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How to reveal various aspects of regulation in group-transfer pathways

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Abstract

For an 'ideal' metabolic pathway, genetic modulation of the enzyme concentration and titration with different types of specific inhibitor all leads to the determination of the same quantitative indicator of the extent to which an enzyme controls the flux and metabolite concentrations: the control coefficient with respect to the enzyme concentration. By contrast, for a group-transfer pathway these methods reveal various modes of the control exerted by an enzyme on the flux and on the concentrations of pathway components. Modulation of gene expression allows one to determine the (classical) control coefficient with respect to the enzyme concentration. Titration with inhibitors (competitive or uncompetitive) that do not bind to enzyme-enzyme complexes leads to information on the classical control coefficient of the inhibited enzyme and on the relative concentrations of its different forms. Should such inhibitors be irreversible, the classical control coefficients can be measured directly. Titration with a purely non-competitive inhibitor (binding to all the complexes of the target enzyme) reveals the *impact* control coefficient, a measure of the total kinetic effect of that enzyme on the system. Combined approaches applied to intact systems will detect an expected variety of control properties that cannot be measured after the system has been disassembled.

Keywords: Group-transfer pathway; Control coefficient; Inhibitor analysis; Metabolic control analysis

1. Introduction

Recent developments in metabolic control theory have addressed highly organized cellular structures involving enzyme–enzyme interactions and complex formation [1–6]. The control properties of these cellular systems may differ drastically from those of 'ideal' metabolic pathways. In particular, it was shown [5], that the control exerted by enzymes on the flux in a group-transfer (or relay) pathway can add up to 2 rather than the 1 expected by the traditional theory.

Group-transfer pathways are important features of cellular metabolic and signal transduction systems (see [5] and references therein). A group-transfer pathway can be considered as a perfect dynamic channel in which a transferred group is not released into the bulk aqueous phase until it reaches the end of the reaction sequence. Upon bimolecular collisions the adjacent enzymes may form the enzyme-enzyme complexes. In a simple control analysis treatment of [5], however, the mean lifetime of these complexes was considered as negligibly small.

In the companion paper [7] we developed a comprehensive control theory for such pathways and showed the functional consequences of changes in the lifetime of enzyme-enzyme complexes for the control. In the present paper we show how, in principle, the control properties of the group-transfer pathway can be measured. We consider genetic modulation of the concentrations of pathway components, titration with specific inhibitors of the enzymes, as well as other external modulations. Importantly, the information on control obtained using different methods is not duplicative but complementary: an extended control analysis of a relay pathway should reveal aspects both of regulation and of mechanism.

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2. Results

2.1. Diverse modes of flux control as revealed by specific inhibitors

Titrations with inhibitors specific for single enzymes have been used to determine the control coefficients of enzymes in a pathway [8–12]. In 'ideal' metabolic pathways (see [27] as a review), the control coefficients determined in this manner do not depend on the particular mechanism of the inhibitor action [8,13]. That is, if the derivative of the flux with respect to the inhibitor concentration is normalized by the elasticity of the 'isolated' reaction to this inhibitor, one obtains the same value of the enzyme control coefficient for different types of specific inhibitor. Moreover, in such ideal pathways (in which reaction rates are proportional to enzyme concentrations) the latter value coincides with the value of the control coefficient measured by a direct modulation of the enzyme concentration.

We shall analyze the response of group-transfer flux to different types of specific enzyme inhibitor. There are various mechanisms of inhibition that are commonly described in terms of their effects on the initial rate of the enzyme reaction (see, e.g., [14,15]). For the pathways considered here this variety is even greater than for the enzyme reaction in 'isolation'. Since any enzyme in a group-transfer pathway interacts with the adjacent enzymes, the response of the flux depends strongly on how an inhibitor affects the enzyme-enzyme complexes.

Specific inhibitors may often not be available. Yet, the analysis presented here will clarify why the control of group transfer flux appears in different modes and how these modes can be related to each other and to the classical enzyme(-concentration) control coefficient.

Purely non-competitive inhibitors

In the traditional case where roles of enzyme and substrate are clearly separable, a non-competitive inhibitor does not displace metabolites from their binding sites in the enzyme molecule. A *purely* non-competitive inhibitor is usually considered to affect only the V_{max} in the steady-state rate equation (cf. [15]). Extrapolating this meaning of non-competitiveness to group-transfer pathways involving enzyme-enzyme interactions we shall consider such a purely non-competitive inhibitor to possess the following properties: (i) it binds to all the enzyme forms (the different states in the kinetic scheme) with the same binding constant, (ii) its binding to any enzyme form transforms the latter into an inactive state, (iii) its binding does not change the ability of the enzyme to form complexes with the adjacent enzymes of the group-transfer pathway.

Fig. 1 shows the group-transfer pathway where the enzyme i is affected by such a purely non-competitive inhibitor (I_i^{nc}, the upper index 'nc' specifies the type of the inhibitor). A group P is transferred between r pathway enzymes from the donor SP to the ultimate acceptor W. The enzyme-enzyme complexes are designated by $Q_i = E_iPE_{i+1}$. We use the same notations as in the companion paper [7], to which the reader is also referred for more detailed definitions. The expressions for the rates of the elemental steps (processes) are:

$$v_{2i-1} = k_{2i-1}^{+} \cdot E_{i-1}P \cdot E_i - k_{2i-1}^{-} \cdot Q_{i-1}$$

$$v_{2i} = k_{2i}^{+} \cdot Q_{i-1} - k_{2i}^{-} \cdot E_{i-1} \cdot E_iP, \ (i = 1,...,r+1) \qquad (1)$$

Here and below, the concentrations of different enzyme forms are denoted by the same symbols as the forms themselves (Fig. 1). The concentrations of the boundary substrates (which are taken to be constant) are designated

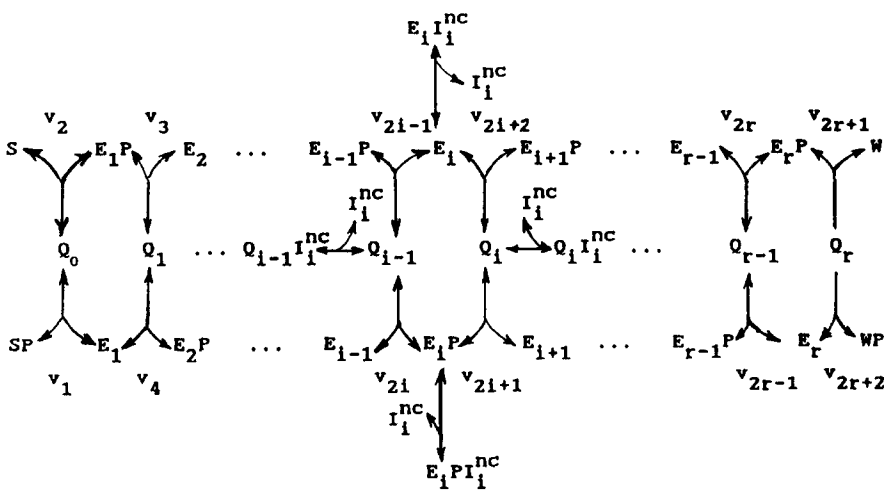

Fig. 1. Group transfer pathway where a single enzyme (i) is affected by a purely non-competitive inhibitor (I_i^{nc}). A group P is transferred between r enzymes ($i = 1, 2,..., r$) from the initial donor (SP) to the ultimate acceptor (W). E_i and E_iP are the forms respectively free and complexed with P, of enzyme i; $Q_i = E_iPE_{i+1}$ are the enzyme-enzyme complexes. $E_iI_i^{nc}$, $E_iPI_i^{nc}$, $Q_iI_i^{nc}$ are the corresponding inactive forms which have bound the inhibitor. v_1, v_2, ..., v_{2r+2} are the rates of the elemental processes.

as $E_0 = [S]$, $E_0P = [SP]$, $E_{r+1} = [W]$, $E_{r+1}P = [WP]$. At steady state, all rates are equal:

$$v_i = J, \quad \textit{for all } i = 1,2,...,2r+2 \tag{2}$$

There are r moiety-conserved cycles in a group-transfer pathway each of which corresponds to the conservation of the total concentration (e_i) of one of the r enzymes. The effect of a change in the enzyme concentration (e_i) on the group-transfer can be quantified by the enzyme(-concentration) control coefficient [16]:

$$C^J_{e_i} = \frac{dJ/J}{de_i/e_i} = \frac{d\ln|J|}{d\ln e_i}, \; C^x_{e_i} = \frac{dx/x}{de_i/e_i} = \frac{d\ln x}{d\ln e_i} \tag{3}$$

where J and x are the steady-state flux and the concentration of a pathway component, respectively.

Determination only of the enzyme(-concentration) control coefficients does not suffice for the description of the control properties of group-transfer pathways [17,27]. Also, the control coefficients of the elemental processes should be considered [7,17]:

$$C^J_{v_i} = \frac{(d\ln|J|/dp_i)_{sys}}{(\partial\ln|v_i|/\partial p_i)_{proc}}, \; C^{x_k}_{v_i} = \frac{(d\ln x_k/dp_i)_{sys}}{(\partial\ln|v_i|/\partial p_i)_{proc}} \tag{4}$$

where parameter p_i affects the process v_i only. Subscripts *sys* and *proc* refer to the different differentiation conditions; i.e., allowing all variables to change until the new steady-state is attained (*sys*) versus keeping all the variables that affect the process v_i constant (*proc*).

