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DOI
10.1111/j.1432-1033.1994.00179.x
Publication date
1994

Published in
European Journal of Biochemistry

Link to publication

Citation for published version (APA):
Kholodenko, B. N., Sauro, H. M., & Westerhoff, H. V. (1994). Control by enzymes, coenzymes and conserved moieties. *European Journal of Biochemistry*, *225*, 179-186. https://doi.org/10.1111/j.1432-1033.1994.00179.x

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Download date:26 Jul 2022

Eur. J. Biochem. 225, 179–186 (1994)
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Control by enzymes, coenzymes and conserved moieties

A generalisation of the connectivity theorem of metabolic control analysis

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(Received May 2/July 13, 1994) – EJB 94 0620/1

The control and regulation of metabolic systems are determined by their responses to changes in the internal metabolites (the internal state) and parameters of the system. In many cases, the concentrations of the intermediates are constrained by moiety conservations, for example those requiring that all intermediate forms of any enzyme sum to the conserved total concentration of that enzyme. In this study, we show how responses to changes in the internal state are related to responses to changes in the total amounts of conserved moieties. The relationship between these two different measures of control leads to a generalisation of the connectivity theorems. The results have important implications for the study of a variety of phenomena such as metabolite (coenzyme) sequestration, group-transfer and channelling. The relationships we derive make it possible to determine the control features of these pathways. As an illustration, two examples are chosen. The first shows the effect of sequestration of substrate moiety while the second deals with the sequestration of the enzyme moieties and enzyme/enzyme interactions.

Moiety-conserved cycles play a special role in metabolism. Coenzymes involved in redox metabolism (such as the NAD/NADH couple) or free energy metabolism (such as ATP, ADP and AMP) are important examples (Reich and Selkov, 1981; Westerhoff and Van Dam, 1987). These coenzymes shuttle redox or free-energy equivalents between metabolic pathways. Over short time scales, the total amount of adenine nucleotide remains essentially constant, although over the long term there will inevitably be the slow turnover of the coenzymes by other metabolic processes. A coenzyme such as NAD/NADH carries a common chemical group whose amount is conserved during its interconversions; such a chemical group is termed a conserved moiety. The pathway of conversions between the different moiety forms is termed a moiety-conserved cycle. In such cases, the total amount of moiety in a cycle can be considered a parameter of the system. Thus, a pertinent question in the analysis of moiety-conserved cycles is how a steady-state pathway flux or metabolite concentration is affected by changes in the total amount of moiety.

In metabolic control analysis, the question of how moiety-conserved cycles relate to the control properties of pathways was first investigated by Westerhoff (1983), Fell and Sauro (1985) and in much more detail by Hofmeyr et al. (1986). These initial studies were largely confined to pathways with a single conserved moiety. In later studies, Sauro et al. (1987), Meiske and Reich (1987), and, in more detail, Kholodenko (1988) and Reder (1988), examined the more general case of pathways with multiple moiety-conserved cycles that could be linked to other conserved cycles via a common metabolite.

A conserved moiety that is often considered implicit in studies of metabolism is the enzyme moiety. The enzyme moiety forms include the free unbound enzyme, the enzyme/substrate complexes and the complexes formed by the association of two or more different enzymes. The enzyme moiety can be considered conserved because the turnover of enzyme by net synthesis and breakdown is slow compared to the pathway interconversions in which it takes part.

It is common in metabolic control analysis to neglect the existence of the internal enzyme cycles when analysing pathways. The usual reason for this is that the concentration of any enzyme metabolite complex is assumed to be small compared to the bulk-phase concentration of the corresponding metabolite. This assumption has the useful consequence that each enzyme cycle in a pathway can be treated as a single block reaction which simplifies the control analysis. Potential problems can arise however, if the pathway under study contains a conserved cycle rather than just a linear or branched system. In such a case, the pathway contains $n + 1$ conserved cycles where n is the number of enzymes, each enzyme contributing a single internal enzyme cycle. Ignoring the enzyme cycles in such cases becomes unacceptable if a significant proportion of the pathway mass exists bound (i.e. sequestered) as enzyme/substrate complex. Such a situation can occur if the concentrations of enzyme are high relative to the free metabolite concentrations and their affinities for metabolites allow a significant fraction of the conserved moiety to be bound to enzymes. The analysis of conserved cycles where the catalysing enzymes are at high concentrations has been the recent focus of interest in the analysis of conserved

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cycles (Fell and Sauro, 1990; Kholodenko et al., 1992; Sauro, 1994).

There is also an additional interest in the general problem of dealing with multiple linked enzyme cycles owing to the close association with the problem of metabolic channelling and group transfer. In channelled pathways, an enzyme cycle can no longer be treated as a single block reaction inside the metabolic pathway, instead one should consider the whole network of elemental processes (steps) where the enzyme-enzyme complexes form 'bridges' between the different enzyme cycles. The steps in which two or more enzyme moieties participate each make a contribution to the control exerted by each of the moiety totals (Kholodenko and Westerhoff, 1993; Van Dam et al., 1993; Sauro, 1994).

The aim of this study is to develop further the general analysis of complex linked moiety-conserved cycles, especially those related to linked enzyme cycles. Such an analysis allows us to establish the relationship between the response of the system to a change in internal metabolites and the response of the system to changes in the conservation totals. This theoretical work forms part of the analytical machinery necessary for treating a whole range of biochemical problems, such as the effect of sequestration, group transfer and channelling (Kholodenko et al., 1993; Sauro, 1994) which up to now have been difficult to treat. In addition, the theory makes certain predictions which are particular to these systems and so can be used to test experimentally whether such systems occur or do not occur *in vivo*. Finally, we illustrate our results by considering problems in metabolite sequestration and group transfer.

