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Control Analysis of Periodic Phenomena in Biological Systems

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Principles of the control and regulation of steady-state metabolic systems have been identified in terms of the concepts and laws of metabolic control analysis (MCA). With respect to the control of periodic phenomena MCA has not been equally successful. This paper shows why in case of autonomous (self-sustained) oscillations for the concentrations and reaction rates, time-dependent control coefficients are not useful to characterize the system: they are neither constant nor periodic and diverge as time progresses. This is because a controlling parameter tends to change the frequency and causes a phase shift that continuously increases with time. This recognition is important in the extension of MCA for periodic phenomena. For oscillations that are enforced with an externally determined frequency, the time-dependent control coefficients over metabolite concentration and fluxes (reaction rates) are shown to have a complete meaning. Two such time-dependent control coefficients are defined for forced oscillations. One, the so-called periodic control coefficient, measures how the stationary periodic movement depends on the activities of one of the enzymes. The other, the so-called transient control coefficient, measures the control over the transition of the system between two stationary oscillations, as induced by a change in one of the enzyme activities. For forced oscillations, the two control coefficients become equal as time tends to infinity. Neither in the case of forced oscillations nor in the case of autonomous oscillations is the sum of the time-dependent control coefficients time-independent, not even in the limit of infinite time. The sums of either type of control coefficients with respect to time-independent characteristics of the oscillations, such as amplitudes and time averages, do fulfill simple laws. These summation laws differ between forced oscillations and autonomous oscillations. The difference in control aspects between autonomous and forced oscillations is illustrated by examples.

Introduction

Quantitative approaches have led to significant advances in the understanding of the control of metabolic and information pathways under stationary conditions.[1−9] In a biochemical/biophysical reaction system such as a metabolic pathway in a living cell the control exerted by any enzyme on any steady-state flux (reaction rate) or concentration can be quantified in terms of the corresponding control coefficient defined by metabolic control analysis (MCA). The (stationary) control coefficient is the relative difference between the two steady-states in pathway flux or metabolite concentration, divided by the causative fractional change in the enzyme's activity, extrapolated to infinitesimally small change.[4−6] This quantifies the ability of an enzyme to influence the steady-state pathway rate or the concentration of a metabolite. It also makes it possible to assess the relative importance of different enzymes to the control of the flux, since in an ideal pathway[10] the sum of the control coefficients of all the enzymes is equal to 1[4−6] (see ref 11 for a review). Basic relationships of MCA, i.e. so-called summation and connectivity theorems, enable one to calculate the control coefficients in terms of the (local) kinetic properties of single reactions, i.e. their elasticity coefficients.[12−14] Consequently, the control of steady-state phenomena in metabolic networks is fairly well understood at the theoretical level.

Living cells also exhibit various important time-dependent phenomena however.[15] For instance, fluctuations in metabolic variables give rise to time-dependent transient processes which are ultimately responsible for the stability of the steady state.[8,16] Permanent perturbations of parameter values lead to transitions to new steady states. Some stationary movements, i.e., self-sustained (limit cycle) oscillations, are themselves time-dependent and stable to fluctuations.[16,17] In physics and chemistry, oscillations have been observed in many complex reaction mixtures.[18] The study of various features of oscillations appeared to be useful for determining the essential parts of complex reaction mechanisms.[18−20] In biology, limit cycle systems are of particular interest, since some of them provide the mechanisms for various biological clocks, including the one governing the cell cycle.[21,22] Metabolic oscillations occur in yeast extracts, in populations of yeast cells (see, for example, refs 23−32), and in photosynthesis.[33] There is a growing interest in the yeast oscillations because they involve active cell−cell synchronization.[32,34] Oscillations have been proposed to be functional, as they may increase thermodynamic efficiency.[35] Baconier et al.[36] and Teusink et al.[37] calculated the control exerted by enzymes on the period of oscillation using particular models of glycolytic oscillations. They found subtly distributed control. Yet, the most intriguing issue about the control of oscillations in biological systems has not been studied in detail, e.g., which laws govern the magnitudes and distribution of the control.

In close vicinity of the (asymptotically) stable steady state the control over a relaxation process has been analyzed by

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Heinrich and Reder[38] (see also refs 39, 40). Definitions of time-dependent control coefficients were generalized to include perturbations of any parameter affecting the enzyme rate, i.e. not necessarily of enzyme concentrations. For relaxations to asymptotically stable steady states, it was shown that time-dependent control coefficients tend to steady-state control coefficients when time tends to infinity.[38] An elegant attempt to extend control analysis to arbitrarily time-dependent trajectories was made by Acerenza et al.[41] These authors introduced an "operational" definition of a time-dependent control coefficient as the relative difference between the reaction rates (concentrations) in the original and the perturbed system observed at time *t*, after a perturbation of a parameter at time 0, divided by the relative change in that parameter. A number of relationships considered to be analogous to the summation and connectivity theorems for systems at steady states[41] were derived. However, below we shall see that for stationary periodic phenomena, e.g., for self-sustained ("autonomous") oscillations (limit cycles), neither these theorems nor the "operational" definitions of time-dependent control coefficients[41] apply as time tends to infinity.

The present paper addresses these problems and develops metabolic control theory for stationary periodic phenomena in biological systems. It first defines time-dependent control coefficients for forced oscillations. Then it shows that for autonomous oscillations the same definitions are not useful. The control of autonomous oscillations can only be quantified by the control coefficients with respect to those characteristics of oscillations that do not depend on the phase of periodic movement. For both autonomous and forced oscillations, summation theorems for control coefficients with respect to the phase-independent properties are then derived. In a simple example of forced oscillations we show that the contributions of different enzymes to the control of the fluxes can change dramatically during the period.

Results

A. Definitions of Time-Dependent Control Coefficients. *A1. Forced Oscillations.* Let us suppose that a system under study is exposed to periodic changes in the environment, resulting in periodic changes of some system parameters, e.g., kinetic constants and the concentration of "external" metabolites. Such a situation can also be described in terms of some periodic external force influencing the system.[42] We shall assume that at fixed parameter values a single (asymptotically) stable steady state of the system exists. When kinetic parameters change periodically, metabolic concentrations (x_i) and reaction rates, J_k (called fluxes in the framework of MCA), become periodic functions of time:

$$x_i = x_i^{\mathrm{per}}(t,\mathbf{e}) = x_i^{\mathrm{per}}(t+T,\mathbf{e}),$$

$$J_k = J_k^{\mathrm{per}}(t,\mathbf{e}) = J_k^{\mathrm{per}}(t+T,\mathbf{e}) \qquad (1)$$

Here T is the period of the external force and $\mathbf{e} = (e_1, e_2, ..., e_n)$ is the vector of the enzyme concentrations. Stationary periodic behavior caused by a periodic external force is called a forced oscillation. During the period of such an oscillation (T), the vector of metabolite concentrations ($\mathbf{x}$) follows a closed trajectory.

