Molecular control analysis: Control within proteins and molecular processes.

Brown, G.C.; Westerhoff, H.V.; Kholodenko, B.N.

DOI
10.1006/jtbi.1996.0178
Publication date
1996

Published in
Journal of Theoretical Biology

J. theor. Biol. (1996) **182,** 389–396

Molecular Control Analysis: Control within Proteins and Molecular Processes*

GUY C. BROWN†, HANS V. WESTERHOFF‡ AND BORIS N. KHOLODENKO§

† *Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.*, ‡ *Department of Microbial Physiology, Free University, De Boelelaan* 1087, *NL*-1081 *TV Amsterdam, The Netherlands, and the* § *A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University*, 119899 *Moscow, Russia*

Molecular control analysis is a method for analysing the extent to which the different elementary steps or rate constants within a molecular process limit the steady-state rate (or other variables) of that process. Any process which may be described by a kinetic diagram of transitions between states of the system may be analysed by molecular control analysis, and this approach has previously been used to analyse control within enzymes, transporters, enzyme complexes, channelled pathways, and group-transfer pathways. We outline the theory of molecular control analysis here, and illustrate its use by analysing control within enzymes (three β-lactamases). Further potential applications include signal-transduction processes, protein folding, and chemical reactions.

1. Introduction

Metabolic control analysis is mainly used to analyse the extent to which different enzymes in a pathway limit the pathway flux. However, the same principles may be used to analyse control within an enzyme, transporter, or any other molecular processes which can be described with a kinetic diagram and rate constants. Molecular control analysis is a method of analysing the extent to which the different rate constants or kinetic steps within a molecular process limit the steady-state rate of that process, or control the level of some intermediate. Potential applications include analysis of rate-limitation within single, isolated enzymes (Brown & Cooper, 1993, 1994; Kholodenko & Westerhoff, 1994), transporters (Brown, 1995), enzyme complexes, interacting proteins (Kacser *et al*., 1990), channelled pathways (Welch *et al*., 1988; Sauro & Kacser, 1990; Sauro, 1994; Kholodenko *et al*., 1995), group-transfer pathways (Van Dam *et al*., 1993; Kholodenko & Westerhoff, 1995a), ion-transporting ATPases, G-proteins, actinomyosin-ATPase, ribosome function, protein folding, receptor function, signal-transduction pathways, DNA supercoiling, and chemical reactions. Control over intermediate levels may also be analysed, for example control over levels of enzyme intermediates, ligand binding, active G-protein, active receptor, DNA supercoiling, and phosphorylated protein. Also control over enzyme K_M and V_{max}/K_M, and many other protein parameters may be analysed. A connected group of theories has been developed to analyse control over molecular processes (Ray, 1983; Brown & Cooper, 1993, 1994; Kholodenko *et al*., 1994a; Kholodenko & Westerhoff, 1994; Sauro, 1994), mostly derived from metabolic control analysis, and we shall refer to this bundle of theory as molecular control analysis.

The distinction between metabolic control analysis and molecular control analysis is both subtle and striking. The first author (GCB) did not think it possible to apply control analysis to rate-limitation within an enzyme for several years. One factor that changed his mind was an off-the-cuff remark by Henrik Kacser in 1990 to the effect that it was

* This paper is dedicated to the memory of Henrik Kacser.
† Author to whom correspondence should be addressed.

0022–5193/96/190389 + 08 $25.00/0

possible. That gave him the confidence to try it out. However, although it was clearly obvious to Kacser, it still took him a year or two (together with Chris Cooper at UCL) to convince himself that is was both possible and useful. HVW and BNK came to realise this possibility in the minibus to the 1992 BioThermo-Kinetics meeting, where Kacser in his opening lecture declared that there was nothing left to develop on the theoretical side of metabolic control analysis! HVW and BNK got to this realisation after understanding that this is how metabolic control analysis could deal with channelling and group-transfer pathways. Indeed protein-protein interactions are another area where the applications of metabolic control analysis are not obvious, and have been regarded as essentially a hindrance to metabolic control analysis. Kacser *et al*. (1990) and Sauro & Kacser (1990) produced some of the first papers in this area showing that some of the effects of protein-protein interactions could be quantified within metabolic control analysis.