DEFINITION OF TERMS

Conservation constraints

Given a simple conserved cycle (Fig. 1) with m metabolites, S_1, S_2, ..., S_m and assuming that the stoichiometry at each reaction is unity, the total amount, T, of moiety in the cycle is given by the simple sum:

$$\sum_{i=1}^{m} S_i = T.$$

We assume in this case that each of the metabolites, S_i, is present in the same volume space. In the more general case, where a pathway may contain any number of conserved cycles and removing the restriction of unit stoichiometry, the total amount of each moiety, T_l, is given by:

$$\sum_{k=1}^{m} \gamma_{lk} S_k = T_l, \quad l = 1, 2, \ldots m - m_0. \tag{1}$$

There will be $(m - m_0)$ values of these equations, corresponding to the number of different types of moiety in the system. This number can be obtained from the rank, m_0, of the stoichiometry matrix $\boldsymbol{N}$ and corresponds to the number of dependent rows of $\boldsymbol{N}$. The sum, T_l, is the amount of moiety in the l^{th} moiety conserved cycle and γ_{lk} can in most cases be interpreted as the number of moieties of type l in the metabolite, S_k. In matrix form, γ_{lk} are the coefficients from the l^{th} row of the $(m - m_0)$ rows by m columns of the conservation matrix, γ.

If we let S_k be the k^{th} element of the m dimensional vector $\boldsymbol{S}$ of metabolite concentrations and T_l, the l^{th} element of the $(m - m_0)$ dimensional vector $\boldsymbol{T}$ of conservation total, then Eqn (1) can be written more compactly as follows:

$$\gamma \boldsymbol{S} = \boldsymbol{T}.$$

The problem of determining γ is briefly discussed in the Appendix.

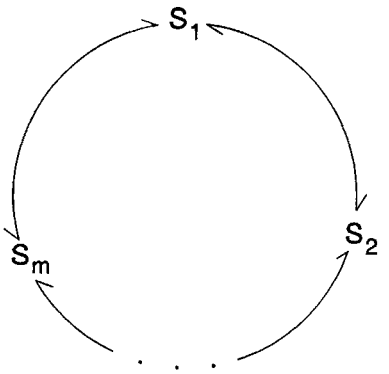

Fig. 1. A conserved cycle with m metabolites, S_1, S_2, ..., S_m.

Metabolic control analysis

For a general metabolic pathway, there will be n reaction steps, their rates being designated by v_i, $i = 1, \ldots, n$. In terms of system properties, the rates v_i at steady-state are referred to as the steady state fluxes J_i.

In the context of metabolic control analysis one question that is of interest is how perturbations to the internal state affect the system variables such as the flux and metabolite concentrations. To answer this, we define the flux response coefficient to an initial perturbation in S_k as follows (Westerhoff and van Dam, 1987; Reder, 1988; Sauro, 1994):

$$R^J_{S_k} = \frac{(\delta J/J)_{t \to \infty}}{(\delta S_k/S_k)_{t=0}}, \tag{2}$$

where J is any steady-state flux. Similarly, the response of a metabolite, S_j, to an initial change in S_k is defined as:

$$R^{S_j}_{S_k} = \frac{(\delta S_j/S_j)_{t \to \infty}}{(\delta S_k/S_k)_{t=0}}. \tag{3}$$

These response coefficients measure the steady-state response of the system (i.e. as $t \to \infty$) to a transient perturbation in one of the metabolites (Westerhoff and Chen, 1984; Westerhoff and Van Dam, 1987; Sauro, 1994). The operational meaning of the definitions given in Eqn (2) and Eqn (3) corresponds to the definition in terms of the eventual response of an intensive thermodynamic variable to a fluctuation of another variable (Westerhoff and Van Dam, 1987). In the case of Eqn (3), the movements in S_j and S_k are not independent but linked by moiety conservation [from Eqn (1), δS_k implies a change in T]. If the perturbed metabolite, S_k, is not part of a conserved cycle then the perturbation will decay to zero, i.e. the starting steady-state concentration is restored. This assumes of course that the steady-state is stable (Westerhoff and Van Dam, 1987). If, however, the metabolite is a member of a conserved cycle, then a change in the total amount of moiety will be affected by the perturbation and, as Hofmeyr et al. (1986) have shown the system will evolve to a different but nearby steady-state.

There is another way of describing the response of a pathway to changes in the internal state and that is by con-

sidering a change to T. Since T, the total amount of moiety, is related to S_k by Eqn (1), a change in S_k will have an effect on T. We can therefore define a response coefficient (Hofmeyr et al., 1986) with respect to T as follows:

$$R_T^J = \frac{(\delta J/J)_{t\to\infty}}{(\delta T/T)}. \tag{4}$$

Note that the effect of δT is permanent and does not itself decay, therefore the measurement of T can be made at any time. Similarly, we can define the response of a concentration to a perturbation in the moiety-conserved sum as:

$$R_T^S = \frac{(\delta S/S)_{t\to\infty}}{(\delta T/T)}. \tag{5}$$

ANALYSIS OF FLUX AND CONCENTRATION RESPONSE

We now wish to investigate how changes to the internal state, S_k, and the total moiety, T_l, affect the steady-state flux and metabolite concentrations. Given a perturbation δS_k to S_k, it is possible to calculate the resulting change in flux in two ways. The first way is to use the definition of R_S^J given in Eqn (2) so that the change in flux can be written as:

$$\frac{\delta J}{J} = R_{S_k}^J \cdot \frac{\delta S_k}{S_k}. \tag{6}$$

The second way of computing the change in flux is from the effect of a change in T. Given that the change in T_l is δT_l, then from Eqn (4), the change in flux as a result in a change in T_l is given by:

$$\frac{\delta J}{J} = \sum_{l=1}^{m-m_0} R_{T_l}^J \cdot \frac{\delta T_l}{T_l}. \tag{7}$$