The system behavior described by eq 1, as well as the system behavior outside that closed trajectory is dictated by chemical processes developing in time according to the kinetic rate equations. Combination of these rates with the map of the chemical network leads to differential equations for all of the independent metabolic variables ($\mathbf{x}$), the so-called chemical kinetics differential equations (eq A1 in Appendix A). The correspondence between the physical system and the mathematical equations allows one to use the work "solution" to refer to "system behavior".

We shall assume that the eigenvalues of the Jacobian of this system of differential equations have negative real parts at all the points of the periodic trajectory. Under these conditions, the periodic solution, $x_i^{\mathrm{per}}(t)$, to eq A1 is unique and asymptotically stable.[17] So-called "conservative" systems (often considered in physics) lack the dissipation of free energy. Such systems usually have an infinitely large number (continuum) of periodic solutions determined by the initial conditions and will not be considered here. Here we consider isothermal, isobaric systems that continuously dissipate free energy, as found in chemical reaction systems[16] and living cells.[8]

Let t^* be an arbitrary time moment and $\mathbf{x}^*$ be a point on the periodic solution corresponding to that moment t^* (such a correspondence is unambiguously determined by the periodical external force, see Appendix A). Once t^* and $\mathbf{x}^*$ have been chosen as the initial condition, the corresponding periodic solution is designated as $\mathbf{x}^{\mathrm{per}}(t,\mathbf{e};t^*,\mathbf{x}^*)$. If now another point ($\mathbf{x}$) in close vicinity to $\mathbf{x}^*$ is chosen as the initial condition, i.e. $\mathbf{x}(t^*) = \mathbf{x}^* + \Delta\mathbf{x}$, then due to the asymptotical stability of $\mathbf{x}^{\mathrm{per}}$, the ensuing trajectory ($\mathbf{x}^{\mathrm{tr}}$) tends to $\mathbf{x}^{\mathrm{per}}$ as time tends to infinity:

$$\lim_{t\to\infty}(\mathbf{x}^{\mathrm{tr}}(t,\mathbf{e};t^*,\mathbf{x}^*+\Delta\mathbf{x}) - \mathbf{x}^{\mathrm{per}}(t,\mathbf{e};t^*,\mathbf{x}^*)) = 0 \qquad (2)$$

Considering (fractional) changes in a steady-state periodic solution caused by a change in a particular enzyme concentration (e_j), one can define (steady-state) "periodic" control coefficients over metabolite concentrations and reaction rates (fluxes) as follows:

$$C_y^{x_i}(t) = (\mathrm{d}x_i^{\mathrm{per}}(t,\mathbf{e})/\mathrm{d}e_j)(e_j/x_i^{\mathrm{per}}) = \mathrm{d}\ln x_i^{\mathrm{per}}(t,\mathbf{e})/\mathrm{d}\ln e_j$$

$$C_j^{J_k}(t) = (\mathrm{d}J_k^{\mathrm{per}}(t,\mathbf{e})/\mathrm{d}\mathbf{e}_j)(e_j/J_k^{\mathrm{per}}) = \mathrm{d}\ln J_k^{\mathrm{per}}(t,\mathbf{e})/\mathrm{d}\ln e_j \quad (3)$$

Since the period T does not depend on system parameters, it follows from eq 1 that the control coefficients, $C_j^x(t)$, are periodic functions of T. If the periodic solutions for the reaction rates can assume zero values at some time values, one should consider the non-normalized flux control coefficients[13] in eq 3.

In eq 3 periodic control coefficients are defined as formal derivatives of the asymptotically stable periodic solution (eq 1) with respect to a parameter of choice (e.g., e_j) (cf. ref 43). This definition corresponds to the comparison of two steady-state periodic solutions (closed trajectories) that differ in e_j by an infinitesimal change, Δe_j. Most importantly, these two solutions are synchronized by the periodic external force. In fact, a one-to-one correspondence exists between any point of either closed trajectory and a value of the periodic force. Hence, also between pairs of the points of the two different trajectories, a one-to-one correspondence exists. This synchronization makes it possible to assign an operational meaning to the (steady-state) periodic control coefficients in terms of (infinitesimal) perturbations (see below and Appendix A).

Alternativley, let us consider the periodic solution $\mathbf{x}^{\mathrm{per}}(t,\mathbf{e};t^*,\mathbf{x}^*)$ and the other solution $\mathbf{x}^{\mathrm{tr}}(t,\mathbf{e}+\Delta\mathbf{e};t^*,\mathbf{x}^*)$ that occurs when a particular enzyme concentration (e_j) is perturbed by Δe_j at the moment t^* (here and below the superscript "tr" specifies the transition process). The function $\mathbf{x}^{\mathrm{tr}}(t,\mathbf{e}+\Delta\mathbf{e};t^*,\mathbf{x}^*)$ is not periodic. It describes the transition process from the periodic solution corresponding to the value e_j to the periodic solution corresponding to the value $e_j + \Delta e_j$. Initially ($t = t^*$), the two

solutions coincide. At any time $t > t^*$, the relative difference between $\mathbf{x}^{tr}$ and $\mathbf{x}^{per}$ divided by $\Delta e_j/e_j$ shows how the particular enzyme e_j affects the metabolite concentration or flux during the transition. The resulting function, obtained in the limit of infinitesimally small Δe_j, is called a transient (time-dependent) control coefficient:

$$ {}^{tr}C_j^{x_x}(t) = \lim_{\Delta e_j \to 0} \frac{x_i^{tr}(t,e_j+\Delta e_j;t^*,\mathbf{x}^*) - x_i^{per}(t,e_j;t^*,\mathbf{x}^*)}{\Delta e_j} \times (e_j/x_i^{per}(t,e_j;t^*,\mathbf{x}^*)) $$

$$ {}^{tr}C_j^{J_k}(t) = \lim_{\Delta e_j \to 0} \frac{J_k^{tr}(t,e_j+\Delta e_j;t^*,\mathbf{x}^*) - J_k^{per}(t,e_j;t^*,\mathbf{x}^*)}{\Delta e_j} \times (e_j/J_k^{per}(t,e_j;t^*,\mathbf{x}^*)) \quad (4) $$

Contrary to periodic control coefficients transient control coefficients do not depend on time periodically, although they do depend on time.