After a certain amount of a purely non-competitive inhibitor (I_i^{nc}) of the enzyme i has been added to a system (see Fig. 1), and the system has relaxed to a new steady state the concentrations of the inactive enzyme forms are related to the corresponding active forms by:

$$E_iI_i^{nc}/E_i = E_iPI_i^{nc}/E_iP = Q_{i-1}I_i^{nc}/Q_{i-1} = Q_iI_i^{nc}/Q_i = I_i^{nc}/K_i^{nc},$$
$$(i = 1,2,...,r) \tag{5}$$

where K_i^{nc} is the inhibition constant, and I_i^{nc} is the free inhibitor concentration. Thus, adding I_i^{nc} effectively results in the following decrease of the total active concentrations (e_{i-1}, e_i and e_{i+1}) of the enzymes i-1, i and $i+1$:

$$e_{i-1}(I_i^{nc}) = e^0_{i-1} - Q_{i-1}\cdot I_i^{nc}/K_i^{nc},$$
$$e_i(I_i^{nc}) = \frac{e_i^0}{1 + I_i^{nc}/K_i^{nc}},$$
$$e_{i+1}(I_i^{nc}) = e^0_{i+1} - Q_i\cdot I_i^{nc}/K_i^{nc},$$
$$(i = 2,3,...r-1) \tag{6}$$

where e^0_{i-1}, e_i^0 and e^0_{i+1} designate the conserved total concentrations of all the forms of the enzymes $i-1$, i and $i+1$, respectively (i.e., their total active concentrations at zero inhibitor concentration). For specific inhibitors of the initial ($i = 1$) and ultimate ($i = r$) enzymes of the pathway only the total concentrations (e_1, e_2) and (e_{r-1}, e_r), respectively, decrease according to Eq. (6), see Fig. 1.

In the steady state the flux J is a unique function of the parameters e_i (and the rate constants of the elemental steps). Hence, the response of the flux to a perturbation caused by the addition of an inhibitor can be represented in terms of the control coefficients of the perturbed enzymes, (Eq. (6)), and the corresponding elasticity coefficients ($\partial\ln e_i/\partial I_i^{nc}$), [18]:

$$R^J_{I_i^{nc}} = \frac{d\ln|J|}{dI_i^{nc}} = C^J_{e_{i-1}}\cdot\frac{\partial\ln e_{i-1}}{\partial I_i^{nc}} + C^J_{e_i}\cdot\frac{\partial\ln e_i}{\partial I_i^{nc}} + C^J_{e_{i+1}}\cdot\frac{\partial\ln e_{i+1}}{\partial I_i^{nc}} \tag{7}$$

To avoid ambiguity connected with the log derivative at $I_i^{nc} = 0$ here we use the derivative with respect to the inhibitor concentration itself rather than the logarithm thereof. Considering the initial slope (dJ/dI_i^{nc} at $I_i^{nc} \to 0$) of the titration curve ($J(I_i^{nc})$) we have from Eqs. (6), (7):

$$R^J_{I_i^{nc}}\Big|_{I_i^{nc}=0} = \left(C^J_{e_{i-1}}\cdot\frac{Q_{i-1}}{e_{i-1}} + C^J_{e_i} + C^J_{e_{i+1}}\cdot\frac{Q_i}{e_{i+1}}\right)\cdot\left(\frac{-1}{K_i^{nc}}\right),$$
$$(i = 2,3,...,r-1) \tag{8}$$

where all the concentrations correspond to zero inhibitor concentration, $I_i^{nc} = 0$. This equation shows that, even if a non-competitive inhibitor is specific for an enzyme (i), its effect on group-transfer flux is not just determined by the control coefficient of that enzyme ($C^J_{e_i}$). In fact, it may be considerably larger, as it also comprises control exerted by other enzymes complexing with the former enzyme ($C^J_{e_{i-1}}$ and $C^J_{e_{i+1}}$). This reflects the feature of group-transfer pathways that the elemental transitions usually involve two enzymes.

For the responses of the flux to specific inhibitors of the initial ($i = 1$) and ultimate ($i = r$) enzymes, we have:

$$R^J_{I_1^{nc}}\Big|_{I_1^{nc}=0} = \left(C^J_{e_1} + C^J_{e_2}\cdot\frac{Q_1}{e_2}\right)\cdot\left(\frac{-1}{K_1^{nc}}\right) \tag{9}$$

$$R^J_{I_r^{nc}}\Big|_{I_r^{nc}=0} = \left(C^J_{e_{r-1}}\cdot\frac{Q_{r-1}}{e_{r-1}} + C^J_{e_r}\right)\cdot\left(\frac{-1}{K_r^{nc}}\right), \tag{10}$$

When the group transfer does not involve significant formation of complexes of the participating enzymes, the Q's in the above equations may be neglected. Then, the response coefficient to a non-competitive inhibitor does return to the control coefficient of the target enzyme (multiplied by the inhibition constant). Notably, this is the case where the sum of the enzyme control coefficients becomes 2 [5,7].

Mimicking the effect of a purely non-competitive inhibitor as an apparent perturbation in unidirectional rate constants. Binding of an inhibitor followed by the formation of inactive enzyme-inhibitor complexes has been described

above as an apparent decrease in the total concentration of the enzyme. Appendix A shows an alternative way of viewing such an inhibition, i.e., as an apparent perturbation in the rate constants of the elemental processes, the total active concentrations of the enzymes remaining unchanged, as if no inactive enzyme forms were present. To apply such an approach to a purely non-competitive inhibitor (see Fig. 1), we represent the active concentration of all forms of the enzyme i by the following functions of the inhibitor concentration, I_i^{nc},

$$E_i/E_i^* = E_iP/E_iP^* = Q_{i-1}/Q_{i-1}^* = Q_i/Q_i^* = 1/(1+I_i^{nc}/K_i^{nc}),$$
$$(i = 1,2,...,r) \qquad (11)$$

where the concentrations denoted by asterisks are the sums of the active (e.g., Q_i) and inactive ($Q_iI_i^{nc}$) concentrations of the corresponding enzyme forms. The following properties of the 'asterisk' concentrations are important: (i) being substituted into the moiety-conservation relations they contribute to the non-perturbed total enzyme concentrations, e_i^0, at any inhibitor concentration, (ii) at $I_i^{nc} = 0$ they coincide with the non-perturbed concentrations of the corresponding forms.

Substituting the active concentrations as represented by Eq. (11) into the rate expressions (Eq. 1), one can see that only the rates v_{2i-1}, v_{2i}, v_{2i+1}, v_{2i+2} will depend on I_i^{nc}. Notably, this dependence is equivalent to decreasing all the forward and backward rate constants, k_j^+, k_j^-, $j = 2i-1$, $2i$, $2i+1$, $2i+2$, by the same factor $(1+I_i^{nc}/K_i^{nc})$:

$$k_j^+(I_i^{nc}) = \frac{k_j^+}{1+I_i^{nc}/K_i^{nc}},\ k_j^-(I_i^{nc}) = \frac{k_j^-}{1+I_i^{nc}/K_i^{nc}} \qquad (12)$$

Then the corresponding elasticities of these rates with respect to the inhibitor are equal to:

$$\frac{\partial \ln|v_j|}{\partial I_i^{nc}} = \frac{1}{1+I_i^{nc}/K_i^{nc}} \cdot \left(\frac{-1}{K_i^{nc}}\right)$$
$$(j = 2i-1, 2i, 2i+1, 2i+2) \qquad (13)$$

These Eqns. (12) and (13) demonstrate how one may mimic the effects of a purely non-competitive inhibitor by changing a proper set of unidirectional rate constants.

Relating the control by enzymes to the control by the elemental processes: the impact control coefficient. The procedure outlined above allows one to calculate the elasticity coefficients of the elemental processes with respect to the inhibitor, Eq. (13). Now we shall make use of Eq. (13) to express the response of the flux to inhibitor (I_i^{nc}) in terms of the control coefficients of the elemental processes and their elasticities to this inhibitor. Applying the response theorem, [18], one obtains:

$$R^J_{I_i^{nc}}\Big|_{I_i^{nc}=0} = \sum_{l=1}^{2r+2} C^J_{v_l} \cdot \frac{\partial \ln|v_l|}{\partial I_i^{nc}}\Bigg|_{I_i^{nc}=0}$$
$$= \left(C^J_{v_{2i-1}} + C^J_{v_{2i}} + C^J_{v_{2i+1}} + C^J_{v_{2i+2}}\right) \cdot \left(\frac{-1}{K_i^{nc}}\right)$$
$$(i = 1,2,...,r) \qquad (14)$$

Whilst Eq. (8) expressed the response to a non-competitive inhibitor into the control exerted by three adjacent enzymes, Eq. (14) expresses that response into the control by four elemental reactions (steps) in which the inhibited enzyme is involved. In line with the accompanying paper [7], equating the right-hand sides of Eqs. (8) and (14) gives useful relationships between the control by the enzyme concentrations and by the individual steps (see Appendix B).

The sum in the right-hand side of Eq. (14) represents the *impact* control coefficient of the enzyme (i) [17]. This impact control coefficient was introduced in [17] as a quantitative indicator of the total impact enzyme i has on the flux via all e_i-dependent elemental processes, i.e., v_{2i-1}, v_{2i}, v_{2i+1}, v_{2i+2} in the pathway under consideration. We conclude that titrations with non-competitive inhibitor turns out primarily to lead to this impact control coefficient:

$$^{imp}C^J_{e_i} = C^J_{v_{2i-1}} + C^J_{v_{2i}} + C^J_{v_{2i+1}} + C^J_{v_{2i+2}} = -\frac{K_i^{nc}}{J} \cdot \frac{dJ}{dI_i^{nc}}\Bigg|_{I_i^{nc}=0} \qquad (15)$$

where $dJ/dI_i^{nc}|_{I_i^{nc}=0}$ is determined by the initial slope of the titration curve. Note, that in ideal pathways such titrations would lead to the classical enzyme control coefficient ($C^J_{e_i}$) as it would be measured using the same expression in the right-hand side of Eq. (15), see [8].

