

Unless noted otherwise, we shall modulate the local rates of the elementary steps in the sense of effecting equal relative changes in the forward and the reverse rate constants. For each elementary step, i, we denote such a modulation by $\mathrm{d}\ln\lambda_i$:

$$\mathrm{d}\ln\lambda_i = \mathrm{d}\ln k_{+i} = \mathrm{d}\ln k_{-i} \tag{1}$$

We now consider the modulation of the steps 3, 4, 7 and 8, parametrized by $\mathrm{d}\ln\lambda_3$, $\mathrm{d}\ln\lambda_4$, $\mathrm{d}\ln\lambda_7$, and $\mathrm{d}\ln\lambda_8$, not changing any other parameters, such that:

$$\mathrm{d}\ln\lambda_7 = \mathrm{d}\ln\lambda_8 = -\frac{v_3}{v_7}\cdot\frac{v_{+3}\cdot\mathrm{d}\ln\lambda_3 + v_{-3}\cdot\mathrm{d}\ln\lambda_4}{v_{+4}+v_{-3}} \tag{2}$$

With this modulation, the steady-state concentrations of the enzyme states (E_2, E_2X, E_3, E_3Y, and E_2CE_3) do not vary; neither does the total flux. Any increase in the channel flux is exactly compensated for by a decrease in pool flux. This may be checked by writing for each reaction rate by how much it will change when these modulations and the variations given below occur are

affected. For instance, Eqs. 2–4 allow one to demonstrate that $d\ln v_3 = v_3 d\ln \lambda_3 - v_{-3} \cdot d\ln C = -dv_7 = -v_7 \cdot d\ln \lambda_7$ and $d\ln v_3 = d\ln v_4 = d\ln \lambda_4 + \frac{v_{+4}}{v_4} \cdot d\ln C$. The corresponding variation in the concentration of the intermediate metabolite and in the pool flux amount to

$$d\ln[C] = \frac{d\ln \lambda_3 - d\ln \lambda_4}{v_{+4} + v_{-3}} \cdot v_3 \quad (3)$$

$$d\ln v_3 = \frac{v_{+4} \cdot d\ln \lambda_3 - v_{-3} \cdot d\ln \lambda_4}{v_{+4} + v_{-3}} \quad (4)$$

For this modulation, the co-response coefficient of the intermediate metabolite concentration with the channel flux is obtained by realising that $d\ln v_7 = d\ln \lambda_7$ and employing Eqs. 2 and 3:

$$\frac{d\ln[C]}{d\ln v_7} = -v_7 \cdot \frac{d\ln \lambda_3 - d\ln \lambda_4}{v_{+4} \cdot d\ln \lambda_3 + v_{-3} \cdot d\ln \lambda_4} \quad (5)$$

When λ_4 is not modulated ($d\ln \lambda_4 = 0$), this co-response is negative. Eq. 4 shows that then, decreasing λ_3 ($d\ln \lambda_3 < 0$) results in a decrease of pool flux, hence the channel flux increases (because the total flux remains constant). Consequently, this equation proves that for any set of kinetic parameters, there is *always* at least one way of increasing channelling with an associated decrease in the concentration of the intermediate metabolite.

There are other types of modulation, where the concentration of the intermediate metabolite decreases with increasing channelling. For example it may be seen by inspection of Eq. 5 that this occurs if λ_4 increases and λ_3 decreases, such that

$$d\ln \lambda_3 < \frac{v_{-3}}{v_{+4}} \cdot d\ln \lambda_4, \ (d\ln \lambda_4 > 0) \quad (6)$$

In fact, modulations where λ_4 is decreased can also lead to a decrease in [C], provided that λ_3 is decreased more than λ_4:

$$d\ln \lambda_3 < d\ln \lambda_4, \ (d\ln \lambda_4 \leq 0) \quad (7)$$

Depending on the relative magnitudes of v_{+4} and v_{-3}, this may be accompanied by an increase or a decrease in channel flux. Eq. 5 also shows that (i) for the special modulation that alters λ_3 and λ_4 by the same factor (as in [21]), [C] does not change with the extent of channelling, and (ii) there are many modulations that will increase [C] with increasing channelling flux.

3.1.2. Proof that there are always ways to increase the extent of channelling such that the concentration of the intermediate metabolite decreases: the case of a branch from the pathway