Appendix A shows that in the case of forced oscillations the transient control coefficients (eq 4) tend to the corresponding periodic control coefficients (eq 3) as time tends to infinity:

$$ \lim_{t\to\infty} ({}^{tr}C_j^{x_i}(t) - C_j^{x_i}(t)) = 0 $$

$$ \lim_{t\to\infty} ({}^{tr}C_j^{J}(t) - C_j^{J}(t)) = 0 \quad (5) $$

This clarifies the operational meaning of the formal periodic control coefficients defined by eq 3. They quantify the control when a system has already relaxed to a new oscillation pattern after a change in the activity of a particular enzyme.

Appendix B shows that the periodic and the transient control coefficients satisfy the same variation equation. Periodic control coefficients are given by the unique periodic solution of the variation equation, whereas transient control coefficients are determined as a time-dependent solution, assuming the initial conditions equal to zero. To come to grips with this result, one may revisit the definitions given by eqs 3 and 4 and note that both the periodic and the transient solutions for metabolite concentrations and fluxes must satisfy the same kinetic equations (see Appendix A, eq A1). For explicit expressions for the periodic and transient control coefficients see Appendix B (cf. ref 38).

It is instructive to compare these periodic and transient control coefficients, which describe the control of forced oscillations, to the corresponding control coefficients defined for perturbations near asymptotically stable steady states.[38,44] The periodic control coefficients (eq 3) defined by the formal differentiation of steady-state periodic solution correspond to the traditional steady-state control coefficients. Indeed, in standard MCA, eq 3 will define the usual control coefficients if the steady-state concentrations and fluxes are substituted in this equation for the periodic ones. The transient control coefficients of an oscillating system (eq 4) correspond to the time-dependent control coefficients, as introduced by Acerenza et al.[41] and Heinrich[44] and by Heinrich and Reder[38] for relaxation behavior around steady states after infinitesimal perturbations in parameters. These coefficients can be obtained if in eq 4 steady-state concentrations (x_i^0) and fluxes (J_k^0) are substituted for the periodic time-dependent solutions, x_i^{per} and J_k^{per}, or if the amplitude of the forcing function approaches zero. Definition (4) and the corresponding definition of time-dependent control coefficients in refs 38 and 41 have the clear operational meaning of relating fractional changes in a particular enzyme concentration at given initial conditions to resulting changes in metabolic concentration or flux at any subsequent point in time. It has been shown in ref 41 that time-dependent control coefficients describing a relaxation process near a steady state tend to the corresponding control coefficients over the steady-state (time-independent) metabolite concentrations and fluxes, as time tends to infinity (cf. eq 5).

A2. Autonomous Oscillations (Limit Cycles). The forced oscillations considered above arose from the influence of a periodic external force on a system that exhibited asymptotically stable steady states in the absence of that force. By contrast, autonomous oscillations occur at time-independent (internal and external) parameter values in systems in which the corresponding steady states are unstable.[16,17,45] In mathematical terms, the latter systems are called autonomous, whereas the former are nonautonomous, as their right-hand sides depend on time periodically and explicitly (see eq A1 in the Appendix A). Physically, a principal difference between forced and autonomous oscillations is that an influence that synchronizes with respect to an external clock is lacking in the latter case, whereas in the former case a periodic external force synchronizes the oscillations in the systems with the initial parameter values to the oscillations in the system with the perturbed values of parameters. Due to lack of a synchronization influence, a stable periodic solution of an autonomous system cannot be asymptotically stable in the strictest sense of convergence to a single $\mathbf{x}(t)$. An initial difference in the phase of oscillations cannot vanish with time in the general case. Although there is convergence to a trajectory of a single form, there is no convergence to a certain phase.

For autonomous oscillations one can define formally the control coefficients, $C_j^x(t)$ and $C_j^J(t)$, analogously to eq 3, i.e., as the log−log derivatives of a unique periodic solution with respect to the enzyme concentrations (or as the non-normalized derivatives if periodic reaction rates assume zero values at some time moments). However, in contrast to the case of forced oscillations these control coefficients do not depend on time strictly periodically. Moreover, they do not exist when time tends to infinity.

To illustrate this, let us present a periodic solution (x_k^{per}) by its Fourier series. We emphasize that both the Fourier coefficients and the frequency (ω) depend on systemic parameters, i.e., on enzyme concentrations ($\mathbf{e}$):

$$ x_k^{per}(t,\mathbf{e}) = \sum_{h=-\infty}^{\infty} x_k^h(\mathbf{e}) \exp(ih\omega(\mathbf{e})t) \quad (6) $$

Here $x_k^h(\mathbf{e})$ denotes hth Fourier coefficient and i is the imaginary unity. Differentiating the Fourier series (6) with respect to a particular enzyme concentration, e_j (see eq 3), one obtains for the control coefficients, $C_j^{x_k}(t)$,

$$ C_j^{x_k}(t) = (e_j/x_k^{per})\Big(\sum_{h=-\infty}^{\infty} (dx_k^h(\mathbf{e})/de_j) \exp(ih\omega(\mathbf{e})t) + it \sum_{h=-\infty}^{\infty} x_k^h(\mathbf{e})(d\omega(\mathbf{e})/de_j) \exp(ih\omega(\mathbf{e})t)\Big) \quad (7) $$

Because of the second term on the right-hand side of this

equation, which is proportional to t, the control coefficients, $C_j^{x_k}(t)$, do not depend on time periodically. Since this term becomes unlimited with time, the coefficients $C_j^{x_k}(t)$ cannot be defined when time tends to infinity.

The transient control coefficients, ${}^{\mathrm{tr}}C_j^{x_k}(t)$, are defined according to eq 4. Hence, they can be found by solving the variation equation (cf. ref 38 and Appendix B). mor the case of autonomous oscillations it has been proved[46] that no solution of the variation equation exists at time tending to infinity. Therefore, also the transient control coefficients with respect to metabolic concentrations or fluxes during the transition from the initial to a perturbed closed trajectory can only be defined for limited time intervals. In the limit of infinite time this control coefficient does not exist either.

The question arises why both control coefficients, $C_j^x(t)$ and ${}^{\mathrm{tr}}C_j^x(t)$, fail to exist when the time of the observation of the periodic or transition process tends to infinity. For the control coefficients, $C_j^x(t)$, this is explained by the phase difference between the original and perturbed oscillations, which continues to increase with time. For the control coefficients ${}^{\mathrm{tr}}C_j^x(t)$, the time-dependent phase difference between the transition and the periodic movements is the culprit. Although the initial and the perturbed trajectories are very close in concentration space, due to the dependence of oscillation frequency on a perturbed parameter, e_j, the phase difference does not vanish with vanishing of Δe_j when time tends to infinity; the infinitesimal difference in phase is amplified infinitely.