Combining Eqs. (8) and (14) allows one to express the *impact* control coefficient into the classical control coefficients. Whenever the enzyme associates with its neighboring enzymes, the control exerted by the latter contributes to the impact of the former. Consequently, the *impact* control coefficient can differ drastically from the control coefficient with respect to the enzyme concentration [6,19]. Importantly, the sum of the impact control coefficients (as can be measured by using purely non-competitive inhibitors) always equals 2 for group-transfer pathways independently of the extent of formation of enzyme-enzyme complexes [6].

Purely competitive inhibitors

A purely competitive inhibitor competes with a substrate (product) for the binding site of the latter at the free

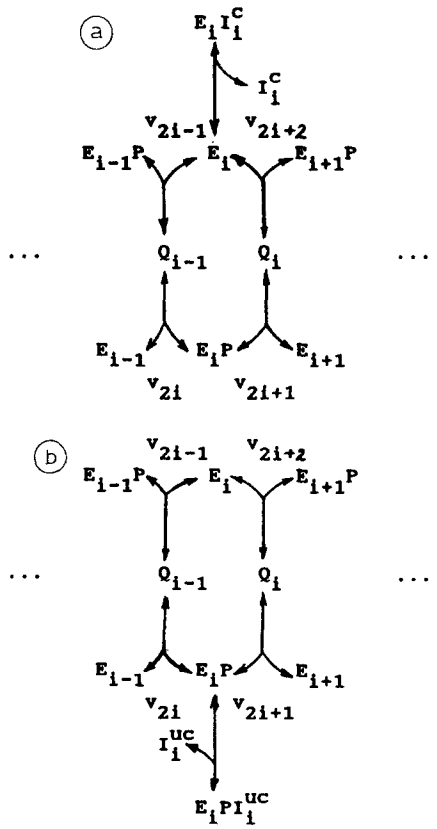

Fig. 2. Schemes of binding (a) a purely competitive inhibitor (I_i^c) and (b) a purely uncompetitive inhibitor (I_i^{uc}) to the enzyme i. $E_iI_i^c$ and $E_iPI_i^{uc}$ are the corresponding inactive forms which have bound the inhibitor.

enzyme molecule and affects only the parameter K_m of the steady-state rate equation (cf., [15]). Generalizing this definition to group-transfer pathways we shall consider such a purely competitive inhibitor (I_i^c) of the enzyme i so as to bind only to the free form, E_i, which is not complexed with any other pathway 'metabolite' (Fig. 2a). It will be assumed that binding I_i^c to E_i transforms the latter into an inactive state (which is unable to participate in the corresponding reactions of the group transfer).

Since a single inactive form exists (Fig. 2a),

$$E_iI_i^c = E_i \cdot I_i^c/K_i^c, \tag{16}$$

only one total concentration, e_i, depends on the concentration of the purely competitive inhibitor, I_i^c, with the inhibition constant, K_i^c,

$$e_i = e_i^0 - E_i \cdot I_i^c/K_i^c, \tag{17}$$

Representing the response of the flux to the perturbation (17) in terms of the control coefficient of the enzyme i we have:

$$R^J_{I_i^c}\big|_{I_i^c=0} = C^J_{e_i} \cdot \frac{E_i}{e_i} \cdot \left(\frac{-1}{K_i^c}\right) \qquad (i = 1,2,...,r) \tag{18}$$

Comparison to the corresponding effect for the non-competitive inhibitor (Eq. (8)) reveals the central aspect of this paper: even though both inhibitors are specific for the same enzyme, they do not measure the same control. The competitive inhibitor does specifically reflect the control exerted by its own target enzyme, modified by the fraction of free enzyme (Eq. 18), whereas the non-competitive inhibitor also reflects control by adjacent enzymes in the group-transfer pathway (Eq. 8).

Mimicking the effect of a purely competitive inhibitor as an apparent perturbation in two unidirectional rate constants. To analyze the inhibition of the flux by a purely competitive inhibitor, I_i^c, (Fig. 2a) as it would be caused by a perturbation in the rate constants, we write the concentration of the active form, E_i, as (see Appendix A and Eq. (16)):

$$E_i/E_i^* = 1/(1 + I_i^c/K_i^c) \tag{19}$$

and substitute it into the rate Eqn. (1) (the concentration denoted by asterisk is the sum of the active and inactive concentrations). In this case, only the rates v_{2i-1} and v_{2i+2} will depend on I_i^c, effectively through decreasing the rate constants k^+_{2i-1} and k^-_{2i+2} by the factor $(1 + I_i^c/K_i^c)$:

$$k^+_{2i-1}(I_i^c) = \frac{k^+_{2i-1}}{1 + I_i^c/K_i^c},\ k^-_{2i+2}(I_i^c) = \frac{k^-_{2i+2}}{1 + I_i^c/K_i^c},$$

so that the corresponding elasticities read:

$$\left.\frac{\partial \ln|v_{2i-1}|}{\partial I_i^c}\right|_{I_i^c=0} = \frac{k^+_{2i-1} \cdot E_{i-1}P \cdot E_i}{v_{2i-1}} \cdot \left(\frac{-1}{K_i^c}\right),$$

$$\left.\frac{\partial \ln|v_{2i+2}|}{\partial I_i^c}\right|_{I_i^c=0} = \frac{k^-_{2i+2} \cdot E_i \cdot E_{i+1}P}{v_{2i+1}} \cdot \left(\frac{-1}{K_i^c}\right)$$

Expressing the response of the flux to the perturbation of these rate constants and using the same principles as above, we have:

$$R^J_{I_i^c}\big|_{I_i^c=0} = \frac{E_i}{J} \cdot \left(C^J_{v_{2i-1}} \cdot k^+_{2i-1} \cdot E_{i-1}P - C^J_{v_{2i+2}} \cdot k^-_{2i+2} \cdot E_{i+1}P\right) \cdot \left(\frac{-1}{K_i^c}\right),\ (i = 1,2,...,r) \tag{20}$$

Notably, a competitive inhibitor does not reflect the control exerted by all elemental steps in which its target enzyme participates, but just two of these.

Eq. (18) expresses the response of the flux to a competitive inhibitor into the control coefficient of the affected enzyme and the relative fraction of the enzyme which has not bound any metabolite. Eq. (20) relates the same response to the control coefficients and the local kinetic properties of the elemental processes in which this particu-

lar enzyme form (which is affected by the inhibitor) participates (see also Appendix B).

Purely uncompetitive inhibitors

A purely uncompetitive inhibitor is considered usually as an inhibitor which binds exclusively to the enzyme-substrate complex [15]. Here we shall consider such a purely uncompetitive inhibitor (I_i^{uc}) that binds and inactivates only the form, E_iP, of the enzyme i, i.e., to the enzyme which has already bound the transferring group P (see Fig. 2b). Then only a single inactive enzyme form exists (cf. Eq. (16)):

$$E_iPI_i^{uc} = E_iP \cdot I_i^{uc}/K_i^{uc} \tag{21}$$

Similarly as above, considering the response of the flux to the inhibitor (I_i^{uc}) as the response to a decrease in the total concentration (e_i) of the enzyme i, one obtains (cf. Eq. (18)):

$$R^J_{I_i^{uc}}\Big|_{I_i^{uc}=0} = C^J_{e_i} \cdot \frac{E_iP}{e_i} \cdot \left(\frac{-1}{K_i^{uc}}\right), (i = 1,2,...,r) \tag{22}$$

Comparison Eq. (22) to Eqs. (8) and (18) reveals that uncompetitive inhibitors uncover yet another face of flux control, i.e., that of the affected enzyme, multiplied by the fraction at which it carries the transferred group.

Mimicking the effect of a purely uncompetitive inhibitor as an apparent perturbation in unidirectional rate constants

To mimic the inhibition by a purely uncompetitive inhibitor, I_i^{uc}, (Fig. 2b) as it would be caused by a perturbation in the rate constants we express the concentration of the active form, E P, as (see Appendix A and Eq. (21)):

$$E_iP/E_iP^* = 1/(1 + I_i^{uc}/K_i^{uc}), \tag{23}$$

Substituting it into Eq. (1) one can see that only the rates, v_{2i} and v_{2i+1}, will depend on I_i^{uc} through a decrease in the kinetic constants, k^-_{2i} and k^+_{2i+1}. Again using the response theorem [18] one will have (cf. Eq. (20)):

$$R^J_{I_i^{uc}}\Big|_{I_i^{uc}=0} = \frac{E_iP}{J} \cdot \left(C^J_{v_{2i+1}} \cdot k^+_{2i+1} \cdot E_{i+1} - C^J_{v_{2i}} \cdot k^-_{2i} \cdot E_{i-1}\right) \times \left(\frac{-1}{K_i^{uc}}\right), (i = 1,2,...,r) \tag{24}$$

Inhibitors with the inhibition constant comparable with or much smaller than the concentration of the affected enzyme: tight-binding and 'irreversible' inhibitors

Now we focus on the advantages of using so called 'irreversible' inhibitors for studies of the control structure of group-transfer pathways. We shall still consider the different types of inhibitors analyzed above, but now with the inhibition constant, K_i, comparable with the concentration of the 'target' enzyme, so-called tight-binding inhibitors. In addition, we shall consider inhibitors with the inhibition constant tending to zero, $K_i \to 0$, therefore leading to very tight, in the limit to irreversible, binding. Notably, using this approach we can also analyze the effects of 'true' irreversible inhibitors which form covalent bonds with the enzyme molecule. We show, that contrary to the classical view, marked differences exist between the responses of group-transfer pathways (and other 'non-ideal' pathways) to the different types of irreversible inhibitors.