We must sum over all T_l in Eqn (7) because the primary event, namely the change in S_k, may connect to a number of moiety cycles and thus affect more than one total. If for instance S_k corresponds to ATP and both total phosphate and total adenine nucleotide monophosphate are conserved, the sum must include both these totals. The linkage between the total amount of moiety and a metabolite is given by Eqn (1), so that the effect on a particular T_l by a single S_k is given by

$$\delta T_l = \gamma_{lk}\,\delta S_k,$$

or in terms of fractional changes

$$\frac{\delta T_l}{T_l} = \gamma_{lk}\frac{\delta S_k}{S_k}\frac{S_k}{T_l}. \tag{8}$$

Substituting Eqn (8) into Eqn (7) gives:

$$\frac{\delta J}{J} = \sum_{l=1}^{m-m_0} R_{T_l}^J \frac{\gamma_{lk}}{T_l}\frac{\delta S_k}{S_k} S_k. \tag{9}$$

The two flux equations, Eqns (6) and (9), must be equivalent since both calculations assume the same primary event as a change in S_k. We can therefore equate their right hand sides and eliminate the common term, $\delta S_k/S_k$, to give finally

$$R_{S_k}^J = \sum_{l=1}^{m-m_0} R_{T_l}^J \frac{\gamma_{lk} S_k}{T_l}. \tag{10}$$

This equation quantifies the relationship between the response to perturbations of the internal state (i.e. the internal metabolite concentration) and its response with respect to a change in the conservation totals. Eqn (10) is a result of the definition in Eqn (1) and the fact that metabolite S_k can be involved in various moiety-conserved cycles.

The corresponding relationship for the concentration response coefficients is:

$$R_{S_k}^{S_i} = \sum_{l=1}^{m-m_0} R_{T_l}^{S_i} \frac{\gamma_{lk} S_k}{T_l}. \tag{11}$$

In matrix form, Eqns (10) and (11) can be re-expressed as follows (Sauro, 1994):

$$\begin{aligned} \mathbf{R}_S^J &= \mathbf{R}_T^J (\text{diag } \boldsymbol{T})^{-1}\, \gamma\, (\text{diag } \boldsymbol{S}), \\ \mathbf{R}_S^S &= \mathbf{R}_T^S (\text{diag } \boldsymbol{T})^{-1}\, \gamma\, (\text{diag } \boldsymbol{S}), \end{aligned} \tag{12}$$

where $\mathbf{R}_S^J$ is an n row by m column matrix containing $R_{S_k}^{J_i}$ elements with $i = 1, \ldots, n$ and $k = 1, \ldots, m$. $\mathbf{R}_T^J$ is an n by $m-m_0$ matrix, diag $\boldsymbol{T}$ is an $m-m_0$ by $m-m_0$ square matrix with all off-diagonal elements equal to zero, and $(\text{diag } \boldsymbol{T})_{ii} = T_i$, diag $\boldsymbol{S}$ is an m by m square matrix with all off-diagonal elements equal to zero, and $(\text{diag } \boldsymbol{S})_{ii} = S_i$; $\mathbf{R}_S^S$ is an m by m square matrix, $\mathbf{R}_T^S$ is an m by $m-m_0$ matrix.

Eqns (12) allow one to express the response to perturbations in the internal state, $\mathbf{R}_S^J$, in terms of the response to the changes in the conservation totals, $\mathbf{R}_T^J$. Alternatively, the response, $\mathbf{R}_T^J$, can be expressed in terms of the $\mathbf{R}_S^J$. In order to do this we must be able to form the right inverse of γ, i.e. the m by $m-m_0$ matrix, $\gamma_{\text{right}}^{-1}$.

The rank of the conservation matrix, γ, is equal to $(m-m_0)$, or in other words it has full rank and all its rows are independent. As a result, we can form a right inverse, $\gamma_{\text{right}}^{-1}$ of γ, defined by:

$$\gamma\, \gamma_{\text{right}}^{-1} = \boldsymbol{I}_{m-m_0}.$$

If we assume the standard form for γ, i.e. $[-\boldsymbol{L}_0\ \boldsymbol{I}_{m-m_0}]$ (see Appendix), then the simplest right inverse is:

$$\gamma_{\text{right}}^{-1} = \begin{bmatrix} \mathbf{0} \\ \boldsymbol{I}_{m-m_0} \end{bmatrix},$$

where $\mathbf{0}$ is the m_0 by $(m-m_0)$ zero matrix.

However, for the most general case where γ can be any form, and not necessarily the standard form, the following strategy can be used. Post-multiplying both sides of $\gamma\, \gamma_{\text{right}}^{-1} = \boldsymbol{I}^{m-m_0}$ by $\gamma\gamma^T$ where γ^T signifies the transpose, and rearranging yields a second form for the right inverse:

$$\gamma_{\text{right}}^{-1} = \gamma^T (\gamma\, \gamma^T)^{-1}.$$

Eqns (12) can be rearranged by post-multiplying both sides by

$$(\text{diag } \boldsymbol{S})^{-1}\, \gamma_{\text{right}}^{-1}\, \text{diag } \boldsymbol{T},$$

so that

$$\mathbf{R}_T^J = \mathbf{R}_S^J (\text{diag } \boldsymbol{S})^{-1}\, \gamma_{\text{right}}^{-1}\, \text{diag } \boldsymbol{T}, \tag{13}$$

$$\mathbf{R}_T^S = \mathbf{R}_S^S (\text{diag } \boldsymbol{S})^{-1}\, \gamma_{\text{right}}^{-1}\, \text{diag } \boldsymbol{T}. \tag{14}$$

We have now described in some detail the relationship between the response to changes in the total amount of moiety and the response to perturbations of the internal state. To complete this analysis we now consider the relationship of the R_T coefficients to the theorems of metabolic control analysis and hence the control coefficients and elasticities.