One of the reasons why channelling of a metabolite could be 'useful' for an organism is if that metabolite is unstable or if it would be consumed by an unwanted, competing pathway. In those cases, a reduction of the concentration of the intermediate metabolite (pool) would reduce the amount of material diverted to the competing pathway (it could be a simple side-reaction). This will be even more important if the branch pathway produces some toxic substance. If channelling is able to decrease the concentration of intermediate metabolite available for the competing pathway this would reduce the flux in that pathway. Consequently, it is important to consider the generalization of Fig. 1a to the case in which the metabolite C is removed by an additional step (competing with step 4). For simplicity we shall assume this reaction to be of first order in the concentration of C and effectively irreversible, i.e.,

$$v_B = \lambda_B \cdot k_B \cdot [C] \quad (8)$$

Steady state requires:

$$v_4 = v_3 + v_B \quad (9)$$

We now consider the modulation analogous to that given by Eq. 2:

$$d\ln \lambda_7 = d\ln \lambda_x = -\frac{v_3 \cdot v_{-4} \cdot d\ln \lambda_3 + v_4 \cdot v_{-3} \cdot d\ln \lambda_4}{v_7 \cdot (v_{-4} + v_{-3})} \quad (10)$$

with additionally:

$$d\ln \lambda_B = \frac{v_4 \cdot d\ln \lambda_4 - v_3 \cdot d\ln \lambda_3}{v_{+4} + v_{-3}} \quad (11)$$

i.e., the branching rate constant λ_B is modulated such that the branch flux does not change, hence:

$$dv_3 = dv_4 \quad (12)$$

and

$$d\ln[C] = -d\ln \lambda_B \quad (13)$$

This modulation is again constructed in such a way that the steady-state concentrations of the enzyme forms and the net rate of any chemical species (metabolites and enzyme forms) remains zero. For instance, that $dv_4 = -dv_8$ can be shown as follows: the rate of step 4 is given (see Fig. 1) by:

$$v_4 = v_{+4} - v_{-4} = \lambda_4 (k_{+4}[C][E_3] - k_{-4}[E_3Y]) \quad (14)$$

By differentiation of Eq. 14 one finds:

$$d\ln v_4 = d\ln \lambda_4 + \frac{v_{+4}}{v_4} d\ln[C] \quad (15)$$

Using Eq. 10, Eq. 11, Eqs. 13 and 14, one finds:

$$d\ln v_8 = d\ln \lambda_8 = -\frac{v_4}{v_8} d\ln v_4 \quad (16)$$

The co-response of v_8 with [C] for this modulation can be written as:

$$\frac{d\ln v_8}{d\ln[C]} = -\frac{v_4}{v_8} \frac{d\ln v_4}{d\ln[C]} \quad (17)$$

using Eq. 15:

$$\frac{d\ln v_8}{d\ln[C]} = -\frac{v_{-4}}{v_8} - \frac{v_4}{v_8}\frac{d\ln\lambda_4}{d\ln[C]} \quad (18)$$

and from Eq. 13:

$$\frac{d\ln v_8}{d\ln[C]} = -\frac{v_{-4}}{v_8} + \frac{v_4}{v_8}\frac{d\ln\lambda_4}{d\ln\lambda_B} \quad (19)$$

Considering the case in which step 4 is not changed ($d\ln\lambda_4 = 0$), this equation proves that there is at least one type of modulation in which channelling flux increases accompanied by decreasing concentration of the intermediate metabolite, viz. $d\ln\lambda_3 < 0$. Together, Eqs. 19 and 11 allow one to select more modulations for which this occurs. This leads to (assuming that $v_{+4} > v_{-3}$):

$$\frac{d\ln\lambda_3}{d\ln\lambda_4} < -\frac{v_{-3}\cdot v_4}{v_3\cdot v_{+4}} \quad (20)$$

or

$$\frac{v_4}{v_3} < \frac{d\ln\lambda_3}{d\ln\lambda_4} \quad (21)$$

A special type of modulation equally affects steps 3 and 4 (i.e., $d\ln\lambda_3 = d\ln\lambda_4$). Eqs. 11 and 13 then lead to:

$$\frac{d\ln[C]}{d\ln\lambda_3} = \frac{v_B}{v_{+4} + v_{-3}} \quad (22)$$

$$\frac{d\ln v_8}{d\ln[C]} = -\frac{v_{-4}}{v_8} - \frac{v_4\cdot(v_{+4} + v_{-3})}{v_8\cdot v_B} \quad (23)$$

$$\frac{d\ln[C]}{d\ln v_8} = -\frac{v_3\cdot v_B}{v_3\cdot v_{+4} + v_4\cdot v_{-3}} \quad (24)$$

For fluxes in the forward direction, the co-response of [C] and v_8 (Eq. 24) is always negative: intermediate metabolite concentration decreases with increasing channelling flux.

3.1.3. The modulation that keeps the concentration of the intermediate metabolite constant while increasing channelling flux is a special case

In Ref. [21], the authors sought to prove analytically that in the model of Fig. 1a variations in the extent of channelling (varied by changing p, see Fig. 2) at constant total flux (maintained by changing q, see Fig. 2) could not in principle affect the concentration of the pool intermediate C, and indeed claimed to have obtained a 'general proof' to this effect. The analytical proofs in the preceding sections seem to be in conflict with this claim. What is then the source of this apparent discrepancy?