The formulas for the response of the pathway flux to inhibitors obtained above are applicable only if the inhibition constants are significantly greater than the concentration of the affected enzyme, $K_i \gg e_i$. If this is not the case, the above formulas for $R^J_{I_i}$ should be modified, as we shall now specify.

In the case of a 'tight-binding' inhibitor with K_i comparable with the enzyme concentration, one can no longer neglect the difference between the total concentration (I_i) of the added inhibitor and its free (I_i^f) concentration. When the dependence of the flux (J) on the inhibitor concentration (I_i) is measured (the titration curve) I_i is considered as the total inhibitor concentration. Also, the response coefficient, $R^J_{I_i}$, is defined in terms of the corresponding derivative with respect to the total inhibitor concentration. However, in the dependencies given above for the enzyme concentrations and reaction rates on the inhibitor concentration, I_i, (see, e.g. Eqs. (6) and (12)), the latter was considered to equal the concentration of the free inhibitor (I_i^f). Therefore, the partial derivatives of the enzyme concentrations and reaction rates with respect to I_i (the elasticity coefficients derived above, see, e.g. Eq. (13)) are actually the derivatives with respect to I_i^f. Hence, to express the response coefficient, $R^J_{I_i}$, in terms of the elasticity coefficients with respect to the free inhibitor concentration, I_i^f, these elasticities should be multiplied by the correction factor $\partial I_i^f/\partial I_i$. Consequently, after multiplying the right-hand sides of the equations above for the response of the flux (e.g., Eqs. (8)–(10), (14)) by this factor, the expressions for $R^J_{I_i}$ will remain correct even when concentrations of the free and total inhibitors cannot be considered to be equal [4]. Note, that the value of the factor $\partial I_i^f/\partial I_i$ depends on the type of the inhibitor added (see below).

In the case of a tight-binding purely non-competitive inhibitor, the relationship between the concentrations $I_i^{nc,f}$ and I_i^{nc} reads (see Eqs. (5)):

$$I_i^{nc} = I_i^{nc,f} + E_iI_i^{nc} + Q_{i-1}I_i^{nc} + E_iPI_i^{nc} + Q_iI_i^{nc} = I_i^{nc,f} \cdot \left(1 + \frac{e_i}{K_i^{nc}}\right), \tag{25}$$

So that,

$$\partial I_i^{nc,f}/\partial I_i^{nc} = \frac{K_i^{nc}}{K_i^{nc} + e_i} \tag{26}$$

The modified equation for the response of the flux to a

tight-binding purely non-competitive inhibitor reads (cf. Eq. (14)):

$$R^J_{I_i^{nc}}\Big|_{I_i^{nc}=0} = \left.\frac{d\ln|J|}{dI_i^{nc}}\right|_{I^{nc}=0} = \left(C^J_{v_{2i-1}} + C^J_{v_{2i}} + C^J_{v_{2i+1}} + C^J_{v_{2i+2}}\right) \cdot \left(\frac{-1}{K_i^{nc}+e_i}\right), (i=1,2,...,r) \quad (27)$$

If $K_i^{nc} \gg e_i$ we retrieve Eq. (15). In the case of an irreversible purely non-competitive inhibitor ($K_i^{nc} \to 0$) one obtains from Eq. (27) (cf. Eq. (15)):

$${}^{imp}C^J_{e_i} = -\frac{{}^{end}I_i^{nc}}{J} \cdot \left.\frac{dJ}{dI_i^{nc}}\right|_{I_i^{nc}=0} \quad (28)$$

where index 'end' refers to the inhibitor concentration required for the complete inhibition of the enzyme i (in practice this concentration is determined from the titration curve as an intersection of two extrapolated lines, see, for example, the figures in [8,11]). Again, similar to the case of Eq. (15), one can see that the right-hand side of Eq. (28) is identical to the expression for the enzyme control coefficient as it would be measured using an *irreversible* inhibitor in the classical approach [8]. However, for a group-transfer pathway it follows from Eq. (28) that with an *irreversible purely non-competitive* inhibitor (which binds also to enzyme-enzyme complexes) the same measurement gives the *impact* control coefficient of the enzyme.

In the case of a tight-binding purely competitive inhibitor, the following relationship between the concentrations $I_i^{c,f}$ and I_i^c holds true (see Eq. (16)):

$$I_i^c = I_i^{c,f} + E_i I_i^c = I_i^{c,f} \cdot \left(1 + \frac{E_i}{K_i^c}\right), \quad (29)$$

so that,

$$\partial\left[I_i^c\right]^f / \partial\left[I_i^c\right] = \frac{K_i^c}{K_i^c + E_i} \quad (30)$$

Hence, the response of the flux to a tight-binding purely competitive inhibitor is equal to (cf. Eq. (18)):

$$R^J_{I_i^c}\Big|_{I_i^c=0} = -C^J_{e_i} \cdot \frac{1}{e_i} \cdot \frac{E_i}{K_i^c + E_i} \quad (31)$$

For the case of an irreversible purely competitive inhibitor (i.e., $K_i^c \ll E_i$) one obtains from Eq. (31):

$$C^J_{e_i} = -\frac{{}^{end}I_i^c}{J} \cdot \left.\frac{dJ}{dI_i^c}\right|_{I_i^c=0} \quad (32)$$

Comparing Eq. (32) with Eq. (28) we conclude that the same experimental procedure with the same calculations (which for an purely *non-competitive* irreversible inhibitor led to the impact control coefficient) now using a purely *competitive* irreversible inhibitor allows one to measure the (classical) control coefficient with respect to the enzyme concentration. Such a difference between the experimental estimations of the control coefficients has already been emphasized for the case of pathways with high enzyme concentrations and moiety-conservations [20]. The differences that exist for such 'complex' pathways when titrating with different types of reversible inhibitors persist in case of irreversible ones [4,20].

The modified equation for the response of the flux to a tight-binding purely uncompetitive inhibitor reads (cf. Eq. (22)):

$$R^J_{I_i^{uc}}\Big|_{I_i^{uc}=0} = -C^J_{e_i} \cdot \frac{1}{e_i} \cdot \frac{E_i P}{K_i^{uc} + E_i P} \quad (33)$$

One can see from Eq. (33), that in the case of an irreversible purely uncompetitive inhibitor (i.e., $K_i^{uc} \ll E_i P$), Eq. (32) remains valid,

$$C^J_{e_i} = -\frac{{}^{end}I_i^{uc}}{J} \cdot \left.\frac{dJ}{dI_i^{uc}}\right|_{I_i^{uc}=0} \quad (34)$$

We conclude that in group-transfer pathways the (classical) control coefficient with respect to the enzyme concentration can be directly measured using irreversible inhibitors which do not bind to enzyme-enzyme complexes. Either irreversible purely *competitive* or irreversible purely *uncompetitive* inhibitors (but not *non-competitive* inhibitor which binds to enzyme-enzyme complexes) can be used. For the pathway under consideration no differences exist between these two types of inhibitors.

Differences between the various modes of control exerted by the same enzyme in a relay pathway: an illustration

We shall illustrate the differences between the control coefficients measured by different inhibitors in an example of a group-transfer pathway involving two enzymes (Fig. 3). We shall simulate numerically the effects of different inhibitors specific to the enzyme 1: (i) a purely *non-competitive* inhibitor in order to estimate the *impact* control coefficient (see Eqs. (15) and (28)), and (ii) an irreversible purely *competitive* or irreversible purely *uncompetitive* inhibitor to estimate the (classical) control coefficient with respect to the enzyme concentration (Eqs. (32) or (34)).

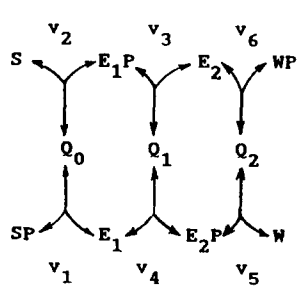

Fig. 3. An example of a group-transfer pathway of two enzymes. (Notations are the same as in Fig. 1.)

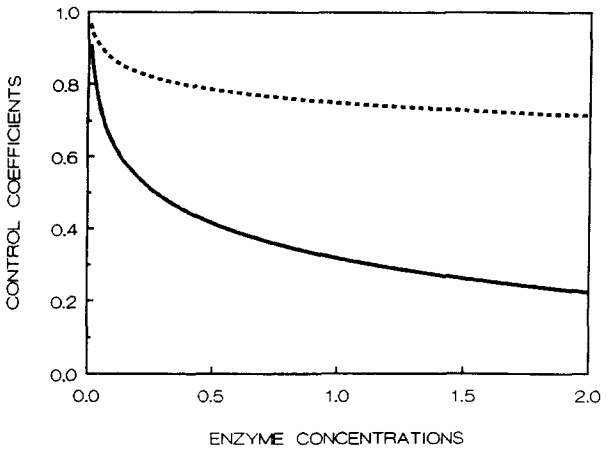

Fig. 4. Various control coefficients of the same enzyme can differ drastically in a group-transfer pathway. The *impact* control coefficient (dotted line) was estimated from the dependence of the pathway flux on the concentration of a purely *non-competitive* inhibitor. The control coefficient with respect to the enzyme concentration (solid line) was estimated from this dependence for irreversible purely *competitive* and irreversible purely *uncompetitive* inhibitors. The concentrations of the enzymes were taken to be equal and were changed proportionally. The parameter values were chosen as follows (dimensionless units): $k_1^+ = 100$, $k_1^- = 50$, $k_2^+ = k_2^- = 20$, $k_3^+ = 10$, $k_3^- = 0.1$, $k_4^+ = 1$, $k_4^- = 0.2$, $k_5^+ = 2$, $k_5^- = 1$, $k_6^+ = 20$, $k_6^- = 10$; $SP = 1$, $S = 0.1$, $W = 1$, $WP = 0.1$, $K_i^{nc} = 100$. Calculations used SCAMP [26] and were checked analytically.