GENERALISED CONNECTIVITY THEOREMS

Flux connectivity theorem

The following will apply to networks of enzyme reactions which are traditionally treated by metabolic control

analysis but also to networks which consist of elemental chemical conversions. A consideration of metabolic systems in terms of their elemental chemical transformation is important in those cases which involve high enzyme concentrations, multiple moiety conservations, group transfer or metabolic channelling. In order that the results will be applicable to this more general case we define n parameters ξ_i, $i = 1, \ldots, n$ each modulating the activity of only the i^{th} elemental process (Kholodenko et al., 1993):

$$v_i(S, \xi_i) = \xi_i \, v_i(S). \tag{15}$$

In relation to the classical pathways, the parameter ξ_i is equivalent to the activity or concentration of an enzyme.

According to the definition given by Eqn (15), the flux and concentration control coefficients of any process v_i can be defined in the following way:

$$C^J_{v_i} = \frac{d \ln |J|}{d \ln \xi_i}, \quad C^{S_j}_{v_i} = \frac{d \ln S_j}{d \ln \xi_i}.$$

In the case where ξ_i is chosen as the concentration of enzyme i, the control coefficient $C^J_{v_i}$ is equivalent to the 'classical' enzyme control coefficient $C^J_{e_i}$. In the initial steady-state of the system, the concentration S_k is perturbed by an amount δS_k. If no additional perturbations are made, the system will relax to a different steady-state (since the parameters T are changed according to Eqn (1) and we assume the system to have structurally stable steady-states), and the initial value of $S + \delta S_k$ will evolve to the final steady-state value. Following the procedure described by Kacser and Burns (1973), let us suppose that after a perturbation in S_k we simultaneously change the parameters ξ_i of all reactions (or the elemental processes) which depend on S_k, to such an extent that all the rates are returned to their initial non-perturbed values, i.e.

$$0 = \frac{\delta v_i}{v_i} = \frac{\delta S_k}{S_k} \varepsilon^{v_i}_{S_k} + \frac{\delta \xi_i}{\xi_i}. \tag{16}$$

The newly attained state is again steady and notwithstanding the perturbed parameters values T_l and ξ_i, all the fluxes will be the same as in the initial steady-state, i.e. there is no change in flux. Since the fluxes, J, are functions of T_l and ξ_i one can write:

$$0 = \frac{\delta J}{J} = \sum_{l=1}^{m-m_0} R^J_{T_l} \frac{\delta T_l}{T_l} + \sum_{i=1}^{n} C^J_{v_i} \frac{\delta \xi_i}{\xi_i}. \tag{17}$$

It follows from Eqns (8) and (16) that (Kholodenko, 1988),

$$\sum_{l=1}^{m-m_0} R^J_{T_l} \frac{\gamma_{lk} S_k}{T_l} = \sum_{i=1}^{n} C^J_{v_i} \varepsilon^{v_i}_{S_k}. \tag{18}$$

The right side of Eqn (18) will be recognised as half of the flux-control connectivity theorem, i.e. as the sum of all the connectivities between S_k and J. If we replace the left side of Eqn (18) with the left side of Eqn (10) we obtain

$$R^J_{S_k} = \sum_{i=1}^{n} C^J_{v_i} \varepsilon^{v_i}_{S_k}. \tag{19}$$

This is the same equation as presented by Westerhoff and Van Dam (1987), and Reder (1988). Noting that for any truly independent variable S_k (e.g. not restrained by moiety conservations), $R^J_{S_k}$ must equal zero, Westerhoff and Van Dam (1987) rederived the flux-control connectivity theorem from this equation. It should be noted that because $C^J_{v_i}$ can be expressed in terms of elasticity coefficients and the network structure, the above equation also allows one to express the responses to metabolite concentrations perturbations with these terms. Consequently, Eqn (19) and Eqn (18) may be considered a generalisation of the flux connectivity theorem.

Concentration connectivity theorem

To derive the response of the metabolite concentration changes in moiety-conserved totals, we will use the same approach as was used for the flux response. After making a perturbation in S_k, we simultaneously change the parameters ξ_i of all reactions which depend on S_k so that all the reaction rates are undisturbed. This means that all the metabolites other than S_k will also be undisturbed under these conditions. This last observation can be stated formally as:

$$\frac{\delta S_j}{S_j} = \delta_{jk} \left(\frac{\delta S_k}{S_k} \right).$$

Here $\delta_{jk} = 1$ if $k = j$ and $\delta_{ij} = 0$ if $k \neq j$.

Using the same approach as before, that is considering the steady-state concentrations as the functions of T_l and ξ_i one obtains (Kholodenko, 1988):

$$\sum_{l=1}^{m-m_0} R^{S_j}_{T_l} \frac{\gamma_{lk} S_k}{T_l} = \delta_{jk} + \sum_{i=1}^{n} C^{S_j}_{v_i} \varepsilon^{v_i}_{S_k}, \tag{20}$$

clearly analogous to Eqn (18). The right side of this equation will be recognised as half the concentration-control connectivity theorem. Replacing the left side of Eqn (20) by the left side of Eqn (11) one obtains:

$$R^{S_j}_{S_k} = \delta_{jk} + \sum_{i=1}^{n} C^{S_j}_{v_i} \varepsilon^{v_i}_{S_k}.$$

Noting that for truly independent variables S_j and S_k, $R^{S_j}_{S_k}$ must equal zero, Westerhoff and Chen (1984) derived the concentration-control connectivity theorem from this equation.