From the reasoning above one may conclude that in the case of autonomous oscillations due to divergence of the initial and the perturbed movements, the control coefficients determined by either eq 3 or eq 4 cannot describe the control exerted by enzymes over periodic values of metabolic concentrations and reaction rates. However, the same reasoning shows that the control over those characteristics of self-sustained oscillations that do not depend on the phase of the movement can be defined (at any time of observation). The log−log derivatives of these time characteristics (Y) with respect to enzyme concentrations determine the coefficients, C_j^Y, that describe the control of (stationary) self-sustained oscillations. For instance, the control coefficients over the amplitude and period of oscillations and over various mean values do exist.

B. Properties of Time-Dependent Control Coefficients in an Example of Forced Oscillations. We shall consider a simple example where a periodic solution (eq 1) and, hence, periodic control coefficients (eq 3) can be found analytically, as functions of time and parameters. This will make it possible to illustrate a number of the control properties, such as the variations of the control distribution with time, and to test whether the summation theorem that is true for steady-state control coefficients continues to apply in the case of forced oscillations.

Scheme 1 shows a metabolic chain of two reactions,

$$\mathrm{S} \rightarrow \mathrm{X} \rightarrow \mathrm{P} \qquad \text{(scheme 1)}$$

We shall suppose that the substrate concentration (S) changes periodically, and the product concentration (P) is kept zero:

$$S(t) = S_0(1 + a \sin(\omega_0 t)) \tag{8}$$

Here S_0 is the substrate concentration in the absence of (external) periodic force, ω_0 is the frequency of the periodic force, and $a < 1$ is the amplitude of the oscillation of the substrate concentration. Let us assume that the reaction rates, v_1 and v_2, are linear functions of metabolite concentrations:

$$v_1 = e_1(k_1 S - k_{-1} x)$$
$$v_2 = e_2 k_2 x \tag{9}$$

Here $k_{\pm i}$, $i = 1, 2$, are the kinetic constants; e_1, e_2 are the total enzyme concentrations; and x is the concentration of the intermediate. Thanks to the linearity of eq 9 with respect to x, its periodic solution (under the influence of the periodic force described by eq 8) can be found readily (see Appendix C):

$$x^{\mathrm{per}}(t) = x^0 + \frac{k_1 S_0 a e_1}{[(k_{-1}e_1 + k_2 e_2)^2 + {\omega_0}^2]^{1/2}} \sin(\omega_0 t - \varphi)$$
$$x^0 = \frac{k_1 s_0 e_1}{k_{-1}e_1 + k_2 e_2} \tag{10}$$

Here $x^0 = x^0(e_1, e_2)$ is the concentration at the asymptotically stable steady state in the neighborhood where the forced oscillations occur. $-\varphi$ is the initial (at $t = 0$) phase of oscillations, for which an explicit expression is given in Appendix C. With respect to the input oscillation (eq 8) the resulting oscillation in x is deformed as a function of the frequency of the former. This is due to the capacitive effect of the concentration of x.

Substituting eq 10 into the rate equations (9), the periodic solution for the fluxes through the first (J_1) and the second (J_2) reactions are obtained:

$$J_1(t) = J_0 + A_1(\mathbf{e}, \omega_0) \sin(\omega_0 t + \chi)$$
$$J_2(t) = J_0 + A_2(\mathbf{e}, \omega_0) \sin(\omega_0 t - \varphi)$$
$$J_0 = \frac{e_1 e_2 k_1 k_2 S_0}{k_{-1}e_1 + k_2 e_2} \tag{11}$$

Here J_0 is the steady-state flux. A_1 and A_2 are the amplitudes of the oscillations of the reaction rates, and χ is the initial phase of oscillations of the J_1 (the explicit expressions for A_1, A_2, and χ are given in Appendix C). From eqs 11 it follows that the fluxes through the first and the second reactions differ at most times. Only their averages are equal. Consequently, in contrast to the case of systems at steady states, the control coefficients over the time-dependent fluxes through sequential reactions in oscillating systems will differ (see Figure 1).[47]

The periodic control coefficients of enzymes 1 and 2 over the flux through either reaction can be obtained by differentiating eq 11 with respect to ln e_1 and ln e_2 (see Appendix C). Figure 2 exemplifies the behavior of enzyme control coefficients for the case when the amplitude of the oscillation of the concentration of the pathway substrate (a in eq 8) is comparable with its average concentration (S_0). One can see that in some time intervals during the period of the oscillation the time-dependent control coefficients are above, whereas in the other intervals they are below the corresponding control coefficients at steady state (when $a = 0$). Moreover, these periodic control coefficients of enzymes 1 and 2 cross several times. As a consequence, in some intervals of time the control exerted by enzyme 1 exceeds the control by enzyme 2, whereas during other intervals the opposite is the case. It should be noted that when the ampli-

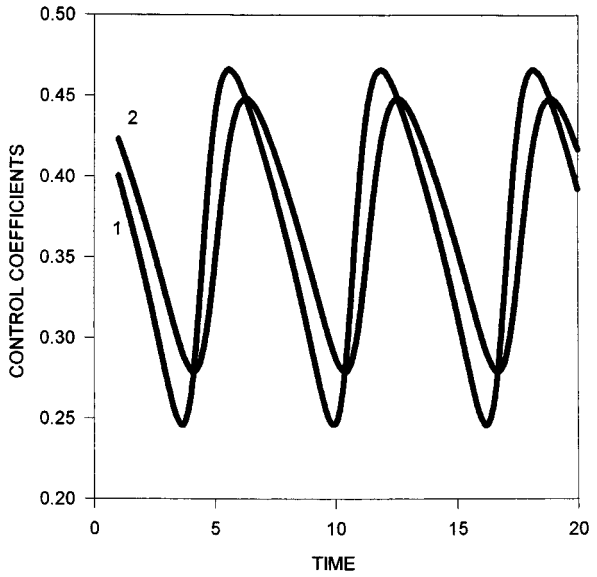

Figure 1. Comparison of periodic control coefficients over the time-dependent fluxes through sequential reactions in the oscillating system depicted by scheme 1: (1) control coefficient over flux J_1 with respect to concentration of the first enzyme; (2) control coefficient over flux J_2 with respect to concentration of the first enzyme. The magnitudes of the constants were $k_1 = 35$, $k_2 = 30$, $k_{-1} = 25$, $k_{-2} = 1$, $S_0 = 20$, $e_1 = 0.1$, $e_2 = 0.05$, $a = 0.5$, and $\omega = 1$.