Fig. 4 shows the results of estimations of these two control coefficients and also, how they depend on the total concentration of the pathway enzymes. At very low concentrations the amount of enzyme-enzyme complexes (e.g., E_1PE_2) can be neglected as compared to the total enzyme concentrations, and the classical (enzyme-concentration) control coefficient approaches the *impact* control coefficient [7,17]. Under these conditions (and for the particular rate constants) the two control coefficients of the enzyme 1 assume high values (Fig. 4). With a proportional increase in the concentrations of pathway enzymes both the *impact* control coefficient (the dotted line in Fig. 4) and the control coefficient with respect to the concentration of the enzyme 1 (the solid line) decrease. However, the latter control coefficient decreases to drastically lower values than the former. We conclude that in a group-transfer pathway the differences between various modes of the control increase with the total amount of enzymes.

2.2. Diverse modes in the control of concentrations

Similarly as for the control of the flux one can analyze the control of the concentrations of pathway components by determining their response to specific inhibitors. Depending on the inhibitor type some of the enzyme forms will be affected directly by added inhibitor, while concentrations of others will change only due to the transition to the new steady state. For example, for a purely non-competitive inhibitor (I_i^{nc}) the directly affected forms are E_i, Q_{i-1}, E_iP, Q_i, and for a purely competitive inhibitor (I_i^c) only E_i (cf. Fig. 1 and Fig. 2a). For the control of the concentrations that are not directly affected by an inhibitor the formulas derived above for the flux responses can be readily transformed into those for the concentration responses. For example, for a purely non-competitive inhibitor (I_i^{nc}) for all the pathway concentrations (x) except E_i, Q_{i-1}, E_iP and Q_i, Eq. (27) takes the form:

$$R^x_{I_i^{nc}}\Big|_{I_i^{nc}=0} = \left.\frac{d\ln x}{dI_i^{nc}}\right|_{I_i^{nc}=0} = \left(C^x_{v_{2i-1}} + C^x_{v_{2i}} + C^x_{v_{2i+1}} + C^x_{v_{2i+2}}\right) \cdot \left(\frac{-1}{K_i^{nc}+e_i}\right) = {}^{\mathrm{imp}}C^x_{e_i} \cdot \left(\frac{-1}{K_i^{nc}+e_i}\right),$$
$$x \neq E_i, Q_{i-1}, E_iP, Q_i, \ (i = 1,2,...,r) \qquad (35)$$

For a purely competitive inhibitor, I_i^c, one obtains for all the concentrations except E_i,

$$R^x_{I_i^c}\Big|_{I_i^c=0} = \frac{E_i}{J} \cdot \left(C^x_{v_{2i-1}} \cdot k^+_{2i-1} \cdot E_{i-1}P - C^x_{v_{2i+2}} \cdot k^-_{2i+2} \cdot E_{i+1}P\right) \cdot \left(\frac{-1}{K_i^c + E_i}\right), \ x \neq E_i, \ (i = 1,2,...,r) \qquad (36)$$

and for a purely uncompetitive inhibitor, I_i^{uc}, one obtains for all the concentrations except E_iP,

$$R^x_{I_i^{uc}}\Big|_{I_i^{uc}=0} = \frac{E_iP}{J} \cdot \left(C^x_{v_{2i+1}} \cdot k^+_{2i+1} \cdot E_{i+1} - C^x_{v_{2i}} \cdot k^-_{2i} \cdot E_i\right) \cdot \left(\frac{-1}{K_i^{uc} + E_iP}\right), \ x \neq E_iP, \ (i = 1,2,...,r) \qquad (37)$$

(the correction factors $\partial I_i^{c,f}/\partial I_i^c$ and $\partial I_i^{uc,f}/\partial I_i^{uc}$ were taken into account in these equations).

The situation partly changes for those pathway components which are directly affected by I_i. In this case, representing the effect of inhibition as a perturbation in the elemental rates, one should consider the sums of the active and inactive concentrations of the enzyme forms, i.e., 'asterisk' concentrations (x^*), see Eqs. (11), (19). Hence, in the flux response formulas above, the response and control coefficients of the flux should be replaced by the response and control coefficients with respect to these 'asterisk' concentrations. The response of x^* always includes the direct inhibitor effect:

$$R^{x^*}_{I_i}\Big|_{I_i=0} = \left.\frac{d\ln x^*}{dI_i}\right|_{I_i=0} = \left.\frac{d\ln x}{dI_i}\right|_{I_i=0} + \left(\frac{1}{K_i}\right) \cdot \left.\frac{dI_i^f}{dI_i}\right|_{I_i=0} = R^x_{I_i}\Big|_{I_i=0} + \left(\frac{1}{K_i}\right) \cdot \left.\frac{dI_i^f}{dI_i}\right|_{I_i=0} \qquad (38)$$

In the case of a purely non-competitive inhibitor (I_i^{nc}), using Eqs. (27) and (38), (26) one obtains for the re-

sponses of the concentrations E_i, Q_{i-1}, E_iP and Q_i (cf. Eq. (35)):

$$R^x_{I_i^{nc}}\big|_{I_i^{nc}=0} = \left(1 + C^x_{v_{2i-1}} + C^x_{v_{2i}} + C^x_{v_{2i+1}} + C^x_{v_{2i+2}}\right) \cdot \left(\frac{-1}{K_i^{nc} + e_i}\right) = \left(1 + {}^{imp}C^x_{e_i}\right) \cdot \left(\frac{-1}{K_i^{nc} + e_i}\right),$$

$$x = E_i, Q_{i-1}, E_iP, Q_i, \ (i = 1,2,...,r) \tag{39}$$

Also here, a purely non-competitive inhibitor does not measure the concentration control of the target enzyme. Rather, it measures the *impact* control coefficient (${}^{imp}C^x_{e_i}$) of that enzyme (i) with respect to any concentration, see Eqs. (35) and (39).

For a purely competitive inhibitor (I_i^c) one obtains for the response of the concentration, E_i:

$$R^{E_j}_{I_i^c}\big|_{I_i^c=0} = \left(1 + \frac{E_i}{J} \cdot \left(C^{E_i}_{v_{2i-1}} \cdot k^+_{2i-1} \cdot E_{i-1}P - C^{E_i}_{v_{2i+2}} \cdot k^-_{2i+2} \cdot E_{i+1}P\right)\right) \cdot \left(\frac{-1}{K_i^c + E_i}\right), \ (i = 1,2,...,r) \tag{40}$$

and for a purely uncompetitive inhibitor (I_i^{uc}) one obtains for the the response of the concentration, E_iP,

$$R^{E_iP}_{I_i^{uc}}\big|_{I_i^{uc}=0} = \left(1 + \frac{E_iP}{J} \cdot \left(C^{E_iP}_{v_{2i+1}} \cdot k^+_{2i+1} \cdot E_{i+1} - C^{E_iP}_{v_{2i}} \cdot k^-_{2i} E_i\right)\right) \cdot \left(\frac{-1}{K_i^{uc} + E_iP}\right), \ (i = 1,2,...,r) \tag{41}$$

However, if one represents the inhibition effect as a change in the total active enzyme concentrations and expresses the response coefficients in terms of the enzyme control coefficients, only the active concentrations of the enzyme forms should be considered. Therefore, the response of any concentration expressed in terms of the concentration control coefficients can be readily obtained from the corresponding formula for the flux response above. For example, in the case of a purely non-competitive inhibitor (I_i^{nc}), one obtains for any pathway concentration (x), cf. Eq. (8)):

$$R^x_{I_i^{nc}}\big|_{I_i^{nc}=0} = \left(C^x_{e_{i-1}} \cdot \frac{Q_{i-1}}{e_{i-1}} + C^x_{e_i} + C^x_{e_{i+1}} \cdot \frac{Q_i}{e_{i+1}}\right) \cdot \left(\frac{-1}{K_i^{nc} + e_i}\right), \ (i = 2,3,...,r-1), \tag{42}$$

x is the concentration of any pathway component (E_j, Q_{j-1}, E_jP, Q_j), $j = 1,2,...,r$.

In the cases of purely competitive (I_i^c) and purely uncompetitive inhibitors (I_i^{uc}) one can write for any concentration (x):

$$R^x_{I_i^c}\big|_{I_i^c=0} = -C^x_{e_i} \cdot \frac{1}{e_i} \cdot \frac{E_i}{K_i^c + E_i} \ (i = 1,2,...,r), \tag{43}$$

$$R^x_{I_i^{uc}}\big|_{I_i^{uc}=0} = -C^x_{e_i} \cdot \frac{1}{e_i} \cdot \frac{E_i}{K_i^{nc} + E_iP} \ (i = 1,2,...,r) \tag{44}$$

We conclude that as with flux control, different inhibitors reveal different aspects of the concentration control by an enzyme in a group-transfer pathway. The (classical) control coefficient of any pathway concentration with respect to the enzyme concentration ($C^x_{e_i}$) can be measured using tight-binding or irreversible purely *competitive* and purely *uncompetitive* inhibitors that do not bind to enzyme-enzyme complexes. *Non-competitive* inhibitors rather reveal the impact control of the enzyme. As was shown before for the pathway under consideration, no differences exist between former two types of inhibitor if they are irreversible.