Relationship between the connectivities and responses

Expressing the above relationships, Eqns (18) and (20), in matrix form and combining them with Eqns (12) we obtain the main result of this study:

$$\mathbf{R}^J_S = \mathbf{R}^J_T (\text{diag } \boldsymbol{T})^{-1} \gamma (\text{diag } \boldsymbol{S}) = \mathbf{C}^J_v \varepsilon^v_S, \tag{21}$$

$$\mathbf{R}^S_S = \mathbf{R}^S_T (\text{diag } \boldsymbol{T})^{-1} \gamma (\text{diag } \boldsymbol{S}) = \mathbf{C}^S_v \varepsilon^v_S + \boldsymbol{I}_m. \tag{22}$$

In addition, Eqns (21) and (22) can be rearranged in the manner described previously to yield:

$$\mathbf{R}^J_T = \mathbf{C}^J_v \varepsilon^v_S (\text{diag } \boldsymbol{S})^{-1} \gamma^{-1}_{\text{right}} (\text{diag } \boldsymbol{T}), \tag{23}$$

$$\mathbf{R}^S_T = (\mathbf{C}^S_v \varepsilon^v_S + \boldsymbol{I}_m) (\text{diag } \boldsymbol{S})^{-1} \gamma^{-1}_{\text{right}} (\text{diag } \boldsymbol{T}). \tag{24}$$

Equating the first and third expressions from Eqns (21) and (22) gives Reders' equations [compare Eqn (19)] of responses to changes in the internal state. These equations, which have been obtained by a formal mathematical method, have an interpretable meaning by equating the terms which enter the connectivity theorem to the response to changes of all moiety conserved cycles which a particular metabolite links. Again, when S is an independent metabolic variable, the responses are 0 or -1 and Eqns (21) and (22) reduce to the standard connectivity theorems (Burns, 1971; Westerhoff and Chen, 1984).

SPECIFIC APPLICATIONS

Linked enzyme/substrate moiety-conserved cycles

Fig. 1 illustrated the simplest possible conserved cycle where each reaction step of the cycle was assumed to be

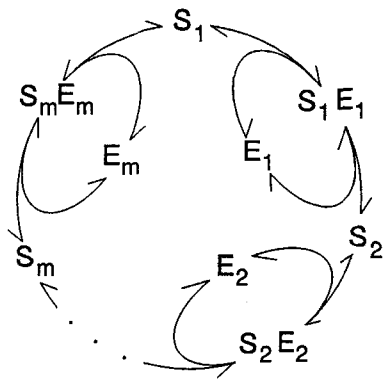

Fig. 2. Metabolic cycle with internal enzyme cycles. Each enzyme cycle is shown in the form of the Michaelian mechanism, i.e. only enzyme states E_i and S_iE_i are present.

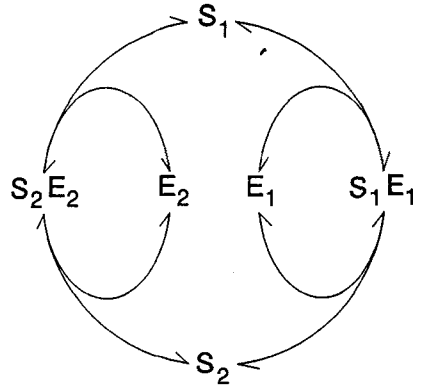

Fig. 3. The pathway of two enzyme cycles involving enzymes, E_1 and E_2, linked via the coenzyme couple, S_1 and S_2.

catalysed by an individual enzyme. In Fig. 2 we represent the same cycle but with the internal enzyme cycles shown (for simplicity) in the form of Michaelian mechanisms; this model is clearly much more realistic. In the first example we will analyse this model, but without loss of generality, we will reduce the cycle to just two enzymes, as shown in Fig. 3. The pathway in Fig. 3 is composed of two enzyme cycles involving enzymes, E_1 and E_2, linked via the coenzyme couple, S_1 and S_2. For such a system, the traditional approach would be to condense the two enzyme cycles into two single reactions for each. In such an approximation, the fact that each enzyme is capable of sequestering free substrate from the bulk phase pools is ignored. Condensing the enzyme cycles into a single reaction is quite valid when the degree of sequestration is small compared to the total substrate mass in the system or when S_1 and S_2 are not part of a single moiety-conserved cycle. However, we wish to consider the case when there is significant sequestration, and for this reason an explicit recognition of the enzyme cycles is necessary. Further comment and an analysis of such pathways will be found in Fell and Sauro (1990), Kholodenko et al. (1992) and Sauro (1994). We begin by writing down the mass conservation relationships:

$$E_1 + ES_1 = T_{e_1}, \quad E_2 + ES_2 = T_{e_2}, \qquad S_1 + ES_1 + ES_2 + S_2 = T_S. \tag{25}$$

To simplify the notation we will designate the enzyme totals, T_{e_1} and T_{e_2} as e_1 and e_2 so that the response coefficients with respect to the total amounts of E_1 and E_2 moiety are written as R_{e_1} and R_{e_2}. The aim of the following analysis is to compute the form of the classical flux summation theorem, i.e. the sum $C^J_{e_1} + C^5_{e_2}$. The response coefficients, $R^J_{e_1}$ and $R^J_{e_2}$ are operationally equivalent to $C^J_{e_1}$ and $C^J_{e_2}$ so that we must concentrate on computing $R^J_{e_1}$ and $R^J_{e_2}$. To do this we will treat the individual enzyme reactions at the level of the elemental steps which involve the turnover of free and complexed enzyme forms.