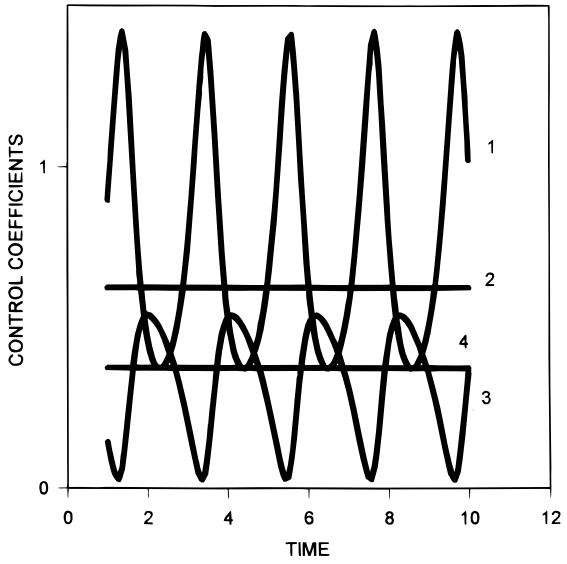

Figure 2. Dependencies of periodic and steady-state enzyme control coefficients of the linear pathway of scheme 1 on time. Lines 1 and 3 refer to the periodic control coefficients over flux J_1 with respect to the first (1) and the second (3) enzyme. Lines 2 and 4 refer to steady-state control coefficients over flux J_1 with respect to the first (2) and the second (4) enzyme. The magnitudes of the parameters were $k_1 = 35$, $k_2 = 30$, $k_{-1} = 25$, $k_{-2} = 1$, $S_0 = 20$, $e_1 = 0.1$, $e_2 = 0.05$, $a = 0.1$, and $\omega = 1$.

tude of the substrate oscillation increases further, even the direction of reaction rates changes during the period and such that they equal zero at some moments. In this case the control can become infinite. If the amplitude of the oscillation of the pathway substrate is small ($a \ll S_0$), the periodic control coefficients do not cross, but oscillate near the corresponding steady-state values (not shown).

Using the explicit expressions for periodic control coefficients (see Appendix C), one can show readily that the summation theorem, which governs the steady-state control coefficients[4] and time-dependent control coefficients for the relaxation near steady states[38] (it requires the sum of these coefficients to be equal to 1), is not valid for periodic control coefficients:

$$C^{J_2}_{e_1}(t) + C^{J_2}_{e_2}(t) = 1 + (A_2/J_2(t))\{[(k_2e_2)^2 + \omega_0{}^2]^{1/2} \sin(\omega_0 t + \zeta_1) + [(k_{-1}e_1)^2 + \omega_0{}^2]^{1/2} \sin(\omega_0 t + \zeta_2)\} \quad (12)$$

C. Summation Theorems. *C1. Summations in the Case of a Forced Oscillation.* Since the reactions rates depend linearly on the enzyme concentrations (activities), simultaneous transformation of these concentrations, of the time, and of the frequency of a periodic external force,

$$e_i^* = \lambda e_i, \quad t^* = t/\lambda, \quad \omega_0^* = \lambda\omega_0 \quad (13)$$

leads to a new equation system that coincides with the initial system after eliminating the superscript (*). Therefore, if the initial conditions are the same, metabolite concentrations of the transformed system at the moment t/λ will coincide with concentrations of the initial system at the moment t, whereas the fluxes will increase by factor λ (proportional to the new enzyme activities):

$$x_i(t/\lambda, \lambda\mathbf{e}) = x_i(t, \mathbf{e})$$

$$J_k(t/\lambda, \lambda\mathbf{e}) = \lambda J_k(t, \mathbf{e}) \quad (14)$$

Applying to eq 14 Euler's theorem on homogeneous functions, one arrives at

$$\sum_j C_j^{x_i}(t) - \mathrm{d}\ln x_i/\mathrm{d}\ln t + \mathrm{d}\ln x_i/\mathrm{d}\ln \omega_0 = 0 \quad (15)$$

$$\sum_j C_j^{J}{}_k(t) - \mathrm{d}\ln J_k/\mathrm{d}\ln t + \mathrm{d}\ln J_k/\mathrm{d}\ln \omega_0 = 1 \quad (16)$$

Although for forced oscillations the control coefficients with respect to the frequency of the external force ($C^{x}_{\omega_0}$, $C^{J}_{\omega_0}$) can be defined formally as the derivative of the periodic solution with respect to ω_0, they become unlimited as time tends to infinity. This is explained by the phase divergence of the initial periodic movement with the frequency ω_0 and the perturbed one with the frequency $\omega_0 + \mathrm{d}\omega_0$, corresponding to the infinitesimal change in ω_0. This phase difference does not remain infinitesimal at infinite times (cf. the case of autonomous oscillations above). Moreover, also the transient control coefficients defined by eq 4, in which the derivatives should be taken with respect to ω_0 (instead of e_j), become unlimited at infinitely large time. Hence, in a general case the summation theorems given by eqs 15 and 16 have no operational meaning as t tends to infinity. Note, however, that the sums given by the first terms in eqs 15 and 16 do exist at infinitely large times. For the above example of forced oscillations, eq 12 shows that the sum of flux control coefficients (the first term in eq 16) depends periodically on time.