Why is it that metabolic control analysis seemed unable to deal with control within enzymes, and with protein-protein interactions? There are at least four problems: (1) no free intermediate, (2) thermodynamics, (3) no direct correspondence between enzymes and processes, and (4) experimental/practical usefulness. Enzymes within a normal metabolic pathway are linked by freely diffusible metabolic intermediates. Thus inactivation of 10% of the protein molecules of one enzyme within the pathway will cause the level of the enzyme's substrate to rise and that of its product to fall, leading to a stimulation of the 90% of the enzyme population that is not inactivated. Thus, the control coefficient of the enzyme may be less than one. In contrast the different kinetic steps within an enzyme are linked by enzyme-bound intermediates or different states of the enzyme which are not freely-diffusible. Inactivation of a kinetic step within 10% of the protein molecules of an isolated enzyme will cause complete inhibition of those protein molecules, but have no effect on the 90% of protein molecules not inactivated. Thus, the control coefficient of the step (and any other step), changed in this non-homogenous fashion, will always be one; and thus a control analysis based on such changes is not useful. However, if instead of inhibiting a step by 100% in 10% of the population of enzyme molecules (non-homogenous inhibition), one inhibits the same step by only 10% in 100% of the population of molecules (a homogenous inhibition), then the enzyme intermediates that act as substrate and product of that step will rise and fall respectively leading to a stimulation of the step. Thus, the control coefficient of the step (when defined for homogenous change) will be smaller than one, and obey summation, branching and connectivity theorems analogous to those of metabolic pathways (see below).

A second objection to applying control analysis to isolated enzymes is that there are thermodynamic restrictions on the type of perturbation that can be made to the kinetics of an enzyme. For example a single rate constant cannot normally be changed alone, as this would result in a change in the equilibrium constant of the reaction, which is not possible. Thus, rate constants must be changed in proportion such that the equilibrium constant is unchanged. However, this does not prevent control coefficients of single rate constants being defined and measured, but it does mean that these coefficients must be used in appropriate combinations to predict real changes (see Brown & Cooper, 1993, 1994; Kholodenko & Westerhoff, 1994; Van Dam *et al*., 1993).

A more general objection to molecular control analysis is one of practical application and usefulness. It may be difficult to measure control coefficients within enzymes (or protein complexes) without measuring rate constants, and if rate constants can be measured, control coefficients may be unnecessary, since all rate changes can be predicted from rate constants alone. We have suggested various methods of measuring control coefficients within proteins (Brown & Cooper, 1994; Kholodenko *et al*., 1994a), and demonstrated that the control coefficients may contain information that is not at all obvious from the rate constants (Brown & Cooper, 1993; Brown, 1995).

In the following sections we will outline the application of molecular control analysis to single enzymes, and then illustrate this by analysing control within three β-lactamases. We will then show how it can be used to analyse protein interactions.

2. Control Analysis Within Single Isolated Enzymes

The theory of enzyme kinetics has been developed in terms of enzyme intermediates linked by steps, and these steps are characterised by rate constants. The flux control coefficient of a step (i) over the steady-state flux (J) of an enzyme can be defined as:

$$C_i^J = \frac{\partial J}{\partial v_i}\cdot\frac{v_i}{J} = \frac{\partial J}{J}\Big/\frac{\partial k_i}{k_i} \qquad (1)$$

where v_i is the local rate of step i, and both the forward (k_{+i}) and reverse (k_{-i}) rate constants of step i are changed in proportion (i.e. $\mathrm{d}k_i/k_i = \mathrm{d}k_{+i}/k_{+i} = \mathrm{d}k_{-i}/k_{-i}$). The flux control coefficient of a step

is just the percentage change in steady-state enzyme rate divided by the percentage change in rate constants of the step (changed proportionately), extrapolated to infinitesimally small change. This is closely approximated by the percentage change in rate brought about by a 1% change in the step. Similarly the extent to which a rate constant (k_{+i}) within an enzyme limits the steady-state rate (J) of that enzyme can be defined as a control coefficient of that rate constant over the steady-state flux (J):

$$C^J_{+i} = \frac{\partial J}{\partial k_{+i}} \cdot \frac{k_{+i}}{J} \tag{2}$$

The control coefficient of a rate constant is just the percentage change in steady-state enzyme rate divided by the percentage change in rate constant, extrapolated to infinitesimally small change. This is closely approximated by the percentage change in rate brought about by a 1% change in the rate constant.