We begin by describing the responses of the fluxes to perturbations to the internal states of the free and complexed forms of both enzymes. Thus using Eqns (18) and (19) we obtain a relation for $R^J_{E_1}$ and $R^J_{ES_1}$:

$$R^J_{E_1} = \frac{E_1}{e_1} R^J_{e_1} \equiv \frac{E_1}{e_1} C^J_{e_1} = C^J_{v_1} \varepsilon^{v_1}_{E_1} + C^J_{v_2} \varepsilon^{v_2}_{E_1}, \tag{26}$$

$$R^J_{ES_1} = \frac{ES_1}{e_1} R^J_{e_1} + \frac{ES_1}{T_S} R^J_{T_S} \equiv \frac{ES_1}{e_1} C^J_{e_1} + \frac{ES_1}{T_S} R^J_{T_S} = C^J_{v_1} \varepsilon^{v_1}_{ES_1} + C^J_{v_2} \varepsilon^{v_2}_{ES_1}. \tag{27}$$

Note that we have replaced the notation, $R^J_{e_1}$, with the equivalent, $C^J_{e_1}$ as this will make further interpretation clearer. It is also worth mentioning that the additional term in the second equation, namely, $R^J_{T_S}$, reflects the fact that ES_1 occurs in two conservation relationships, the totals for T_{e_1} and T_S.

Noting that the rates of the elemental steps with respect to the enzyme forms are first-order, it is straightforward to show that the internal elasticities are related by the simple relations:

$$\varepsilon^{v_1}_{E_1} + \varepsilon^{v_1}_{ES_1} = 1 \quad \text{and} \quad \varepsilon^{v_2}_{E_1} + \varepsilon^{v_2}_{ES_1} = 1. \tag{28}$$

Combining Eqns (26) and (27) and using Eqn (28) yields:

$$C^J_{e_1} = C^J_{v_1} + C^J_{v_2} - \frac{ES_1}{T_S} R^J_{T_S}. \tag{29}$$

Using the same reasoning as was used above, the equation for the second enzyme cycle can also be found:

$$C^J_{e_2} = C^J_{v_3} + C^J_{v_4} - \frac{ES_2}{T_S} R^J_{T_S}. \tag{30}$$

In contrast to the classical expectation where the sum of the microscopic control coefficients is expected to equal the enzyme control coefficient, in the case of the linked substrate cycles the sum of the microscopic control coefficients is reduced as a result of sequestration $\left(\text{by the amount of } \frac{ES_2}{T_S} R^J_{T_S}\right)$. These equations show that the microscopic control coefficients do not sum to the enzyme control coefficients but an additional term indicates that titration with enzyme would underestimate the true value provided the enzyme concentration is sufficiently high and comparable to the free substrate concentrations.

The summation theorem can be obtained by simply summing Eqns (29) and (30),

$$C^J_{e_1} + C^J_{e_2} = 1 - \frac{T_b}{T_S} R^J_{T_S},$$

where T_b is equal to the sum of the complexed forms, ES_1 + ES_2. Once again, we see that the value of the summation theorem is reduced by the sequestration term, $R^J_{T_S}$ (T_b/T_S). Operationally this means that titrating with both enzymes

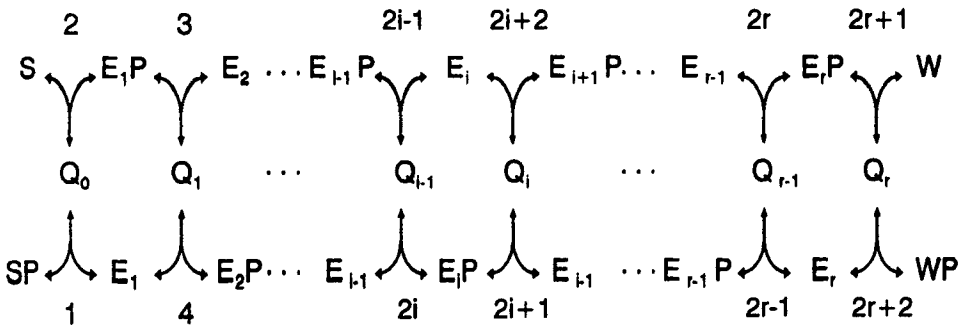

Fig. 4. Group-transfer pathway. A group P is transferred between r enzymes from the donor SP to the ultimate acceptor W. The enzyme/enzyme complexes are designated by Q_i. The numbers of the elemental steps are shown.

will result in a non-proportional attenuated response. This analysis is not limited to two enzymes, but can be easily extended in the same manner with cycles having an arbitrary number of enzymes. For n enzymes, the equation is (Kholodenko et al., 1992):

$$\sum_{i=1}^{n} C_{e_i}^J = 1 - \frac{T_b}{T_S} R_{T_S}^J. \tag{31}$$

Group-transfer pathway

This second example deals with pathways involving the transfer of a chemical group through a series of proteins, i.e. group-transfer pathways (or relay pathways). Examples are the bacterial phosphotransferase system transferring a phosphate group from phospho*enol*pyruvate to a sugar molecule whilst transporting the latter across the membrane (Postma et al., 1993), and the electron transport chain in mitochondrial and bacterial membranes transferring an electron from, e.g. NADH to oxygen. Such a group transfer pathway can be considered as a perfect, dynamic channel in which the transferred group is not released into the bulk aqueous phase until it reaches the end of the reaction sequence.

Fig. 4 shows a group-transfer pathway where a group P is transferred between r enzymes from the donor SP to the ultimate acceptor W. The enzyme-enzyme complexes are designated by $Q_i = E_iPE_{i+1}$, where i and $i+1$ are the numbers of adjacent enzymes (for $i = 0$ and $i = r$, $Q_0 = SPE_1$, $Q_r = E_rPW$). There are r moiety-conserved cycles which correspond to the conservation of the total concentration (e_i) of any enzyme i:

$$E_i + E_iP + Q_{i-1} + Q_i = e_i,\ i = 1, 2, \ldots, r. \tag{32}$$

Note, that these cycles are linked since the enzyme-enzyme complexes Q_i enter $i-1$ and i moiety conservation totals simultaneously. The four elemental steps (processes) in which enzyme i forms participate will be called the reaction cycle of the enzyme i (Fig. 4).