One may note that if the form (amplitude) of the oscillation in x were independent of the forcing frequency ω_0, x could be written as

$$x(\omega_0, t) = x(\omega_0 t)$$

In this case, the second and third term of eqs 16 disappear and the classical summation theorems, but now for periodic control coefficients, are retrieved. This condition holds in electrical networks without capacitances, and in linear chemical networks where the variable metabolites occur in such small volumes that the corresponding relaxation times are much smaller than the period of the applied oscillation. As illustrated by eq 10,

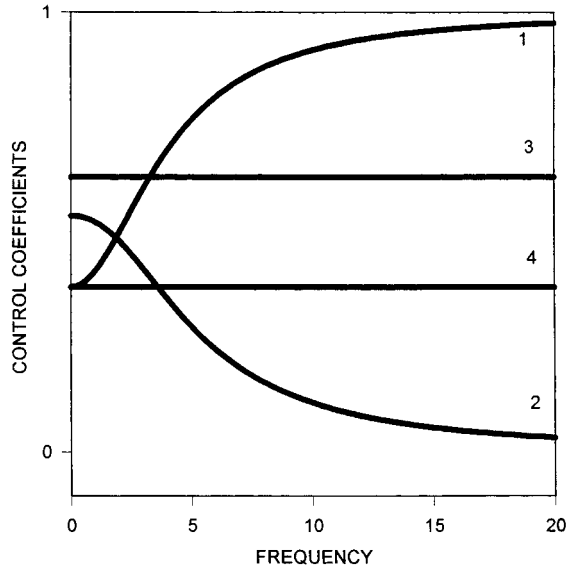

Figure 3. Comparison of the amplitude control coefficients with the steady-state control coefficients for the linear pathway depicted in scheme 1. Curves 1 and 2 are dependencies of control coefficients over the amplitude A_1 of the periodic flux J_1 with respect to concentrations of enzymes 1 and 2 on frequency of external periodic force. Curves 3 and 4 are steady-state control coefficients with respect to enzymes 1 and 2, respectively. The magnitudes of the constants were $k_1 = 35$, $k_2 = 30$, $k_{-1} = 25$, $k_{-2} = 1$, $S_0 = 20$, $e_1 = 0.1$, and $e_2 = 0.05$.

through the frequency dependence of the amplitude of the oscillation in x^{per}, the form of oscillations in a metabolic network often depends on the frequency of the applied oscillations.[48] Accordingly, in the more general case, the second and third term of eq 16 do not cancel, and the simple summation theorems do not apply.

As in the case of autonomous oscillations (below), one should go to functions of metabolic concentrations and reaction rates that do not depend on the phase of the movement for summation theorems to become practicable. In particular, the control coefficients with respect to the frequency can be defined for time-independent characteristics, such as for the averages over the period or amplitudes of oscillations.

The following summation theorems hold for the control coefficients of enzymes over the amplitude of stationary oscillations of metabolic concentrations (A_x) or fluxes (A_J) and their average values over the period ($\bar{x}$, $\bar{J}$) in the case of oscillations forced at a frequency ω_0:

$$\sum_j C_j^{A_x} + C_{\omega_0}^{A_x} = 0, \quad \sum_j C_j^{\bar{x}} + C_{\omega_0}^{\bar{x}} = 0$$

$$\sum_j C_j^{A_J} + C_{\omega_0}^{A_J} = 1, \quad \sum_j C_j^{\bar{J}} + C_{\omega_0}^{\bar{J}} = 1 \qquad (17)$$

In the example considered above (section B) the control coefficients over the amplitudes of metabolic concentrations and fluxes with respect to the frequency of the external force and of the enzyme concentrations can be calculated readily (see Appendix C). One can see that they satisfy the summation theorems given by eq 17. Figure 3 compares the control coefficients over the amplitude A_11 of the periodic flux J_1 (curves 1 and 2) with the flux control coefficients for the steady-state system (curves 3 and 4). One can see that the relative distribution of the amplitude control between the enzymes of oscillating systems depends dramatically on the frequency of the periodic external force. Moreover, no predictions for this

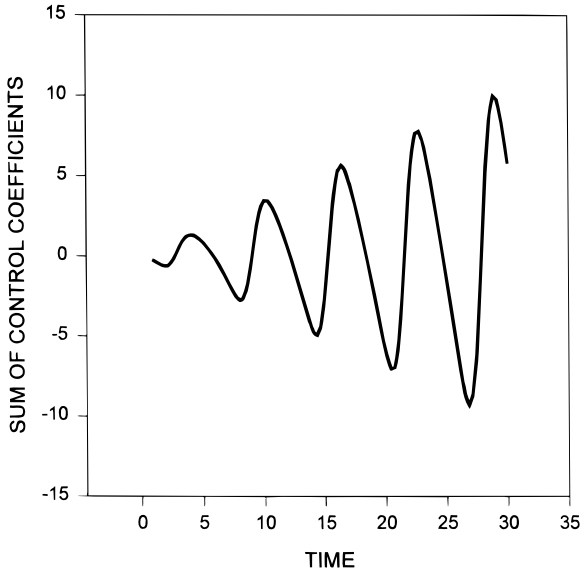

Figure 4. Dependency of the sum of the time-dependent control coefficients on time in the case of sinusoidal autonomous oscillations.

distribution can be made on the basis of the magnitudes of the corresponding flux control coefficients at the steady state.

C2. Summation Theorems for Autonomous Oscillation. As was the case for forced oscillations, simultaneous transformation (13) of the enzyme concentrations (activities) and of the time,

$$e_i^* = \lambda e_i, \quad t^* = t/\lambda \qquad (18)$$

leads to a new equation system that coincides with the initial system after eliminating the superscript (*). Therefore, eqs 14 continue to apply. This results in the following relationships for the control coefficients defined by eq 3:[41]

$$\sum_j C_j^{x_i}(t) - \mathrm{d}\ln x_i/\mathrm{d}\ln t = 0$$

$$\sum_j C_j^{J_k}(t) - \mathrm{d}\ln J_k/\mathrm{d}\ln t = 1 \qquad (19)$$

where the control coefficients are defined as formal derivatives of concentrations and fluxes with respect to e_j at the moment t (eq 3). However, for autonomous oscillations the relationships (19) are not very useful because the control coefficients become unlimited with time, unless it is emphasized that the enzymes have an excessive and oscillatory control on the metabolite concentrations. We shall illustrate this by the simplest possible example, i.e. that of the sinusoidal oscillation in the concentration (x),

$$x^{per}(t) = A[2 + \sin(\omega t + \varphi)]$$

Equation 19 implies

$$\sum_j C_j^x = (\omega t)\cos(\omega t + \varphi)/[2 + \sin(\omega t + \varphi)]$$

which, as illustrated by Figure 4, is quasi periodic with a continuously increasing amplitude. This unlimited increase in the amplitude of the sum of the control coefficients is because any difference in frequency between the perturbed and unperturbed system causes a phase difference increasing continuously with time, as if the clock speed of the two systems differs. Note that in steady states the second term in eq 19 is zero and the traditional summation theorems are found.