The two types of control coefficient (C_i and C_{+i}) defined here differ in that the former quantifies the effect of a possible experimental operation, which does not affect any equilibrium constant, while the latter definition cannot correspond to any real experimental operation (unless the step involves ligand binding) because changing a single rate constant within an enzyme is not possible thermodynamically. Any real experimental operation will change a set of two or more rate constants, but the effect of these changes can be analysed as the sum of the effects due to the individual rate constants, i.e. using these control coefficients of rate constants. The control coefficient of a step is equal to the sum of the control coefficients of its rate constants, i.e.:

$$C^J_i = C^J_{+i} + C^J_{-i}. \tag{3}$$

These coefficients provide convenient and unambiguous quantifiers of rate limitation, and in addition obey many theorems which help to relate these coefficients to other parameters and variables (Ray, 1983; Brown & Cooper, 1993, 1994; Kholodenko *et al*., 1994; Kholodenko & Westerhoff, 1994; Van Dam *et al*., 1993). The most important of these theorems are outlined below.

For enzyme reactions with unbranched mechanisms the control coefficients of forward rate constants are positive ($C_{+i} \geqslant 0$). The control coefficients of reverse rate constants are negative ($C_{-i} \leqslant 0$), and the absolute value is always smaller than that of the corresponding forward rate constant ($C_{+i} \geqslant -C_{-i}$). The sum of the control coefficients of all steps or rate constants is one:

$$\sum_{i=1}^{n} C^J_i = 1. \tag{4}$$

The ratio of the absolute control coefficients of the reverse and forward rate constants of a step is equal to the disequilibrium ratio of that step, that is the ratio of the mass action ratio (Γ) to the equilibrium constant (K) of the step (i), or the ratio of reverse (v_{-i}) and forward (v_{+i}) unidirectional rates:

$$C^J_{-i}/C^J_{+i} = v_{-1}/v_{+i} = -\Gamma_i/K_i. \tag{5}$$

The fractional level of an enzyme intermediate [i.e. the concentration of the intermediate (E_j) divided by the total enzyme concentration (e)] is equal to the sum of the control coefficients of all rate constants depleting that intermediate. Thus for an intermediate (E_j) in a linear pathway:

$$\overset{i}{\rightleftharpoons} E_j \overset{j}{\rightleftharpoons}$$

with rate constants k_{-i} and k_{+j} leading away from it, the control coefficients of these rate constants are given by:

$$C^J_{-i} + C^J_{+j} = E_j/e. \tag{6}$$

The control coefficients of rate constants involving binding of enzyme substrates or products are equal to the sensitivity (elasticity ϵ) of the enzyme rate to changes in that reactant concentration. Thus, for example, the control coefficient of the rate constant involved in substrate (s) binding is given by:

$$C^J_{+s} = \epsilon_s. \tag{7}$$

Where the enzyme reaction pathway is branched, the sum of control coefficients for any branch over the common flux is proportional to the relative flux through that branch. Thus, if a reaction pathway (a) with flux J_a divides into two branches (b and c) with fluxes J_b and J_c, then the relative control coefficients of these branches over J_a is given by:

$$C^{J_a}_{J_b}/C^{J_a}_{J_c} = J_b/J_c \tag{8}$$

Control coefficients of steps or rate constants over any enzyme intermediate level, K_M, V_{max}/K_M, or any other variable of the system may also be defined (e.g. the control coefficient of a rate constant over the K_M of an enzyme can be defined as the normalised partial differential of the K_M with respect to the rate constant, which closely approximates to the percentage change in K_M caused by a 1% change in the rate constant). These coefficients obey theorems related to those for

flux control coefficients (Ray, 1983; Brown & Cooper, 1993, 1994; Kholodenko & Westerhoff, 1994).