2.3. Modulating enzyme concentration

Changes in enzyme concentrations can be achieved by genetic means, for instance by comparing various heterocaryons of *Neurospora crassa* [21], by causing a gene to be expressed from a plasmid [22], or by modulating the expression of the chromosomal gene [23]. Measuring the responses ($R^J_{e_i}$ and $R^x_{e_i}$) of the flux and pathway concentrations to such a modulation, the classical control coefficients ($C^J_{e_i}$ and $C^x_{e_i}$) with respect to the concentration of the manipulated enzyme (e_i) are determined (see Eq. (3)):

$$R^J_{e_i} = \frac{d \ln|J|}{d \ln e_i} = C^J_{e_i}, \ R^x_{e_i} = \frac{d \ln x}{d \ln e_i} = C^x_{e_i} \tag{45}$$

Using this approach one should distinguish between 'dictatorial' and 'democratic' hierarchies of the control [24]. In the case of a dictatorial hierarchy the modulation of the gene encoding the enzyme of interest leading to a change in the concentration of this enzyme (and, hence, in pathway metabolites) does not cause changes in the concentration of other enzymes, since there is no feedback from the metabolic level to the higher levels of the control. Hence, Eq. (45) can be applied directly. In the case of a democratic hierarchy the modulation of a single gene can result in changes of the concentrations of several enzymes. Therefore, the flux and concentration response coefficients will correspond to the weighted sum of the enzyme control coefficients each of which multiplied by the ratio of a change in the concentration of the corresponding enzyme to a change in the concentration of the reference enzyme.

2.4. Response of the flux to modulation of pathway boundary substrates

One can obtain additional information about the control exerted on the flux and pathway concentrations by modu-

lating the concentrations of the initial donor (pair SP and S) and ultimate acceptor (pair W and WP) of the transferred group. Because the concentrations of the boundary substrates, [SP] and [S], [W] and [WP], enter the rate equations in the same way as the unidirectional rate constants, k_1^+ and k_2^-, k_{2r+1}^+ and k_{2r+2}^-, respectively (see Eq. (1)), one may write:

$$R_{SP}^J = \frac{d\ln|J|}{d\ln[SP]} = \frac{d\ln|J|}{d\ln k_1^+} = R_{k_1^+}^J$$

$$R_S^J = \frac{d\ln|J|}{d\ln[S]} = \frac{d\ln|J|}{d\ln k_2^-} = R_{k_2^-}^J \tag{46}$$

Analogous equations address the response with respect to the pair W and WP. One may obtain the same equations for the concentration response coefficients by formal substitution of the concentrations for the flux in Eq. (49). When normalized by the elasticities with respect to the corresponding rate constants, the response coefficients transform into the control coefficients of the initial and ultimate elemental processes (see Eq. (4)). For instance:

$$C_{\upsilon_1}^J = \frac{R_{SP}^J}{\partial\ln\upsilon_1/\partial\ln k_1^+} \tag{47}$$

Let us designate $C_{e_0}^J$ and $C_{e_{r+1}}^J$ as the sums of the flux response coefficients of the initial donor pair (SP and S) and ultimate acceptor pair (W and WP), respectively:

$$C_{e_0}^J = R_S^J + R_{SP}^J,\ C_{e_{r+1}}^J = R_W^J + R_{WP}^J \tag{48}$$

These control coefficients, $C_{e_0}^J$ or $C_{e_{r+1}}^J$, describe the control by changes in the total concentrations ([SP] and [S]) of the donor or acceptor ([W] and [WP]) when the ratios [SP]/[S] and [W]/[WP] are clamped. The control measured in this manner is not just equal to sum of the control over the elemental processes in which donor and acceptor pairs participate; it involves also the control exerted by the first and the last enzyme of the group-transfer pathway (see Eqs. (30) and (31) of the companion paper [7]):

$$C_{e_0}^J + C_{e_1}^J \cdot \frac{Q_0}{e_i} = C_{\upsilon_1}^J + C_{\upsilon_2}^J, \tag{49}$$

$$C_{e_r}^J \cdot \frac{Q_r}{e_r} + C_{e_{r+1}}^J = C_{\upsilon_{2r+1}}^J + C_{\upsilon_{2r+2}}^J, \tag{50}$$

Similar equations can be obtained for the concentration control coefficients (note, that the equations referring to the control of Q_0 and Q_r include an additional 1 in the right-hand side (see the companion paper [7]). We conclude, that measurements of the response of the flux and pathway concentrations to changes in boundary substrates can give some information about the control coefficients of the initial and final elemental steps of a group-transfer pathway and also about their elasticities to unidirectional rate constants.

2.5. Combining modulation of gene expression with an inhibitor analysis

Combining inhibitor titrations with genetic methods one can additionally to the enzyme(-concentration) control coefficient determine both the *impact* control coefficients (Eq. (14)) and the concentrations of some enzyme forms (Eqs. (18) and (22)). Moreover, using a multiinhibitor analysis of the group-transfer pathway one can determine also the control coefficients of the elemental processes (see Appendix C).

The enzymes of group-transfer pathways do not only function as catalysts and substrates of the group transfer. They are also at the bottom of a gene expression pathway, leading from the genes that encode them, through the corresponding mRNA to the protein. This has been called a hierarchical control [24], which may be 'dictatorial' or 'democratic' (see also above). In the case of a 'dictatorial' control the control coefficients determined by genetic means coincide with the control coefficients determined at the level of a metabolic pathway (for instance, by titration with some types of inhibitors). It is also instructive to consider systems with a 'democratic' hierarchy of the control, where some intermediate concentrations, or extent of phosphorylation affect gene expression. In this case the control coefficients determined by modulating the gene expression give information on the control in the whole (genetic and metabolic simultaneously) system. A comparison of these control coefficients and those determined by using inhibitors can give an insight into the interplay of genetic and metabolic regulation.

3. Discussion

In this paper we have shown how, in principle, control properties of group-transfer (relay) pathways may be determined experimentally. In simple ('classical') metabolic pathways modulation of enzyme concentration, inhibitor titration and modulation of the concentrations of 'boundary' substrates and products, all lead to a single set of coefficients quantifying the control exerted by the enzymes on flux or pathway concentrations. Control by enzyme concentrations and enzyme activities are synonymous in such simple pathways. Group-transfer pathways involve enzyme-enzyme interactions and such interactions cause the control by enzyme concentration and by any of the enzyme's activities to diverge. In other words, control by an enzyme comes in various modes in such pathways [17,25]. Most importantly, the present paper shows how the different modes of the control exerted by an enzyme in a group-transfer pathway can be determined and distinguished experimentally. Indeed, modulation in enzyme concentration, titration with non-competitive or competitive and uncompetitive inhibitors turned out to lead to the

Table 1
What various methods measure in a group transfer pathway

	'Classical' enzyme control coefficients, $C^J_{e_i}$	Impact control coefficients $\mathrm{imp}_{C^J_{e_i}}$	'Classical' enzyme control coefficients, mixed with concentrations of enzyme forms	Control coefficients of unidirectional rate constants of boundary steps
Modulation of the enzyme concentration	+	−	−	−
Titration with purely non-competitive inhibitor	−	+	−	−
Titration with purely competitive inhibitor: $K_i \geq e_i$,	−	−	+	−
irreversible	+	−	−	−
Titration with purely competitive inhibitor: $K_i \geq e_i$,	−	−	+	−
irreversible	+	−	−	−
Modulation of the pathway boundary substrates	−	−	+	+

determination of the different control modes of enzymes in a group-transfer pathway. Table 1 summarizes some results on different modes of the control which can be measured by different methods.

Notably, the analysis in terms of the effects of different inhibitors has led us to a new theoretical approach to study control and regulation in metabolic pathways. In the Appendix C we show how this new approach can be used to express the control exerted by the enzyme concentrations in terms of the control exerted by the elemental steps of the reaction network and in terms of kinetic properties of those steps. This type of approach can be used for any pathway that deviates from ideal pathways usually analysed in classical metabolic control analysis [27].

Of course, one should realize that in many cases a complete experimental analysis will be hampered by the lack of specific inhibitors, by difficulties in modulating the expression of only the gene encoding the enzyme of interest. Yet, even if such a complete analysis is not possible, it remains important to understand what mode of control is measured by the method that is available.

Because the various methods measure different modes of the control exerted by an enzyme, parallel use of a number of these methods should allow measurement of the differences between the modes. Not only should this be of conceptual interest, it should also reveal mechanistic aspects of the intact pathway that would be difficult to reveal otherwise. For, this paper shows how these differences are related to properties such as concentrations of enzyme forms and extent of formation of enzyme-enzyme complexes. Ultimately, i.e., with the simultaneous use of many methods, it should become possible to establish the magnitudes of the control coefficients of the elemental processes. We conclude that measuring the responses of the flux to the different types of perturbation and using the relationships derived, one obtains valuable information about global and local control properties of group-transfer pathways.