Taking into account that the free enzyme form E_i and its 'loaded' form E_iP enter the single moiety-conserved cycle, one can calculate the control coefficient of any enzyme i as a response either to a change in free enzyme or 'loaded' enzyme concentration. Using Eqns (10) and (19) one obtains:

$$R_{E_i}^J = \frac{E_i}{e_i} C_{e_i}^J = \sum_j C_{v_j}^J \varepsilon_{E_i}^{v_j},\ i = 1, 2, \ldots, r, \tag{33}$$

$$R_{E_iP}^J = \frac{E_iP}{e_i} C_{e_i}^J = \sum_j C_{v_j}^J \varepsilon_{E_iP}^{v_j},\ i = 1, 2, \ldots, r. \tag{34}$$

Since only two rates depend on the concentrations E_i or E_iP (Fig. 4), only two non-zero terms enter the right hand sides of these equations.

For the response coefficients to a change in the concentrations Q_{i-1} and Q_i of enzyme-enzyme complexes one may write using Eqns (10) and (19):

$$R_{Q_i}^J = C_{e_i}^J \frac{Q_i}{e_i} + C_{e_{i+1}}^J \frac{Q_i}{e_{i+1}} = \sum_j C_{v_j}^J \varepsilon_{Q_i}^{v_j},\ i = 1, 2, \ldots, r-1. \tag{35}$$

For the responses to a change in Q_o and Q_r only a single term is present in the left-hand side of Eqns (35), i.e. the term with $C_{e_1}^J$ or with $C_{e_r}^J$, respectively. Summing the four equations describing the responses to change in the concentrations of all the forms of the enzyme i [see Eqn (32)] one obtains (after slight rearrangement):

$$C_{e_{i-1}}^J \frac{Q_{i-1}}{e_{i-1}} + C_{e_i}^J + C_{e_{i+1}}^J \frac{Q_i}{e_{i+1}} = \sum_j C_{v_j}^J (\varepsilon_{E_i}^{v_j} + \varepsilon_{E_iP}^{v_j} + \varepsilon_{Q_{i-1}}^{v_j} + \varepsilon_{Q_i}^{v_j}),\ i = 2, \ldots, r-1. \tag{36}$$

The step rates are first-order functions with respect to any enzyme form. Consequently for any reaction j participating in the reaction cycle of the enzyme i,

$$\varepsilon_{E_i}^{v_j} + \varepsilon_{E_iP}^{v_j} + \varepsilon_{Q_{i-1}}^{v_j} + \varepsilon_{Q_i}^{v_j} = 1,$$

and for any reaction j outside the reaction cycle of the enzyme i,

$$\varepsilon_{E_i}^{v_j} + \varepsilon_{E_iP}^{v_j} + \varepsilon_{Q_{i-1}}^{v_j} + \varepsilon_{Q_i}^{v_j} = 0.$$

Then,

$$C_{e_{i-1}}^J \frac{Q_{i-1}}{e_{i-1}} + C_{e_i}^J + C_{e_{i+1}}^J \frac{Q_i}{e_{i+1}} = \sum_{\substack{\text{rates inside} \\ \text{the enzyme} \\ i \text{ cycle}}} C_{v_j}^J,\ i = 2, \ldots, r-1. \tag{37}$$

For the enzymes at the ends of the chain, i.e. for $i = 1$ and $i = r$, the terms with $C_{e_0}^J$ and with $C_{e_{r+1}}^J$ disappear from Eqn (37).

The sum of C_v control coefficients over all the steps of the enzyme cycle has been termed the 'impact' control coefficient of the enzyme i by Kholodenko and Westerhoff (1993). In classical pathways, the impact control coefficient is equivalent to the classical control coefficient measured with respect to enzyme concentration. In relation to various non-ideal pathways (Kholodenko et al., 1993) the impact control coefficient is an analogue of the control coefficient

measured with respect to the enzyme rate (compare Sauro and Fell, 1990; Sauro and Kacser, 1990; Schuster and Heinrich, 1992).

We will designate the control exerted by the 'boundary' metabolites S, SP and W, WP, i.e. the sum of the corresponding response coefficients, by $C^J_{e_0}$ and $C^J_{e_{r+1}}$ as follows:

$$C^J_{e_0} = R^J_S + R^J_{SP}, \quad C^J_{e_{r+1}} = R^J_W + R^J_{WP}. \tag{38}$$

Expressing response coefficients in Eqn (38) via the control coefficients of the steps times their elasticities with respect to the boundary metabolites and combining with responses to a change in the concentrations Q_0 and Q_r one obtains:

$$C^J_{e_0} + C^J_{e_1}\frac{Q_0}{e_1} = C^J_{v_1} + C^J_{v_2}, \tag{39}$$

$$C^J_{e_{r+1}} + C^J_{e_r}\frac{Q_r}{e_r} = C^J_{v_{2r+1}} + C^J_{v_{2r+2}}. \tag{40}$$

Summing Eqns (37) over all the enzymes in a group transfer pathway and adding Eqns (39) and (40) one finally obtains the new summation theory that is valid for any relay pathway:

$$C^J_{e_0} + \sum_{i=1}^{r} C^J_{e_i}\left(1 + \frac{Q_{i-1} + Q_i}{e_i}\right) + C^J_{e_{r+1}} = 2. \tag{41}$$

Hence, the sum of the enzyme control coefficients always exceeds unity in the system of group transfer (perfect dynamic channel). However, in the general case of channelling it can be less than unity (Sauro and Kacser, 1990; Kholodenko and Westerhoff, 1993; Sauro, 1994).