Whilst we do not take issue with the *reasoning* of the proof in [21], it transpires that the algebraic analysis in [21] is not in fact general for the case where one changes the proportion of flux through the channel while maintaining a constant net flux. The reason why the derivation in [21] is not general is that it possesses the following important and unnecessary set of constraints: one of the modulation parameters (q) has to affect k_{+3}, k_{-3}, k_{+4} and k_{-4} by the same factor (so here $q = \lambda_3 = \lambda_4$), and the other ($p$) to affect k_{+7}, k_{-7}, k_{+8}, and k_{-8}, also by the same factor ($p = \lambda_7 = \lambda_8$); in the language of MCA, as in fact mentioned in [21], the elasticities of each of these steps towards the parameter that affects them is unity:

$$\epsilon_q^3 = \epsilon_q^4 = \epsilon_p^7 = \epsilon_p^8 = 1 \quad (25)$$

where p and q are the two parameters of the double modulation.

There are, however, other perfectly feasible double modulations which are not subject to the constraint of Eq. 25: for instance, we can affect both steps 7 and 8 (and 3 and 4) by changing only k_{+7} and k_{-8} by a factor p (and k_{+3}, k_{-3}, k_4, k_{-4} by a factor q) and *not* k_{-7} and k_{+8}. In this case, the elasticity coefficient of steps 7 and 8 towards p are different from 1. It should be noted that this modulation does not violate microscopic reversibility. Following the same steps taken in [21] (described in their Eqs. (1)–(9)), we obtain the relation (see Appendix 1):

$$\frac{d\ln[C]}{d\ln p} = C_7^{[C]}\varepsilon_p^7 + C_8^{[C]}\varepsilon_p^8 - \frac{\left(C_3^{[C]} + C_4^{[C]}\right)\left(C_7^{J_{net}}\varepsilon_p^7 + C_8^{J_{net}}\varepsilon_p^8\right)}{C_3^{J_{net}} + C_4^{J_{net}}} \quad (26)$$

where $C_7^{[C]}$ is the concentration-control coefficient of metabolite C with respect to the rate of step 7, ε_p^7 is the elasticity coefficient of step 7 with respect to the parameter p, and so on.

In contrast to Eq. 9 of [21], there is nothing we can say about the right-hand side of Eq. 26. While we cannot prove analytically that this expression is generally different from zero, below we shall show by numerical simulation that it is often not zero.

Similarly, we may make a double modulation in which only steps 3 and 7 are affected (by multiplying k_{+3} and k_{-3} with q and by multiplying k_{+7}, k_{-7} with p); thus the elasticity coefficients of step 4 towards q and of step 8 towards p are zero, since they are not affected by these parameters. Given this, a similar derivation to that in [21] results in:

$$\frac{d\ln[C]}{d\ln p} = C_7^{[C]} - C_3^{[C]}\frac{C_7^{J_{net}}}{C_3^{J_{net}}} \quad (27)$$

The reasoning used for Eq. 26 also applies for Eq. 27: the right-hand side cannot be proven always to be zero, but we shall also show by simulation cases in which it is not zero.

There are yet further methods for maintaining the net flux constant, which do not require double modulations; in this case a derivation along the lines of that in [21] does not seem to be possible because there is simply no second

parameter being adjusted! Thus, however superficially persuasive it might appear, the so-called 'general proof' of [21] is not: it only applies to the special cases in which the Eq. 25 holds.

3.1.4. Numerical results at constant net flux

In the analysis in [21], the steady-state flux was engineered to be constant (to several decimal places) by adjusting the rate constants of steps 3 and 4, multiplying them by a factor q for each value of p (this corresponds to $d\ln p = d\ln\lambda_7 = d\ln\lambda_8$ and $d\ln q = d\ln\lambda_3 = d\ln\lambda_4$ in Section 3.1.1 and Section 3.1.2. As discussed above, however, there are other ways of fixing the net flux while modulating the proportion passing through the channel. One such way is to modify the first step to make its rate to be constant (i.e., of zero order with respect to its own substrate and product) such that we effectively fix the input flux, instead of fixing the concentration of the first substrate [50]. This corresponds to ('velocity-induced') pathways that have a constant input of substrate, rather than being 'reservoir-induced' by having a constant concentration of initial substrate, the internal metabolite concentrations adjust to a preset flux. (This distinction is equivalent to the contrast, that is made explicit in electrical circuit theory, between using a constant current source and a constant voltage source; see, for example, [51,52].) Imposing a constant flux in this way can also be done at other points in the pathway, such as the last step. That case corresponds to pathways that have a constant output of product, rather than constant concentration of product. In each case, the net flux in the steady state will be equal to that of the zero-order step.

Other ways of varying the flux through the channel whilst maintaining a fixed value of net flux include changes in some rate constants of the channel branch (steps 7 and 8 in Fig. 1) compensated by changes in other rate constants of the pool branch (steps 3 and 4 in Fig. 1). These will differ from the double modulations of [21] whenever all of the 8 kinetic constants involved are not changed simultaneously. Particularly, we have done this (i) by changing k_{+7} and k_{-8}, and compensating with q, (ii) by changing k_{+3} and k_{-3}, and compensating with k_{+7} and k_{-7}, and (iii) by imposing constant flux as described above.