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Appendix 1

Inhibitors forming inactive enzyme-inhibitor complexes: mimicking a decrease in the active concentration of an enzyme by changes in unidirectional rate constants

We shall consider an arbitrary enzyme mechanism inside any metabolic network which may also include direct interactions of this enzyme with other enzymes. The different states of the enzyme of interest (enzyme intermediates) will be designated by $Y_1, Y_2, ..., Y_n$. We shall suggest that binding a specific inhibitor (I) to some enzyme intermediate form (Y_i) transforms the latter into a form (Y_iI) which is not subject to any catalytic transformation. At any steady state of the reaction the equilibrium exists between Y_i, I and the complex Y_iI, since the latter does not undergo further transformations:

$$Y_iI = Y_i \cdot I/K_i \qquad \text{(A1)}$$

Here K_i is the equilibrium constant (that is identical to the dissociation constant of the complex Y_iI), I is the free inhibitor concentration. Due to the equilibrium (Eq. A1) the concentration, Y_i, can be expressed in terms of the sum (Y_i^*) of the concentrations of the active (i.e., uninhibited) and inactive (inhibited) forms, $Y_i^* = Y_i + Y_iI$, and the free inhibitor concentration, I:

$$Y_i = Y_i^*/(1 + I/K_i) \qquad \text{(A2)}$$

Now we return for a moment to a situation when no inhibitor is added and consider all moiety-conservation relationships in which the concentration Y_i participates. Depending on the metabolic network and on the particular enzyme form, these relationships may have the physical interpretation of conservations of the total concentration of the enzyme under consideration and of some other enzymes or substrates which this particular enzyme form (Y_i) may involve. One may see, that after inhibitor addition, the sum, Y_i^*, of the active *i-th* enzyme intermediate and its complex with the inhibitor, will replace Y_i in these relationships. Most importantly after the replacement of Y_i by Y_i^* all these moiety-conservation relationships remain unchanged.

Now we consider the equations for the unidirectional rates in which the particular enzyme form (Y_i) participates as a reactant. The (mathematical) products of Y_i and the corresponding unidirectional rate constant (k_j) enter any such equation. Hence, after expressing Y_i in terms of Y_i^* and I, see Eq. A2, the dependence on the inhibitor concentration, I, can be transferred and ascribed to the rate constant, k_j, which becomes $k_j(I)$:

$$k_j(I) = k_j/(1 + I/K_i) \tag{A3}$$

Subsequently, after the inhibitor addition the only change in the equations which determine the system steady state is the appearance of the superscript (*) denoting the affected enzyme forms and selective changes in the rate constants of steps in which these forms participate. Removing the superscript, one mimics the effects of inhibition by changing unidirectional rate constants only. Most importantly, for every form Y_i, these changes (Eq. A3) always affect two (or more if Y_i is at a branch point of the mechanism) rate constants. One is the forward rate constant of the step depleting Y_i and the second is the reverse rate constant of the step producing Y_i. Consequently, the Gibbs energy difference for the whole reaction, e.g., from SP to W in a group-transfer pathway (see the main text), does not change.

Appendix B. General relations between the control coefficients of the enzymes and the elemental processes. Expressing the global control properties into the local ones

In this appendix we express the control coefficients of the enzymes in terms of the control coefficients of the elemental processes. We also show that analysis of the flux and concentration responses presented above allows one to obtain general relationships between the different types of the control coefficients in a group transfer pathway and also express the latter in terms of elasticities. We shall first do this for the flux control coefficients.

Equating the respective right-hand sides of Eqs. (8)–(10) to that of Eq. (14) one obtains:

$$C^J_{e_{i-1}} \cdot \frac{Q_{i-1}}{e_{i-1}} + C^J_{e_i} + C^J_{e_{i+1}} \cdot \frac{Q_i}{e_{i+1}} = C^J_{v_{2i-1}} + C^J_{v_{2i}} + C^J_{v_{2i+1}} + C^J_{v_{2i+2}}, \ (i = 2,3,...,r-1), \tag{B1}$$

$$C^J_{e_1} + C^J_{e_2} \cdot \frac{Q_1}{e_2} = C^J_{v_1} + C^J_{v_2} + C^J_{v_3} + C^J_{v_4}, \tag{B2}$$

$$C^J_{e_{r-1}} \cdot \frac{Q_{r-1}}{e_{r-1}} + C^J_{e_r} = C^J_{v_{2r-1}} + C^J_{v_{2r}} + C^J_{v_{2r+1}} + C^J_{v_{2r+2}} \tag{B3}$$

Eqs. (B1)–(B3) are identical to Eqs. (18)–(20) of the companion paper [7]. As was shown in that paper these equations allow one to express the flux control coefficients of the enzymes in terms of the control coefficients of the elemental processes and the relative fractions of enzyme complexes. They were also used to derive new summation theorems for the flux control coefficients of the enzymes of group-transfer pathways. Comparing Eqs. (18) and (20) one obtains:

$$C^J_{e_i} = \frac{e_i}{J} \cdot \left(C^J_{v_{2i-1}} \cdot k^+_{2i-1} \cdot E_{i-1}P - C^J_{v_{2i+2}} \cdot k^-_{2i+2} \cdot E_{i+1}P \right), \ (i = 1,2,...,r) \tag{B4}$$

Eq. (B4) is identical to Eq. (F5) of the companion paper [7]. Eq. (F5) obtained there by an abstract mathematical method, here acquires the transparent meaning of the equivalence of the variations in the flux, caused either by a change in the enzyme concentration or by a change in some rate constants of the elemental processes (corresponding to a particular mechanism of inhibition). A similar relationship (Eq. (F7) of that paper) corresponding to a change in some other elemental rate constants, follows from Eqs. (22) and (24):

$$C^J_{e_i} = \frac{e_i}{J} \cdot \left(C^J_{v_{2i+1}} \cdot k^+_{2i+1} \cdot E_{i+1} - C^J_{v_{2i}} \cdot k^-_{2i} \cdot E_{i-1} \right) \ (i = 1,2,...,r) \tag{B5}$$

One can also consider a type of purely uncompetitive inhibitor that can bind to both enzyme forms, E_iP and Q_i, or a 'hypothetical' type of inhibitor which binds only to the complex Q_i. Combining the effect of such an inhibitor with the influence of purely competitive inhibitor of the adjacent enzyme $i + 1$, we can obtain an additional expression for the control coefficient ($C^J_{e_i}$) of the enzyme i which is identical to Eq. (F13) of the companion paper [7].

Comparing the corresponding expressions for the concentration response coefficients above, one can readily obtain the general equations relating the different types of the concentration control properties of the group-transfer

pathway. For example, equating the right-hand side of Eq. (35) or (39) with that of Eq. (42) one obtains the relationships between the control coefficients of the enzymes and the control coefficients of the elemental processes, see Eqs. (43) and (49) of the companion paper [7]. Comparing Eq. (36) or (40) with Eq. (43) one obtains the equation relating the global and local control coefficients, see Eq. (F25) of the companion paper [7].

Appendix C. The simplest example: a relay pathway involving two enzymes

The differences between control coefficients measured by different inhibitors were illustrated in Section 1e in an example of a group-transfer pathway involving two enzymes (Fig. 3). Here, for the same example we shall illustrate how such data could be used in principle to reveal control and kinetic properties of the group transfer. Depending on the experimental data, there are several ways of determining the global and local control properties of the pathway. The most difficult situation occurs if one can measure only the pathway flux (as the input or output flux) and cannot measure the concentrations of the enzyme forms. Here this specific case will be considered.

Starting with the, too often unrealistic, assumption that all the specific inhibitors possessing the properties described above are available, we show how one can determine all types of control coefficient. Then, we abandon this assumption and show how the number of such inhibitors can be diminished. Finally, we consider the situation when the enzyme(-concentration) control coefficients can be measured by modulating the genes encoding the pathway enzymes and show what additional information can be obtained in case some specific inhibitors (at least one such an inhibitor) are also available.

Using irreversible, purely competitive or purely uncompetitive inhibitors one can measure the 'classical' control coefficients with respect to the enzyme concentration, see Eqs. (32) and (34):

$$C^J_{e_1} = M_1,\ C^J_{e_2} = M_2 \tag{C1}$$

where the terms M_i designate the values resulting directly from the measurements. It is noteworthy, that using irreversible specific inhibitors of any type (see Eqs. (28), (32), (34)) one simultaneously determines the total enzyme concentrations,

$$e_1 = M_3,\ e_2 = M_4 \tag{C2}$$

As was mentioned under Introduction (see also Results) the magnitudes on the enzyme(-concentration) control coefficients do not suffice to describe completely the control structure of the 'channelled' pathways, e.g., to estimate the impact control coefficients of the pathway enzymes. For such a complete description it is also necessary to determine the control and/or elasticity coefficients of the elemental processes. To estimate them in the group-transfer pathway one should obtain some additional experimental data which must be considered together with the relationships derived above.

Using purely non-competitive inhibitors one can measure the impact control coefficients of the enzymes 1 and 2 (see Eqs. (14), (15) for the case of $K_i^{nc} \gg e_i$ and Eq. (28) for an irreversible purely non-competitive inhibitor):

$${}^{imp}C^J_{e_1} = C^J_{v_1} + C^J_{v_2} + C^J_{v_3} + C^J_{v_4} = M_5, \tag{C3}$$

$${}^{imp}C^J_{e_2} = C^J_{v_3} + C^J_{v_4} + C^J_{v_5} + C^J_{v_6} = M_6, \tag{C4}$$

Here the values of the impact control coefficients are represented as the values (M) resulting directly from the measurements. However, in case $K_i^{nc} \gg e_i$ this implies that the inhibition constant, K_i^{nc}, can be measured in an independent experiment. From these data (M_1, M_2, M_4, M_5) and Eq. (B2) (or M_1, M_2, M_3, M_6 and Eq. (B3) with $r = 2$) one can estimate the concentration of the enzyme-enzyme complex, Q_1,

$$Q_1 = e_2 \cdot \left({}^{imp}C^J_{e_1} - C^J_{e_1}\right)/C^J_{e_2} = e_1 \cdot \left({}^{imp}C^J_{e_2} - C^J_{e_2}\right)/C^J_{e_1} \tag{C5}$$

Using purely competitive and purely uncompetitive inhibitors with $K_i \gg e_i$ and taking into account the data (C1),(C2), one can estimate the concentrations of the following enzyme forms (see Eqs. (18), (22)):

$$E_1 = M_7, E_1P = M_8, E_2 = M_9, E_2P = M_{10} \tag{C6}$$

Then, the concentrations of the complexes Q_0 and Q_2 can be determined from the moiety-conservation relationships:

$$Q_0 = e_1 - (E_1 + E_1P + Q_1) \tag{C7}$$

$$Q_2 = e_2 - (E_2 + E_2P + Q_1) \tag{C8}$$

Thus, the experimental procedure described allows one to estimate the concentrations of all the enzyme forms (E_1, Q_0, E_1P, Q_1, E_2, E_2P, Q_2).