If the mechanism and rate constants of an enzyme or other molecular process are known then the control coefficients of those rate constants and steps can be calculated directly, either by differentiating the rate equation with respect to the relevant rate constants or by numerical analysis. The control coefficients within triose phosphate isomerase, carbamate kinase, lactate dehydrogenase, and the sodium-glucose cotransporter have been calculated in a range of conditions from the known rate constants (Brown & Cooper, 1993; Brown, 1995). Interestingly although each of these proteins has been claimed to have a rate-limiting step, the control analysis showed that in most conditions control is shared between a number of steps and rate constants, and control shifts dramatically with conditions. Thus the general assumption that all enzymes have a rate-limiting step is false, and thus all methods of enzyme analysis based on this assumption are misleading. Control is more likely to be located in one or a small number of steps in extreme conditions of very low or very high substrate or product concentrations (V_{max}/K_m or V_{max} conditions). With physiological levels of substrates and products control tends to widely spread through many steps. Enzymologists and protein engineers tend to measure activity in extreme conditions, and this may be misleading if the information is extrapolated back to the physiological situation. Another important implication for protein engineers is that changing almost any set of rate constants within a protein may change its V_{max} or K_m.

If the enzyme intermediate levels can be measured in the steady state then the control coefficients can be determined without knowing the rate constants, using the equations given above. Methods for measuring the control coefficients are suggested in Brown & Cooper (1994) and Kholodenko *et al*. (1994a).

Prior to the advent of molecular control analysis the theoretical and experimental analysis of rate limitation within enzymes and other proteins has been dominated by the paradigm that each enzyme has a rate-limiting step. Thus, the analysis of rate limitation has used a number of methods and intuitive rules intended to identify that step. Such methods include: reactant kinetic-isotope effects, solvent kinetic-isotope effects, viscosity effects on diffusion, and pH and temperature profiles. However, if in fact control is distributed in enzymes as indicated above, then these methods will lead to misleading conclusions if the results are analysed in terms of the paradigm of a rate-limiting step. If thermodynamic or kinetic information on the elemental steps is available it is often assumed that: (a) the step that is furthest from equilibrium is rate limiting, (b) the first step that is far from equilibrium is rate limiting, (c) steps that are close to equilibrium have no control, (d) the step with the lowest forward rate constant is rate limiting, or (e) the step following the intermediate with the highest relative concentration is rate limiting. None of these intuitive rules is strictly true (Brown & Cooper, 1994; Kholodenko *et al*., 1994a).

Various types of free-energy profile or kinetic-barrier diagrams have been devised to help locate rate-limiting steps within enzymes, but in fact none of them actually serve this purpose consistently. The flux control coefficients quantify rate limitation exactly, and thus the true criterion for a rate-limiting step is that the control coefficient of that step is one. When there is no unique rate-limiting step, the extent of rate limitation by different rate constants and steps can be depicted as in Fig. 1.

3. Application of Molecular Control Analysis to β-lactamases

In order to illustrate the application of molecular control analysis to control within enzymes we have calculated the flux control coefficients of three β-lactamases from the rate constants determined by Christensen *et al* (1990). β-lactamases hydrolyse β-lactam antibiotics such as benzylpenicillin, via an acyl-enzyme intermediate, with the following kinetic scheme:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \overset{k_{+2}}{\rightharpoonup} \text{E-acyl} \overset{k_{+3}}{\rightharpoonup} E + P.$$

The rate constants were determined by Christensen *et al* (1990) for β-lactamase I from *Bacillus cereus*, PC1 β-lactamase from *Staphylococcus aureus*, and RTEM β-lactamase from *Escherichia coli*, using benzylpenicillin as substrate. The flux control coefficients of these rate constants and steps were calculated analytically using equations given in Brown & Cooper (1993) for control over k_{cat}/K_M and over k_{cat}, and are plotted in Fig. 1. The control coefficients of a rate constant over k_{cat}/K_M is just the percentage change in k_{cat}/K_M divided by the percentage change in rate constant (extrapolated to infinitesimal change), and is equivalent to the flux control coefficient in the condition of very low substrate concentration; similarly the control coefficient over k_{cat} is equivalent to the flux control coefficient in the condition of very high concentration of substrate (Brown & Cooper, 1993). Note that the control coefficients of either rate constants or steps always sum to one; and the control coefficient of