3.1.4.1. Modulation of complex stability. Another way of modulating the proportion of flux passing through the channel is to vary k_{+7} and k_{-8} (whilst keeping their ratio constant in order to maintain the same thermodynamic constraints). In order to keep the total flux constant, we adjust q, which multiplies k_{+3}, k_{-3}, k_{+4} and k_{-4} (as in [21]). This differs from the modulations described in [21] in that in that paper the proportion of flux passing through the channel was modulated solely by increasing p (which is the same as increasing k_{+7}, k_{-7}, k_{+8} and k_{-8} by the same factor). Changing k_{+7} and k_{-8} affects the stability of the E_2CE_3 complex, in contrast to increasing p which just makes steps 7 and 8 faster (closer to equilibrium) and does not affect the stability of the complex. (Such an effect on k_{+7} and k_{-8} might easily be considered in evolutionary terms to be the result of a single-site mutation in the gene(s) coding for either E_2 or E_3, which would be a simple mechanism for affecting the stability of E_2CE_3 – see Fig. 1a.) Representative data are given in Fig. 3, where it may be seen that it is indeed quite possible to vary the extent of channelling at constant total (net) flux but nonetheless simultaneously effect a decrease in the steady-state value of [C] (cf. [53]). An increased stability of the E_2CE_3 complex, i.e., of the dynamic channel, causes a smaller pool size.

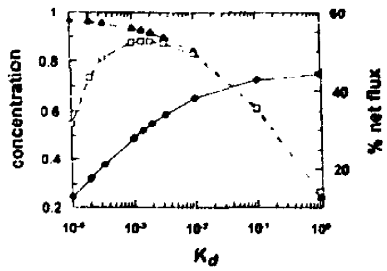

Fig. 3. Steady-state concentration of the pool metabolite C, enzyme complex E_2CE_3, and percentage of flux through the channel as a function of the dissociation constant of the E_2CE_3 complex. The degree of channelling is varied by changing k_{+7} and k_{-8} (obeying the relation $k_{-8} = k_{+7}/10$), simultaneously the net flux is kept constant by adjusting q (which multiplies k_{+3}, k_{-3}, k_{+4} and k_{-4}). Other parameters: $V^f = V^r = K_{mp} = K_{mp} = 1$ (step 1); $p = k_{+2} = k_{-2} = k_{+5} = 1$; $k_{+3} = k_{-3} = k_{+4} = 0.4$; $k_{-4} = 0.04$; $k_{+6} = 10$; $k_{-7} = k_{-8} = 0.1$; $V = 100$, $K_m = 1$ (step 6); $[A] = 10$; $[E_2]_{tot} = [E_3]_{tot} = 1$. The K_d values are for the dissociation of E_2CE_3 by step 7, the dissociation of this species via step 8 has K_d values one order of magnitude lower. The J_{net} values were fixed at 0.169067278 (+/−0.000000059%). q varies from 0.652369577 ($K_d = 1$) to 115.883115 ($K_d = 0.0001$). Closed circles represent $[C]_{ss}$, closed triangles $[E_2CE_3]_{ss}$, and open squares the steady-state channel flux.

3.1.4.2. Modulation of steps 3 and 7. Modulating the proportion of flux passing through the channel whilst maintaining the net flux constant by varying k_{+3} and k_{-3} compensating with an appropriate change in k_{+7} and k_{-7} (leaving the other steps unchanged), could be effected in nature by a suitable point mutation in the gene encoding E_2 or E_3. A simulation in which this was carried out is displayed in Fig. 4. Again it is easy to find conditions in which one can vary the extent of channelling at constant total (net) flux and simultaneously reduce the pool size. In this particular case one may observe that a rather small increase of channel flux (from 74 to 97% of the total flux) is accompanied by a decrease of nearly two orders of magnitude in $[C]_{ss}$.

One can effectively do the complementary type of modulation to that described in the previous paragraph (to vary the proportion of flux passing through the channel whilst maintaining the net flux constant) by varying k_{+4} and k_{-4} and then compensating by an appropriate change in k_{+8} and k_{-8}. This could also be effected in nature by a