The relationships involving the control coefficients over time-independent properties are useful for analyzing the control on

a periodic trajectory. Considering eq 14 on a periodic trajectory, one can conclude that the amplitude (A_x) of oscillations of metabolite concentrations and their average values ($\bar{x}$) over a period remain unchanged under the transformation (18):

$$A_x(\lambda\mathbf{e}) = A_x(\mathbf{e}), \quad \bar{\mathbf{x}}(\lambda\mathbf{e}) = \bar{\mathbf{x}}(\mathbf{e})$$

whereas the amplitude (A_J) of oscillations of fluxes, their averages ($\bar{J}$), and the period (T) of oscillations satisfy the relationships

$$A_J(\lambda\mathbf{e}) = \lambda A_J(\mathbf{e}), \quad \bar{J}(\lambda\mathbf{e}) = \lambda\bar{J}(\mathbf{e}), \quad T(\lambda\mathbf{e}) = (1/\lambda)T(\mathbf{e})$$

Therefore, the control coefficients of enzymes over the amplitude of oscillations of metabolite concentrations and fluxes, their averages, and the period of oscillations obey the simple summation theorems[47] (cf. ref 49):

$$\sum_j C_j^{A_x} = 0, \quad \sum_j C_j^{\bar{\mathbf{x}}} = 0$$

$$\sum_j C_j^{A_J} = 1, \quad \sum_j C_j^{\bar{J}} = 1$$

$$\sum_j C^T{}_j = -1 \qquad (20)$$

Discussion

To calculate the fluxes and concentrations in a chemical reaction system from all the parameter values, numerical integration of complex equations is required especially if it pertains to a living cell (e.g., refs 43, 50). To calculate how steady-state properties are controlled by the parameters of that system, an analytical method exists, called metabolic control analysis (MCA)[4,43] provided that one defines control in terms of infinitesimal influences. The method has a parallel in biochemical systems analysis,[2,3] has had important practical applications (e.g., ref 51), and has increased biochemical insight into what controls metabolic gene expression and signal transduction[9] networks.

Some of the most important biological processes are not (always) at steady state however.[15] Therefore various attempts have been made to extend MCA to time-dependent systems. The phenomenon that may seem most amenable to such an extension is that of stationary oscillations of metabolic oscillating systems. A long known example is that of yeast glycolytic oscillations; under certain conditions yeast extracts and populations of yeast cells exhibit oscillations in NADH and glycolytic intermediates (e.g., refs 23−26). These oscillations are autonomous; that is, they are not driven by an externally imposed oscillation. The same system may also be exposed to relevant imposed oscillations such as in glucose concentration or in the flux. Quite interesting metabolic dynamics then ensues.[52]

Attempts to develop MCA for the analysis of the control of the time dependence of metabolite concentrations and fluxes have led to algebraic expressions (e.g. ref 14) that have had no implementation until now. Various authors including ourselves have embarked on developing a more complete analysis.

Most importantly, the present paper resolves many of the remaining issues by proving that much of what is being attempted is impossible. It is impossible to analyze the control of metabolic concentrations in an autonomously oscillating system in terms of well-defined control coefficients, because the latter tend to infinity as time proceeds. The culprit is that system parameters will control not only the concentrations and fluxes but also the frequency at which they oscillate. Any parameter change may therefore cause a phase shift that continues to increase with time. An important consequence is that theorems such as derived in ref 41 for oscillating metabolite concentrations cannot contribute much to the understanding of how autonomously oscillating systems are controlled. The relationship, considered as the connectivity theorem by Acerenza et al.,[41] is, in fact, the variation equation with respect to the transient control coefficients. As shown in Appendix B when time tends to infinity, the solution to this equation does exist in the case of forced oscillations but does not exist for the case of self-oscillations.

By showing what is impossible, this paper also highlights what is possible. It is possible and useful to define control coefficients and to derive summation theorems for all properties that do not depend on the phase of the oscillations, i.e., all time-independent properties such as amplitude and mean value. The theorems derived here are in line with earlier results for autonomous oscillations.[33,47,49]

So far, control analysis has been applied to autonomous systems in which all the external and internal parameters are assumed to be fixed. A distinction between autonomous and forced oscillations is that in the latter case the equation system is not autonomous mathematically in terms of its time dependence.

Therefore it seemed that if time and phase of the oscillations of the system were defined by the outside of the system, a control analysis of the oscillating metabolite concentrations and fluxes might became possible. This should apply to entrained oscillations:[20] oscillations enforced by a periodic force defined from the outside of the system. Differences between forces being defined by the outside world have earlier been shown to be crucial for the understanding of biological free-energy transduction.[42,53] This paper has shown that, indeed, none of the limitations for the application of MCA to oscillating concentrations and reaction rates in autonomous systems are present in periodically enforced systems.

The importance of entrainment[20] reaches well beyond the homogeneous systems discussed in this paper. Autonomous oscillations in a population of cells are only macroscopic if cells oscillate in phase. For sustained oscillations some continuing mutual entrainment of the oscillations of the individual cells is required. Recently, the conditions required to obtain sustained glycolytic oscillations in yeast cells have been obtained[31] and the chemical identity of the synchronizing intercellular agent has been identified.[32] It is imperative to understand what controls such oscillations in heterogeneous systems. By treating the intercellular communications as small perturbations by an externally oscillating agent, the system can be approached.

For the control analysis of oscillating systems, it has been important to distinguish between two time-dependent control coefficients. Transient control coefficients quantify the control over a transition from the initial periodic trajectory to the trajectory that corresponds to the perturbed enzyme concentration (cf. eq 4). Periodic control coefficients refer to dependence of the stationary time-dependent oscillation on the concentration of an enzyme. As for forced oscillations, the transient control coefficients tend to the control coefficients of steady-state periodic trajectory when time tends to infinity and the control of periodically oscillating metabolic concentrations and reaction rates by parameters of the system is defined properly. Appendices A and B show the mathematical reasons for that: if all the real parts of the eigenvalues of the Jacobian matrix, $\mathbf{N}\cdot(\partial\mathbf{v}/\partial\mathbf{x})$, are negative on the periodic trajectory, $x^{per}(t;e)$, the solution to the variation equation (B7) exists at any (even

infinitely large) time moment. For autonomous oscillations both the operational and the formal definitions of time-dependent control coefficients are possible only in terms of the control over time-independent properties of the oscillations. The reason is lack of asymptotic stability for autonomous oscillations (hence, phase instability), because one of the eigenvalues of a monodromy matrix for a limit cycle (which corresponds to the matrix **F**, see eq B11) equals 1. Explicit expressions (see eqs B10, B11, and B16 in the Appendix B) for the periodic and transient control coefficients with respect to parameters other than the imposed frequency have been derived for the case of forced oscillations.