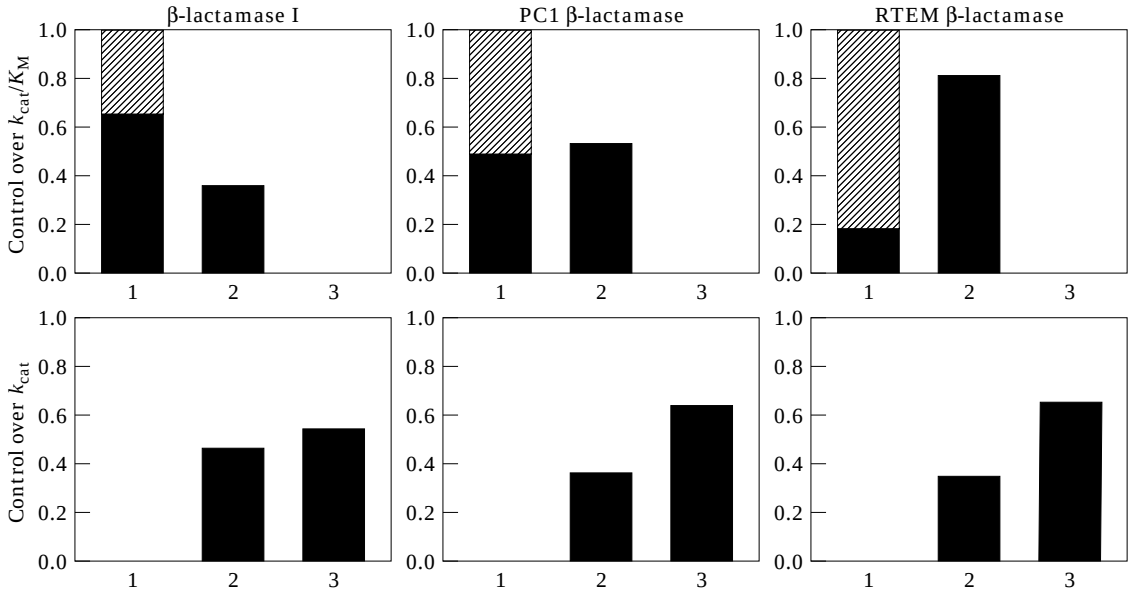

FIG. 1. Control coefficients of steps and rate constants within three β-lactamases over k_{cat}/K_M and k_{cat}. The top row of histograms gives control over k_{cat}/K_M, the bottom row gives control over k_{cat}. The first column gives control within β-lactamase I, the second column control within PC1 β-lactamase, and the third column control within RTEM β-lactamase. In each histogram the control coefficient of the step is indicated by the height of the filled (black) column, the absolute value of the control coefficient of the reverse rate constant is indicated by the striped section of the column (although these coefficients are always negative), and the total height of the column (filled plus striped) indicates the value of the forward rate constant of the step. The numbers (1, 2, 3) indicate the number of the step in the kinetic scheme. The rate constants k_{+1} ($\mu M^{-1}.s^{-1}$), k_{-1} (s^{-1}), k_{+2} (s^{-1}), and k_{+3} (s^{-1}) for β-lactamase I were 41, 2320, 4090, and 3610; and for PC1 β-lactamase were 22, 196, 173, and 96; and for RTEM β-lactamase were 123, 11 800, 2800, and 1500, as determined by Christensen *et al*. (1990).

substrate association (C_{+1}) over k_{cat}/K_M is always one, and control over k_{cat} is always zero. Because the acyl-enzyme intermediate has only one rate constant depleting it in the above scheme the control coefficient of this rate constant (k_{+3}) is equal to the fractional concentration of this intermediate [see eqn (6)]; thus with very low substrate concentrations this control coefficient is zero, and in any other condition the coefficient can simply be measured as the fractional concentration of the intermediate. Since the reverse rate constant of this step (k_{-3}) is assumed to be very small in the kinetic scheme, the control coefficient of the reverse rate constant is close to zero, and thus the control coefficient of the step is equal to that of the forward rate constant.