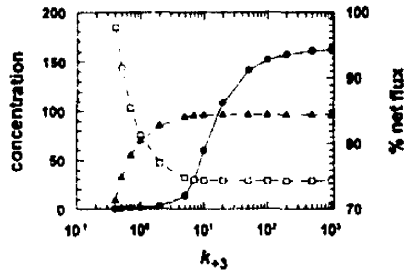

Fig. 4. Steady-state concentration of pool metabolite C, enzyme complex E_2CE_3, and percentage of flux through the channel as a function of k_{+3}. The degree of channelling is varied by changing k_{+3} and k_{-3}, simultaneously the net flux is kept constant by adjusting k_{+7} and k_{-7} ($k_{-3} = k_{+3}$ and $k_{-7} = k_{+7}/10$). Other parameters: $V^f = K_{ms} = 1$, $V^r = 10^{-5}$, $K_{mp} = 100$ (step 1); $p = q = k_{+2} = k_{-4} = k_{+5} = 1$; $k_{-2} = k_{+4} = k_{+8} = k_{-8} = 0.1$; $k_{-5} = 10$; $V = K_m = 1$ (step 6); [A] = 10; $[E_2]_{tot} = [E_3]_{tot} = 1$. The J_{net} values were fixed at 0.277106896 (+/-0.000000027%). Closed circles represent $[C]_{ss}$, closed triangles $100 \times [E_2CE_3]_{ss}$ and open squares the steady-state channel flux.

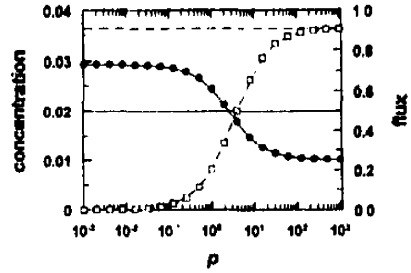

Fig. 5. Steady-state concentration of pool metabolite C and percentage of steady-state flux through the channel as a function of p at constant supply of substrate. The degree of channelling is varied by setting the parameter p to increasing values; the net flux is constant due to the fixed rate of the first step. Other parameters: $v = 0.9088$ (step 1); $k_{+2} = k_{+3} = k_{-3} = k_{+4} = k_{+5} = k_{-5} = k_{+7} = 1000$; $q = k_{-2} = k_{-4} = k_{-7} = k_{+8} = k_{-8} = 1$; $V = K_m = 1$ (step 6); [A] = 10; $[E_2]_{tot} = [E_3]_{tot} = 1$. Closed circles represent $[C]_{ss}$ and open squares the flux through the channel (steps 7 and 8). The solid horizontal line represents the value of $[C]_{ss}$ for the pathway of Fig. 1b (without channelling) for the same parameters as above (except that steps 7 and 8 do not exist, neither does E_2CE_3), the horizontal broken line represents the value of the net flux for the same model.

suitable point mutation in the genes encoding E_2 or E_3. The data from simulations in which this was carried out (not shown) indicate that one can again easily find conditions in which one can vary the extent of channelling at constant total (net) flux but nonetheless simultaneously effect a substantial change in $[C]_{ss}$ (see also Section 1.1).

3.1.4.3. Inflow controlled pathway. We now consider the case in which the pathway is supplied with substrate (via reaction 1) at a constant rate, which we set to an arbitrary value. In the mathematical model (Fig. 2) this is done by substituting the term

$$\frac{\frac{V^f[A]}{K_{ms}} - \frac{V^r[B]}{K_{mp}}}{1 + \frac{[A]}{K_{ms}} + \frac{[B]}{K_{mp}}}$$

by the constant 0.9088 (the desired value of steady-state flux). In this case the net flux of the pathway in the steady state is truly constant at different values of p, under conditions in which, *most importantly*, *no* other parameters of the model need to be adjusted. Since the only parameter that is changing in these simulations is p any effect on any variable that is observed can safely be attributed to channelling. Fig. 5 details the effects of changing p under these conditions on both the pool size and the magnitudes of the fluxes. It is clear that depending on the extent of channelling, the concentration of the pool intermediate can double or halve, relative to the pathway lacking a channel.

A related way of imposing constant flux is by setting the last step (6) of the pathway to a constant rate. This is achieved by substituting the term $\frac{V[D]}{K_m + [D]}$ by the desired value of steady-state flux. Once again, we found circumstances in which when p is varied, the co-response of $[C]_{ss}$ and channel flux was not zero (data not shown).

3.1.5. Effectiveness of channelling in decreasing the pool when the net flux is allowed to vary

Although it has been previously shown that channelling is able to decrease an intermediate metabolite pool size when the flux is allowed to change [20], the significance of the numerical results shown could be gainsaid on the grounds that the concentration that had been decreased by (in that case) three orders of magnitude was already small in the absence of a channel, such that reducing it further would be meaningless in practical terms. In Table 1 we show data for a different numerical case in which the conditions are such that the concentration of the intermediate without the channel is relatively high but which is decreased by one order of magnitude when the channel is operating (with high values of p). As previously [20] we define the coefficient as the ratio of the concentration of the intermediate C at a certain value of p to the concentration of C without the channel ($\alpha = [C]/[C]_0$), similarly the