DISCUSSION

This study describes in detail the general approach that elucidates principles of regulation and control in systems with complex linked moiety-conserved cycles. Such cycles appear frequently in real metabolic pathways and may give these pathways special regulatory properties (Hofmeyr et al., 1986). Moreover, one should recognize that inside any reaction cycle, through which some (substrate) chemical moiety is conserved, there are internal (enzyme) moiety conserved cycles. The latter cannot be ignored if an appreciable amount of pathway metabolites is bound to enzymes, i.e. exists inside these internal cycles. As shown in this study, group transfer, metabolite sequestration and metabolic channelling are all examples of important biological phenomena that depend on linked moiety-conserved cycles. Traditional metabolic control analysis (Fell, 1992) has not dealt with the analysis of such systems. We have treated such pathways by examining their responses to perturbations in the concentration of any internal metabolite (R_S coefficient) and the responses to changes in the total sum of any conserved moiety (R_T coefficient).

When transiently changed concentrations are freely variable, the system will return to its previous steady state (Westerhoff and Chen, 1984; Westerhoff and Van Dam, 1987). However, when imposed changes in metabolite concentrations imply a change in the moiety conservation totals, the system will relax to a different steady state. Using such a reasoning, we developed the relationship between the response of the system to perturbations in the internal state and the response of the system to changes in the total sum of moiety [Eqns (12–14)].

We showed in this study, that the response of the flux (J) to a change in the concentration of the internal metabolite (S_k) coincides with the sum of all the connectivities between (S_k) and (J) that is the right-hand side of the connectivity theorem (compare Reder, 1988). As a consequence, comparing the responses to changes in internal metabolites and conservation totals this paper derives generalised connectivity theorems [Eqns (21–24), compare 'classical' connectivity theorems (Kacser and Burns, 1973; Westerhoff and Chen, 1984)].

For non-ideal systems (Kholodenko et al., 1993) where the classical assumptions of additivity and independence do not apply (Sauro and Kacser, 1990), the results obtained in this study allow one to understand the effects of variations in the enzyme concentrations. For these systems, the theorems of traditional metabolic control analysis (Kacser and Burns, 1973; Heinrich and Rapoport, 1974) cannot be applied to understand the control exerted by the enzymes. This study shows, however, the advantages of considering those systems as networks of elemental chemical conversions (a 'microdescription' of the system; Kholodenko and Westerhoff, 1993). Different forms of any enzyme play the role of the metabolites of such a network, and the control coefficient of an enzyme is the response coefficient to a change in the corresponding moiety conserved total, i.e. in the total concentration of that enzyme. Such an approach allowed us to calculate the control coefficients and the summation theorem for systems, for example, with metabolic sequestration and for group transfer reactions. We show that in contrast to the classical expectation, the sum of the enzyme control coefficients is reduced by the sequestration of metabolites [Eqn (31), compare Fell and Sauro, 1990, Kholodenko et al., 1992) and of the enzymes [Eqn (41), Kholodenko et al., 1993]. However, in group transfer pathways the direct enzyme/enzyme interactions tend to increase that sum [Eqn (37), compare Van Dam et al., 1993].

APPENDIX

One of the problems in representing the $m-m_0$ conservation constraints of a pathway is determining the entries to γ. The task is made more difficult by the fact that more than one solution is possible. Following Reder (1988), we partition the $\boldsymbol{S}$ vector of metabolites into m_0 independent metabolites, $\boldsymbol{S}_I$, and $m-m_0$ dependent metabolites, $\boldsymbol{S}_D$, thus, $\boldsymbol{S} = [\boldsymbol{S}_I\ \boldsymbol{S}_D]^T$. The stoichiometry matrix, $\boldsymbol{N}$, is also partitioned (and reordered if necessary) to match the ordering in the $\boldsymbol{S}$ vector. Given that $\boldsymbol{v}$ is the vector of reaction rates, v_i, then following Reder:

$$\boldsymbol{N}\boldsymbol{v} = \begin{bmatrix}\boldsymbol{N}_r\\ \boldsymbol{N}'_r\end{bmatrix}\boldsymbol{v} = \begin{bmatrix}\dot{\boldsymbol{S}}_I\\ \dot{\boldsymbol{S}}_D\end{bmatrix}.$$

The rows of $\boldsymbol{N}'_r$ are formed from linear combinations of the independent rows in $\boldsymbol{N}_r$. As a result, we are able to define a link matrix (Reder, 1988), $\boldsymbol{L}_0$, that will transform one into the other:

$$\boldsymbol{N}'_r = \boldsymbol{L}_0\boldsymbol{N}_r.$$

We can therefore write

$$\boldsymbol{N}_r\boldsymbol{v} = \dot{\boldsymbol{S}}_I,$$

$$\boldsymbol{L}_0\boldsymbol{N}_r\boldsymbol{v} = \dot{\boldsymbol{S}}_D,$$

from which $\dot{\boldsymbol{S}}_D - \boldsymbol{L}_0\dot{\boldsymbol{S}}_I = 0$ and in integrated form $\boldsymbol{S}_D - \boldsymbol{L}_0\boldsymbol{S}_I = \boldsymbol{T}$.

Thus finally

$$[-\boldsymbol{L}_o\boldsymbol{I}]\begin{bmatrix}\boldsymbol{S}_I\\ \boldsymbol{S}_D\end{bmatrix} = \boldsymbol{T}.$$

Comparing this last equation with Eqn (1) from the main text shows that the term $[-\boldsymbol{L}_o\boldsymbol{I}]$ is equal to the conservation matrix, γ. It is important to appreciate that other row orderings of the stoichiometry matrix are possible which would yield alternative forms for γ and by necessity alternative physical interpretations to $\boldsymbol{T}$. The interpretation problems of different γ and $\boldsymbol{T}$ forms in relation to computer algorithms for determining γ are briefly discussed in Sauro and Fell (1991).

This study was supported by the United Nations Educational Scientific and Cultural Organisation and the Netherlands Organisation for Scientific Research (NWO).

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