The control coefficients depicted in Fig. 1 illustrate that for all the β-lactamases, control (i.e. rate limitation) is shared between steps, and varies with the substrate concentration; thus there is no unique rate-limiting step. However, the control distribution is remarkably similar between the three β-lactamases given the relatively wide difference in rate constants (values in the figure legend, for example the k_{cat} is 1920 s^{-1} for β-lactamase I, 62 s^{-1} for PC1 β-lactamase, and 980 s^{-1} for RTEM β-lactamase). Since control was also widely distributed in triose phosphate isomerase, carbamate kinase, and lactate dehydrogenase (Brown & Cooper, 1993) and the sodium-glucose cotransporter (Brown, 1995) this suggests that: (i) the general belief that an enzyme has a rate-limiting step is wrong, and (ii) there may be some reason that evolution of enzymes results in a wide control distribution. A possible reason for the latter is that if natural selection is acting to increase k_{cat} or k_{cat}/K_M, then selection pressure can only be exerted on steps or sets of rate constants which have significant flux control, since changes in steps or rate constants with no control will have no effect on the rate. Selection will result in increases in those rate constants with significant control and thus decreases in their control coefficients, and consequently increase in the control exerted by other steps or sets of rate constants not previously exerting control. Conversely, if steps have no significant flux control there will be no selection pressure to prevent back mutation to lower rate constants, resulting in higher flux control. Thus, the selection process itself will tend to equalise the control coefficients throughout the enzyme or any other molecular process the rate of which is being maximised by selection. However, other factors, such as catalytic difficulty, will influence the relative rate constants and thus the control distribution.

Calculating the control coefficients gives important information that is not readily visible from the rate constants alone. Thus, for example, Christensen *et al* (1990) concluded from the β-lactamase rate constants which they determined that these β-lactamases were fully efficient enzymes. However, the control coefficients quantifying control over k_{cat}/K_M (Fig, 1) indicate that these enzymes cannot be fully efficient. Maximal efficiency for an irreversible enzyme must mean that k_{cat}/K_M is a maximum due to the enzyme being entirely limited by substrate association and substrate association is entirely limited by diffusion (Albery & Knowles, 1976). However for each of the enzymes in Fig. 1 there is substantial control over the k_{cat}/K_M by step 2, and therefore, if the rate constants of step 2 were to increase (proportionately so the equilibrium constant is unchanged) k_{cat}/K_M would increase, and therefore the enzymes cannot be maximally efficient.

4. Analysis of Multi-protein Complexes and Protein Interactions

In principle the analysis outlined above is applicable to any molecular process, which may be described by a kinetic scheme and rate constants. Examples that have been considered are: channelling of intermediates between enzymes (Kholodenko *et al*, 1995; Sauro, 1994), protein-protein interactions and associations (Kholodenko *et al*, 1994b) and group-transfer pathways (Van Dam *et al*, 1993, Kholodenko & Westerhoff, 1995a). These examples cannot be analysed by normal metabolic control analysis because of: (i) non-diffusible intermediates, (ii) lack of one-to-one correspondence between enzymes and processes, and (iii) failure of the summation and connectivity theorems of metabolic control analysis (reviewed in Kholodenko & Westerhoff, 1995b).

There are a number of special features of kinetic schemes that make them differ from schemes of metabolic pathways and result in special control properties. (1) All states of the system are specified, together with all possible transitions between the states. (2) The elasticities of steps (ϵ_i) are directly related to their thermodynamic properties (Kholodenko & Westerhoff, 1994):

$$\epsilon_y^i = 1/(1 - e^{\Delta G_i/RT}),\ \epsilon_z^i = 1/(1 - e^{-\Delta G_i/RT}) \qquad (9)$$

Here y and z are a substrate and product (the stoichiometric coefficients of which are assumed to be one for the sake of simplicity), ΔG is the Gibbs energy difference across step i, defined as the chemical potential of the product(s) minus the chemical potential of the substrate(s). (3) The elasticities of unidirectional rates with respect to the intermediate levels are all either one or zero. (4) The control coefficients of a step and its rate constants are related through eqn (3) and as follows:

$$C_i^J/v_i = C_{+i}^J/v_{+i} = -C_{-i}^J/v_{-i}. \qquad (10)$$

(5) For molecular processes the level of an intermediate may be regarded as the probability of occupancy of that state, and rate constants may be regarded as the probabilities of the transitions (Hill, 1977). (6) There may be one or more reaction cycles resulting in moiety-conserved cycles.