Table 1
Modulation of the degree of channelling by p at variable net flux

p	$[C]_{ss}$	J_{net}	$J_{channel}$
0	0.6904590	0.5614159	0.0000000
0.001	0.8225023	0.2879963	0.0000599
0.01	0.8221078	0.2883956	0.0005988
0.1	0.8181458	0.2923762	0.0059792
1	0.7769361	0.3309076	0.0588740
10	0.3681878	0.5892023	0.4544255
100	0.0644146	0.7418649	0.7301332
1000	0.0458458	0.7522876	0.7511557
10000	0.0441450	0.7532643	0.7531515

The degree of channelling is modulated by the parameter p (that multiplies k_{+7}, k_{-7}, k_{+8} and k_{-8}) allowing the net flux to act as a true variable. Other parameters: $V^f = V^r = K_{ms} = K_{mp} = 1$ (step 1); $q = k_{+2} = k_{-3} = k_{+3} = k_{-4} = k_{+5} = k_{+7} = 1$; $k_{-2} = k_{-4} = k_{-7} = k_{+8} = k_{-8} = 0.1$; $k_{+5} = 10$; $V = K_m = 1$ (step 6); [A] = 10; $[E_2]_{tot} = [E_3]_{tot} = 1$.

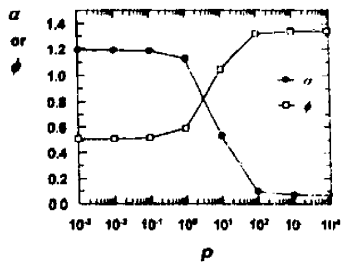

Fig. 6. Effect of parameter p on the relative steady-state concentration of pool metabolite C and relative steady-state J_{net}. As detailed in the text, α is the value of $[C]_{ss}$ divided by $[C]_{ss}$ when $p = 0$ (no channelling) and ϕ is the value of J_{net} divided by J_{net} when $p = 0$.The proportion of flux through the channel is modulated via parameter p. Other parameters as listed in the legend of Table 1.

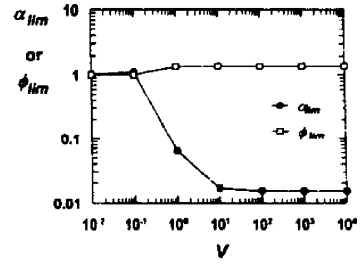

Fig. 7. Maximal relative steady-state concentration of pool metabolite C (α_{lim}) and maximal relative steady-state J_{net} (ϕ_{lim}) as a function of V. At each value of V, α_{lim} and ϕ_{lim} were arbitrarily taken to be α and ϕ at $p = 10000$ which, in this case, slightly underestimates the true value of $\alpha_{p\to\infty}$ and $\phi_{p\to\infty}$. All other parameters as described in Table 1.

coefficient ϕ is defined as the ratio of total (net) flux at a certain value of p to the total flux without the channel. Fig. 6 shows the dependence of α and ϕ on p for the data of Table 1. The value of α when p tends to infinity, α_{lim}, is a function of (but not only of) the catalytic capacity of the last step (V in this model), as shown previously for other numerical cases [20]. The higher V is, the more effectively does the addition of a channel decrease the intermediate pool concentration. Such a dependence is shown in Fig. 7. In this particular case, channelling only has a substantial effect when V is higher than approximately 1; when it is equal to or higher than 10, the reduction in the concentration of the pool intermediate is by two orders of magnitude. Thus we see from the data displayed in Table 1 and Figs. 6 and 7 that it is possible to have a channel that reduces an originally large concentration of the free (pool) metabolite by two orders of magnitude.

4. Discussion

The literature is rich in suggestions for the possible advantages of metabolic channelling for cellular metabolism (e.g., [11–18]). Nonetheless, however attractive the term 'advantage' might be, we think it is more appropriate at this stage to think simply in terms of the 'effects' or 'consequences' of metabolic channelling [54].

More specifically, the issue addressed here is: can an increased proportion of channelling flux be accompanied by decreased concentration of the intermediate metabolite? In a previous paper [20] we showed numerically that for some examples it can.

Our previous results [20] were challenged in [21] by arguing that the changes in pool size that we had observed when modulating the extent of channelling were due to changes in the net flux. This interpretation is based on the fallacy that one variable (in this case the net flux) can control another variable (the pool size). This is a very important issue which (in a biochemical context) has been addressed by Metabolic Control Analysis (e.g., [47]): changes in the initial concentration of any variable cannot affect the steady-state magnitude of any other variable (except for systems which are inherently multi-stable and in which the perturbation pushes the system away from the original basin of attraction – see [45,46]). In other words, no changes in the steady-state magnitude of a variable (in this case the concentration of the intermediate metabolite) can be accounted for in terms of being caused by other variables (the net flux), since they are both effects rather than causes. Effects can only be accounted for by (changes in) parameters. Changes in q, which is also a parameter, can cause changes in intermediate concentrations as well. Simultaneous changes in p and q designed to make the net flux constant means that any effects are due to changes in *both p and q*.