Let these concentrations of the enzyme forms be determined at several concentrations of the boundary substrates S, SP and/or W, WP. Substituting these data into Eqs. (2) for the steady state, one obtains a linear equation system with respect to the rate constants of the elemental processes. Then, the rate constants can be determined by any of the numerical methods of linear algebra, e.g., the linear regression method. Subsequently, the elasticity coefficients of the elemental processes can be calculated. This allows one to estimate the control coefficients ($C^J_{v_i}$) of the elemental processes (in terms of the elasticity coefficients, see [7]).

In practice only few of the required inhibitors are available. The relationships derived above help to decrease the number of required inhibitors. For example, it is sufficient to have a purely non-competitive inhibitor of only one of the enzymes 1 and 2, see Eq. (C5). In the following example, let a purely competitive or purely

uncompetitive inhibitor (with $K_1 \gg e_1$) be not available for each of the enzymes. In this case one can determine only 2 of the 4 concentrations (64). Then, one should measure also the response coefficients of the flux to a change in the concentrations of the boundary substrates, i.e., to estimate directly the control coefficients of the boundary substrates:

$$C^J_{e_0} = \frac{d\ln|J|}{d\ln[S]} + \frac{d\ln|J|}{d\ln[SP]} = M_{11}$$

$$C^J_{e_3} = \frac{d\ln|J|}{d\ln[W]} + \frac{d\ln|J|}{d\ln[WP]} = M_{12} \qquad \text{(C9)}$$

For these control coefficients, the following relationships hold (see Eqs. (49), (50)):

$$C^J_{e_0} + C^J_{e_1} \cdot \frac{Q_0}{e_1} = C^J_{v_1} + C^J_{v_2}, \qquad \text{(C10)}$$

$$C^J_{e_2} \cdot \frac{Q_2}{e_2} + C^J_{e_3} = C^J_{v_5} + C^J_{v_6}, \qquad \text{(C11)}$$

We can estimate the right-hand sides of Eqs. (C10), (C11) as:

$$C^J_{v_1} + C^J_{v_2} = 1 - {}^{imp}C^J_{e_2} = 1 - M_6, \qquad \text{(C12)}$$

$$C^J_{v_5} + C^J_{v_6} = 1 - {}^{imp}C^J_{e_1} = 1 - M_5, \qquad \text{(C13)}$$

Then, the concentrations of the complexes Q_0 and Q_2 can be found:

$$Q_0 = e_1 \cdot \left(1 - {}^{\text{imp}}C^J_{e_2} - C^J_{e_0}\right)/C^J_{e_1}, \qquad \text{(C14)}$$

$$Q_2 = e_2 \cdot \left(1 - {}^{\text{imp}}C^J_{e_1} - C^J_{e_2}\right)/C^J_{e_2}, \qquad \text{(C15)}$$

Using these concentration values, the remaining unknown values in Eq. (C6) can be determined from the moiety-conservation relationships (cf. Eqs. (C7) and (C8)).

A slightly more complicated case arises if in this example apart from 2 purely competitive or purely uncompetitive inhibitors with $K_i \gg e_i$, both irreversible purely competitive and irreversible purely uncompetitive inhibitors of one of the enzymes 1 or 2 are absent. Then the 'classical' control coefficient of one of the enzymes (M_1 or M_2 value) cannot be measured directly. However, this control coefficient can be found simultaneously with the concentration Q by solving Eqs. (B2), (B3) using the measured values of the impact control coefficients (see Eqs. (C3), (C4)).

An even more complicated case arises when irreversible purely competitive and irreversible purely uncompetitive inhibitors of both enzymes and either a purely competitive or purely uncompetitive inhibitor (with $K_i \gg e_i$) of one of the enzymes are absent. Then, one cannot measure directly neither 'classical' control coefficients of both enzymes nor the concentrations E_i and E_iP. In this case, 2 classical control coefficients and all 7 unknown concentrations of the enzyme forms can be simultaneously found by solving the system of 9 equations that is constructed from Eqs. (B2), (B3), (18), (22), (C10), (C11) and 2 moiety-conservation constraints.

Now we consider a situation where the control coefficients, $C^J_{e_i}$, with respect to the enzyme concentrations (e_i) and these concentrations themselves are measured by the genetic (and accompanying) methods. In the notations of this section this means that M_1, M_2, M_3 and M_4 values are known. Let us suppose that one has only three inhibitors, e.g., a purely non-competitive inhibitor of each enzyme and a purely competitive inhibitor of the enzyme 2 (with $K^c_i \gg e$). Then one can measure the impact control coefficients of the enzymes 1 and 2 (M_5 and M_6 values, Eqs. (C3) and (C4)) and the concentration of the free form, E_2, of the enzyme 2 (M_9 value, Eq. (C6)). From these data the concentration of the enzyme-enzyme complex, Q_1, can be estimated (see Eq. (C5)). Also the sums of the control coefficients of the elemental processes, $C^J_{v_1}$ and $C^J_{v_2}$, Eq. (C12), $C^J_{v_5}$ and $C^J_{v_6}$, Eq. (C13), can be found.

According to Eq. (C9) one can measure the control coefficient of the boundary substrates (the initial donor, M_{11} value, and ultimate acceptor, M_{12} value), and using Eqs. (C10)–(C15) the concentrations Q_0 and Q_2 can be found. Then, the concentration E_2P can be determined from the moiety-conservation sum of the enzyme 2 (Eq. (C8)). Similarly, one can determine the concentrations E_2, E_2P, Q_2, at several concentrations of the boundary substrates. Substituting these data into Eqs. (2) for the steady-state flux one can calculate the rate constants, k^+_5, k^-_5, k^+_6, k^-_6.

Now we use general relations between the control coefficients of the enzymes and the elemental processes and the kinetic properties of the latter (see the corresponding section above). With $r = 2$, Eq. (F11) of the companion paper [7] reads:

$$C^J_{e_2} = \frac{e_2}{J} \cdot \left(C^J_{v_6} \cdot k^+_6 - C^J_{v_5} \cdot k^-_5\right), \qquad \text{(C16)}$$

From Eqs. (C13) and (C16) the control coefficients of the elemental processes, $C^J_{v_5}$ and $C^J_{v_6}$, can be found.

To determine the other elemental control and elasticity coefficients (i.e., the rate constants) one should consider additional equations. With $i = 1$ and $i = 2$ Eqs. (B4) and (B5) read:

$$C^J_{e_1} = \frac{e_1}{J} \cdot \left(C^J_{v_1} \cdot k^+_1 \cdot SP - C^J_{v_4} \cdot k^-_4 \cdot E_2P\right), \qquad \text{(C17)}$$

$$C^J_{e_1} = \frac{e_1}{J} \cdot \left(C^J_{v_3} \cdot k^+_3 \cdot E_2 - C^J_{v_2} \cdot k^-_2 \cdot S\right), \qquad \text{(C18)}$$

$$C^J_{e_2} = \frac{e_2}{J} \cdot \left(C^J_{v_3} \cdot k^+_3 \cdot E_1P - C^J_{v_6} \cdot k^-_6 \cdot WP\right), \qquad \text{(C19)}$$

$$C^J_{e_2} = \frac{e_2}{J} \cdot \left(C^J_{v_5} \cdot k^+_5 \cdot W - C^J_{v_4} \cdot k^-_4 \cdot E_1\right), \qquad \text{(C20)}$$

Combining with Eqs. (F10), (F11) and (F13) of the companion paper [7] we obtain:

$$C^J_{e_1} = \frac{e_1}{J} \cdot \left(C^J_{v_2} \cdot k_2^+ - C^J_{v_1} \cdot k_1^- \right), \quad \text{(C21)}$$

$$C^J_{e_1} + C^J_{e_2} \cdot (e_1/e_2) = \frac{e_1}{J} \cdot \left(C^J_{v_4} \cdot k_4^+ - C^J_{v_3} \cdot k_3^- \right), \quad \text{(C22)}$$

Adding to Eqs. (C3),(C7),(C12),(C17)–(C22), Eqs. (2) (with $i = 1, 2, 3, 4$) and the Haldane relation one can calculate the remaining unknown concentrations E_1, E_1P, and the control and elasticity coefficients of the elemental processes ($i = 1, 2, 3, 4$), and also the rate constants. Thus, the complete control and kinetic structure of the group-transfer pathway of two enzymes is determined.

Since in this example only the flux is measured, one needs three inhibitors to determine completely the pathway control and kinetic properties. However, the use of even a single inhibitor, in addition to modulating gene expression can greatly contribute to the analysis of the control structure of 'channelled' pathways. The usefulness of data obtained by inhibitor analysis increases drastically if one is able also to measure the concentration response coefficients. For most group-transfer pathways the number of known inhibitors is far too small to allow a complete analysis. Yet, we think that this Appendix illustrates the diversity of control coefficients and the fact that to really understand control of group-transfer pathways, much more experimental work will be needed.

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