Moiety-conserved cycles of proteins play a special role in molecular control analysis. In the case of a single enzyme eqn (6), which relates the concentration (E_j) of an intermediate state (j) to the control exerted by the rate constants depleting this state, results from moiety conservation within an enzyme (Kholodenko *et al*., 1994b). In the case of multiple interacting proteins, a particular state can be connected to more than one moiety-conserved cycle, when this state is a multi-protein complex, and thus eqn (6) needs to be modified in this case. From eqns (9) and (10) and the generalized connectivity theorem (Kholodenko *et al*., 1994b), it follows that the control exerted by the rate constants depleting a protein-complex state, Z_i, is equal to the sum of the control coefficients of each enzyme in the complex divided by the fractional concentration of each enzyme involved in the complex:

$$\sum_{i=1}^{n} C_{\pm i}^J = \sum_{r=1}^{m} (C_{e_r}^J \cdot Z_i/e_r) \qquad (11)$$

where n is the number of rate constants depleting Z_i, and m is the number of enzymes involved in Z_i. Here, for the sake of simplicity it is assumed that only one molecule of each enzyme is involved in the complex (Z_i) and this complex is not involved in the conservation of any substrate moiety. Thus, for example if the complex state Z_i involves three enzymes and has two rate constants depleting it:

$$C_{-i}^J + C_{+j}^J = Z_i(C_{e_1}^J/e_1 + C_{e_2}^J/e_2 + C_{e_3}^J/e_3). \qquad (12)$$

In the absence of moiety-conserved cycles the normal connectivity theorems of metabolic control analysis may be used in molecular control analysis, and thus the control distribution may be determined by measuring the elasticities.

It may be useful to regard molecular control analysis, that is analysis in terms of a kinetic scheme of all micro-processes, as the basic form of control analysis from which other forms of control analysis may be derived by grouping the rate constants of

elemental steps in particular ways. For example, classical metabolic control analysis is derived by grouping all the rate constants that scale with enzyme concentrations. Other types of control coefficients result from grouping the rate constants in other ways (Kholodenko & Westerhoff, 1995b). We have previously discussed how the control coefficients exerted by proteins involved in channelling, group-transfer, multi-protein complexes and other protein interactions may be derived only by analysing control at the microlevel of elemental processes and then summing these contributions for a whole protein to give a macroscopic control coefficient (Kholodenko & Westerhoff, 1995b).

5. Other Applications

Additional examples of systems that may be studied by molecular control analysis include G-proteins, cascades of protein kinases and phosphatases, receptor-effector interactions, signal transduction networks, actinomyosin, protein translation, and gene transcription. Protein folding, unfolding or assembly could also be analysed, where there is a steady state with intermediates, to identify rate-limitation in the process. For example if a protein-folding pathway were described by the following kinetic diagram:

$$U \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} I_1 \overset{k_{+2}}{\rightharpoonup} I_2 \overset{k_{+3}}{\rightharpoonup} F$$

where U is the unfolded form of the protein, F is the folded form, and I_1 and I_2 are two intermediates in the folding pathway. The concentration of U is regarded as fixed, and the concentrations of I_1 and I_2 are variable. The control in this pathway can be determined by differentiating the rate equation for the above scheme according to eqns (1) and (2), giving the control coefficients in terms of the rate constants:

$$C^J_{+1} = 1;\ C^J_{-1} = \frac{-k_{-1}}{k_{-1}+k_{+2}};$$

$$C^J_{+2} = \frac{k_{-1}}{k_{-1}+k_{+2}};\ C^J_{+3} = 0 \quad (13)$$

$$C^J_1 = \frac{k_{+2}}{k_{-1}+k_{+2}};\ C^J_2 = \frac{k_{-1}}{k_{-1}+k_{+2}};\ C^J_3 = 0. \quad (14)$$

In the absence of knowledge of the rate constants, the control coefficients can be measured directly by measuring the disequilibrium of the first step, and using eqns (4) and (5). Thus, for example, a measurement of v and v_{+1} would be sufficient to determine all the control coefficients in the above scheme.