Consequently, when the interest lies in two variables of the system (i.e., the proportion of channelling flux and the intermediate metabolite concentration) one is really asking for a comparison of the responses of the two variables to the parameter change. Hofmeyr et al. [49] have defined the infinitesimal equivalent of this comparison as the co-response coefficient.

In general, co-responses depend on the parameters that are modulated. By stating that they have given a general proof that channelling has no effect on the intermediate metabolite concentration at constant net flux, the authors of [21] suggested otherwise. Is the co-response of channelling flux and intermediate metabolite concentration indeed an exception and independent of which parameters are changed?

Both analytically and numerically the present paper disproves this possibility: the variation of the intermediate metabolite concentration with channelling depends greatly on how channelling is increased. Indeed, [C] can rise, fall or remain constant, with increased channelling flux (at constant total flux).

Accordingly, we have shown analytically that for any implementation of the model of Fig. 1a, there are ways to increase the channelling flux and decrease the concentration of the intermediate metabolite C at constant net flux. This answers the issue raised (can channelling be accom-

panied by a decreased intermediate metabolite concentration?) in the affirmative.

In [21], it was assumed that the only legitimate way of enhancing channelling flux is to simultaneously decrease the rate constants k_{+3}, k_{-3}, k_{+4}, and k_{-4}, by the same factor and increase the rate constants k_{+7}, k_{-7}, k_{+8}, and k_{-8}, by another factor. In particular the authors criticised calculations in which we had changed k_{+3} and k_{-4}, at constant k_{+3}/k_{-3}, and k_{+4}/k_{-4}, adjusting p to maintain the net flux constant. The authors of [21] took refuge in an incomplete representation of the present Fig. 1b and 'demonstrated' that in that figure, where channelling is absent, the same parameter change would also lead to a decrease in [C]. In [21], this was attributed to "the cross-over effect of changing relative activities of two enzymes". The attribution was necessarily incorrect, since the cross-over effect is defined [23] (and see [24,25,55]) as the relative effect of the modulation of the activity of a *single* enzyme on metabolites upstream and downstream of that enzyme. Such a modulation does change the total flux and is hence inconsistent with the boundary conditions that we imposed and to which the calculations met essentially. In the present paper we have presented additional modulations, that do not correspond even to what were referred to as 'cross-over effects' in [21]. For instance, increasing the stability of the E_2CE_3 complex (Fig. 3) can lead to a negative co-response of channelling flux and intermediate metabolite concentration, and so could simultaneous and equal modulation of k_{+7}, k_{-7}, k_{+8}, and k_{-8} at constant supply of substrate (Fig. 5). Using a temporal analysis, Easterby [60] has also pointed to some of the fallacies of the model in [21].

Perhaps most revealing is our result that even the modulation of channelling proposed in [21] leads to a decrease in intermediate metabolite concentration with increased channelling (see Eq. 24), except in the special case of no consumption of the intermediate C by side-reactions. Notably, the modulation applied here is the same as that proposed in [21] with the provision that, in this more general case of a branch from the pathway, not only the net pathway flux but also the branch step flux is kept constant.

For a genuine study of the effect of channelling on a pool size we must modulate *only* p. If changes in p also affect the net flux this merely confirms that flux is a variable, not a parameter. Therefore the correct conclusion is that channelling *can* decrease the pool size [20] and increase the flux (contrary also to what was stated by [56]). Heinrich and Schuster [57], based on a similar model to that of [21] in which the conservation of enzyme species was ignored, came to the same conclusion as the latter authors (i.e., that channelling would not be able to decrease the pool size at constant overall flux). Nonetheless, they also stated their belief that "(...) flux enhancement is likely to be the most important advantage of channelling (...)" [57]. It seems obvious to us that if the question of interest is whether adding a channel to an otherwise 'naked' pathway affects concentrations or fluxes, cases in which the net flux is arbitrarily forced to be constant by simultaneously modulating other rate constants are rather irrelevant. At all events, we have shown that the way in which the flux is controlled (at a constant value) influences the outcome of the experiment (Figs. 3–5).

A similar argument to that in [21] was previously raised in [57], but this was based on a unrealistic model of dynamic channelling. In the latter paper it was considered that channelling consists of a simple branch separating a pool mechanism from a direct transfer mechanism, converging again further downstream. However this is an incorrect assumption as it ignores mass conservation relations and the fact that a substrate-enzyme complex reacts with a free enzyme (step 7 in our model, see Fig. 1). Also here, only if all the rate constants of one branch were multiplied by the same scalar did the pool size remain constant at variable channel flux. As pointed out in [57], double modulations of the type described in [21] result in an effective 'decoupling' of the branch where the intermediate is released to the 'bulk' solution from the channel [57]; in such conditions varying the parameters on the channel branch cannot affect variables on the pool branch. This results, in fact, in a circular argument in which one ensures that there will be no changes in the pool size (by applying such a special modulation) to prove that the pool size does not change.

In summary, we may conclude that the effectiveness of channelling in decreasing a pool, even at constant flux, is very much a reality.

Acknowledgements

We thank Boris Kholodenko and Athel Cornish-Bowden for discussions. We thank the following for financial support: Junta Nacional de Investigação Científica e Técnologica, Portugal (Grant BD-197/90-IF) (to P.M.), The Wellcome Trust and the Biotechnology Directorate of the Science and Engineering Research Council, U.K. (to P.M. and D.B.K.) and the Netherlands Organization for Scientific Research, N.K.I. (to H.V.W.).

Appendix A

A.1. Derivation of Eqs. 26 and 27

We begin by writing down the relationships between the logarithmic changes in net flux J_{net} and the parameters p and q, and of the the logarithmic changes in the concentration of the pool intermediate C ([C]) and the parameters p and q:

$$\mathrm{d}\ln J_{net} = \left(C_7^{J_{net}}\varepsilon_p^7 + C_8^{J_{net}}\varepsilon_p^8\right)\mathrm{d}\ln p + \left(C_3^{J_{net}}\varepsilon_q^3 + C_4^{J_{net}}\varepsilon_q^4\right)\mathrm{d}\ln q \quad \text{(A1)}$$

$$d\ln[C] = \left(C_7^{[C]}\varepsilon_p^7 + C_8^{[C]}\varepsilon_p^8\right)d\ln p + \left(C_3^{[C]}\varepsilon_q^3 + C_4^{[C]}\varepsilon_q^4\right)d\ln q \quad (A2)$$

where C_i^j represents the control coefficient of variable j with respect to step i defined as follows:

$$C_i^j = \frac{d\ln j}{d\ln v_i} \quad (A3)$$

and ε_k^i the elasticity coefficient of step i with respect to parameter k defined as follows:

$$\varepsilon_k^i = \frac{d\ln v_i}{d\ln k} \quad (A4)$$

For a double modulation to be effected at constant net flux ($d\ln J_{net} = 0$), q must be adjusted following a change in p. Setting the RHS of Eq. A1 to zero and solving for $d\ln q/d\ln p$, one obtains:

$$\frac{d\ln q}{d\ln p} = -\frac{C_7^{J_{net}}\varepsilon_p^7 + C_8^{J_{net}}\varepsilon_p^8}{C_3^{J_{net}}\varepsilon_q^3 + C_4^{J_{net}}\varepsilon_q^4} \quad (A5)$$

Using Eq. A5, one can recast Eq. A2 as:

$$\frac{d\ln[C]}{d\ln p} = C_7^{[C]}\varepsilon_p^7 + C_8^{[C]}\varepsilon_p^8 - \frac{\left(C_3^{[C]}\varepsilon_q^3 + C_4^{[C]}\varepsilon_q^4\right)\left(C_7^{J_{net}}\varepsilon_p^7 + C_8^{J_{net}}\varepsilon_p^8\right)}{C_3^{J_{net}}\varepsilon_q^3 + C_4^{J_{net}}\varepsilon_q^4} \quad (A6)$$

If the elasticities of each of the steps 3 and 4 to q and of the steps 7 and 8 to p are unity, Eq. A5 reduces to:

$$\frac{d\ln[C]}{d\ln p} = C_7^{[C]} + C_8^{[C]} - \frac{\left(C_7^{[C]} + C_8^{[C]}\right)\left(C_7^{J_{net}} + C_8^{J_{net}}\right)}{C_7^{J_{net}} + C_8^{J_{net}}} \quad (A7)$$

which is the same as Eq. (5) of [21], and then (and only then) one could carry on and arrive at Eq. (9) of [21], i.e., $\frac{d\ln[C]}{d\ln p} = 0$.

We obtain Eqs. 26 and 27 by setting the relevant elasticities in Eq. A7 to zero and 1, as follows:If $\varepsilon_p^7 = \varepsilon_q^8 \neq 1, \varepsilon_q^3 = \varepsilon_q^4 = 1$ we have:

$$\frac{d\ln[C]}{d\ln p} = C_7^{[C]}\varepsilon_p^7 + C_8^{[C]}\varepsilon_p^8 - \frac{\left(C_3^{[C]} + C_4^{[C]}\right)\left(C_7^{J_{net}}\varepsilon_p^7 + C_8^{J_{net}}\varepsilon_p^8\right)}{C_3^{J_{net}} + C_4^{J_{net}}} \quad (A8)$$

If $\varepsilon_p^8 = \varepsilon_q^4 = 0, \varepsilon_q^3 = \varepsilon_p^7 = 1$ we have:

$$\frac{d\ln[C]}{d\ln p} = C_7^{[C]} - \frac{C_3^{[C]}C_7^{J_{net}}}{C_3^{J_{net}}} \quad (A9)$$

which are respectively Eqs. 26 and 27 of the main body of the text. In the case of both Eq. A8 and Eq. A9, one cannot use the branch theorems [25,58] to further simplify them, in contrast to the special case of Eq. A7.

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