A system of coupled chemical reactions could also be analysed by molecular control analysis to determine the extent to which each reaction limits the steady-state fluxes. Thus, for example, control over atmospheric reactions, industrial chemical processes, or free radical processes could be analysed using the same approach of molecular control analysis, provided asymptotically stable steady states reign.

We thank the Royal Society (London), Wellcome Trust, and Netherlands Organization for Scientific Research (NWO) for financial support. Thanks to Scott Brogan for suggesting the application to protein folding.

REFERENCES

Albery, W. J. & Knowles, J. R. (1976). Evolution of enzyme function and the development of catalytic efficiency. *Biochemistry* **15,** 5631–5640.

Brown, G. C. (1995). Rate control within the Na^+/glucose cotransporter. *Biophys. Chem.* **54,** 181–189.

Brown, G. C. & Cooper, C. E. (1993). Control analysis applied to single enzymes: can an isolated enzyme have a unique rate-limiting step? *Biochem. J.* **294,** 87–94.

Brown, G. C. & Cooper, C. E. (1994). The analysis of rate-limitation within enzymes: relations between flux control coefficients of rate constants and unidirectional rates, rate constants and thermodynamic parameters of single isolated enzymes. *Biochem. J.* **300**, 159–164.

Christensen, H., Martin, M. T. & Waley, S. G. (1990). β-Lactamases as fully efficient enzymes. *Biochem. J.* **266,** 853–861.

Hill, T. L. (1977). *Free Energy Transduction in Biology*. New York: Academic Press.

Kacser, H., Sauro, H. M. & Acerenza, L. (1990). Enzyme-enzyme interactions and control analysis. 1. The case of non-additivity: monomer-oligomer associations. *Eur. J. Biochem.* **187,** 481–491.

Kholodenko, B. N. & Westerhoff, H. V. (1994). Control theory of one enzyme. *Biochim. Biophys. Acta* **1208,** 294–305.

Kholodenko, B. N. & Westerhoff, H. V. (1995a). Control theory of group-transfer pathways. *Biochim. Biophys. Acta* **1229,** 256–274.

Kholodenko, B. N. & Westerhoff, H. V. (1995b). The macro- versus microworld of biochemical regulation and control. *Trends Biochem. Sci.* **20,** 52–54.

Kholodenko, B. N., Westerhoff, H. V. & Brown, G. C. (1994a). Rate limitation within a single enzyme is directly related to enzyme intermediate levels. *FEBS Lett.* **349,** 131–134.

Kholodenko, B. N., Sauro, H. M. & Westerhoff, H. V. (1994b). Control by enzymes, coenzymes and conserved moieties: a generalization of the connectivity theorem of metabolic control analysis. *Eur. J. Biochem.* **225,** 179–186.

Kholodenko, B. N., Cascante, M. & Westerhoff, H. V. (1995). Control theory of metabolic channelling. *Mol. Cell. Biochem.* **143,** 151–168.

Ray, W. J. (1983). Rate-limiting step: a quantitative definition. Application to steady-state enzymic reactions. *Biochemistry* **22,** 4625–4637.

Sauro, H. M. (1994). Moiety-conserved cycles and metabolic control analysis: problems in sequestration and metabolic channelling. *Biosystems* **33,** 55–67.

SAURO, H. M. & KACSER, H. (1990). Enzyme-enzyme interactions and control analysis. The case of non-independence: heterologous associations. *Eur. J. Biochem.* **187,** 493–500.

VAN DAM, K., VAN DER VLAG, J., KHOLODENKO, B. N. & WESTERHOFF, H. V. (1993). The sum of the flux control coefficients of all enzymes on the flux through a group-transfer pathway can be as high as two. *Eur. J. Biochem.* **212,** 791–799

WELCH, G. R., KELETI, T. & VERTESSEY, B. (1988). The control of cell metabolism for homogenous vs heterogenous enzyme systems. *J. theor. Biol.* **130,** 407–422.