As discussed above, only in the case of forced oscillations, does control of time-dependent properties lend itself to relevant control analysis. An exception here is the control by the imposed frequency. Although for forced oscillations one can define formally the derivative of the periodic solution of the differential equations with respect to the frequency as a periodic control coefficient, the corresponding transient control coefficient will not tend to the latter when the time tends to infinity. Hence, this definition does not reflect the control over the metabolic variables themselves. Again one should go to the averages or amplitude.

This paper also reports some model calculations. Figure 1 illustrates that the (periodic) control coefficients of enzymes over the fluxes through sequential reactions differ significantly, whereas the corresponding steady-state control coefficients are identical. Explicit expressions for the control coefficients of the enzyme 1 over fluxes J_1 and J_2 are given in Appendix C. Similar observations in ref 47 have demonstrated that also metabolic dynamics is subject to subtle control, perhaps even more so than metabolic steady states.

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Appendix

Appendix A. Transient and Periodic Control Coefficients. The kinetic equations of a metabolic network that is exposed to a periodic external force can be written as follows (cf. ref 38):

$$d\mathbf{x}/dt = \mathbf{N}\cdot\mathbf{v}(t,\mathbf{x},\mathbf{e}) \tag{A1}$$

Here $\mathbf{x} = [x_1, ..., x_m]^T$ and $\mathbf{v} = [v_1, ..., v_n]^T$ are the vectors of the metabolite concentrations and the reactions rates, and **N** is the m by n stoichiometry matrix. Due to the periodic dependence of some kinetic parameters (**p**) on time, the reactions rates, $v_j(t,\mathbf{x})$, depend on time explicitly. It is convenient to present each v_j as the product of the concentration (maximal activities) of the corresponding enzyme (e_j) and some function (w_j) that depends on time periodically due to periodic changes of the parameters (**p**) with time:

$$v_j = e_j w_j(\mathbf{p}(t),\mathbf{x}), \quad \mathbf{p}(t) = \mathbf{p}(t+T), \quad \text{hence,}$$

$$v_j(t+T,\mathbf{x}) = v_j(t,\mathbf{x}), \quad j = 1, ..., n \tag{A2}$$

Here T is the period of the external force that is related to the frequency (ω) by the relationship $T = 2\pi/\omega$. This paper is limited to systems in which the eigenvalues of the matrix $\mathbf{N}\cdot\partial\mathbf{v}/\partial\mathbf{x}$ (the Jacobian) have negative real parts at all the points of the periodic trajectory. Under this condition, eq A1 has a unique, asymptotically stable periodic solution, $\mathbf{x}^{per}(t,\mathbf{e})$, determined by the periodic boundary condition:

$$\mathbf{x}^{per}(t^*,\mathbf{e}) = \mathbf{x}^{per}(t^*+T,\mathbf{e}) \tag{A3}$$

where t^* is a particular initial moment of time. The solution for this boundary condition will be denoted by $x_i^{per}(t,\mathbf{e};t^*,\mathbf{x}^*)$. The solution is periodic with period T,

$$\mathbf{x}^{per}(t,\mathbf{e};t^*,\mathbf{x}^*) = \mathbf{x}^{per}(t^*+T,\mathbf{e};t^*,\mathbf{x}^*)$$

Note that $\mathbf{x}^*$ cannot be arbitrary but is uniquely determined by t^* on the periodic trajectory because of an external "clock" provided by the periodic external force. Considering the two periodic solutions that correspond to the initial value **e** and to a perturbed value of the jth enzyme concentration ($e_j + \Delta e_j$), respectively, we present the derivative of x_i^{per} with respect to e_j as

$$C^{x_i}_j(t) = (dx_i^{per}(t,\mathbf{e})/de_j)(e_j/x_i^{per}) = (e_j/x_i^{per}(t,\mathbf{e})) \times \lim_{\Delta e_j \to 0} \frac{x_i^{per}(t;e_j+\Delta e_j,) - x_i^{per}(t;e_j)}{\Delta e_j} \tag{A4}$$

Our immediate goal is to construct one-to-one correspondence between the points of the two periodic solutions $\mathbf{x}^{per}(t,e_j)$ and $x^{per}(t,e_j+\Delta e_j)$. The reason to expect such a correspondence is the identity of the phase of the periodic external force for the two solutions (external clock). During any period, e.g., within the time interval $0 \le t < T$, each point **x** on the closed periodic trajectory in concentration space corresponds to a unique set of values (t, $\mathbf{p}(t)$). Consequently, the following correspondences (χ_1 and χ_2) exist:

$$\chi_1: \qquad (t, \mathbf{p}(t)) \leftrightarrow \mathbf{x}^{per}(t,e_j), \quad 0 \le t < T$$

$$\chi_2: \qquad (t, \mathbf{p}(t)) \leftrightarrow \mathbf{x}^{per}(t,e_j+\Delta e_j), \quad 0 \le t < T \tag{A5}$$

Importantly, these correspondences are one-to-one. Therefore, also a one-to-one correspondence between the two periodic solutions $\mathbf{x}^{per}(t,e_j)$ and $\mathbf{x}^{per}(t,e_j+\Delta e_j)$ exists, as given by the composition $\chi = \chi_1^{-1} \circ \chi_2$:

$$\chi: \qquad \mathbf{x}^{per}(t,e_j) \leftrightarrow \mathbf{x}^{per}(t,e_j+\Delta e_j), \quad 0 \le t < T \tag{A6}$$

Note that this one-to-one correspondence (synchronization), χ, is imposed by the periodic external force. χ will change when a different periodic driving force is applied.

Let us denote by $\mathbf{x}^*$ and $\mathbf{x}^{**}$ the points that belong to closed trajectories $\mathbf{x}^{per}(t,e_j)$ and $\mathbf{x}^{per}(t,e_j+\Delta e_j)$ at time $t = t^*$. According to eq A6, $\mathbf{x}^{**}$ can be presented as

$$\mathbf{x}^{**} = \chi\,(\mathbf{x}^*) \tag{A7}$$

Considering the points (t^*, $\mathbf{x}^*$) and (t^*, $\mathbf{x}^{**}$) as the initial conditions of the two solutions, $\mathbf{x}^{per}(t,e_j,t^*,\mathbf{x}^*)$ and $\mathbf{x}^{per}$-$(t,e_j+\Delta e_j;t^*,\mathbf{x}^{**})$, we refine eq A4 as the following:

$$C^{x_i}_j(t) = e_j/x_i^{per}(t,\mathbf{e}) \times \lim_{\Delta e_j \to 0} \left[\frac{x_i^{per}(t,e_j+\Delta e_j;t^*,\chi(\mathbf{x}^*)) - x_i^{per}(t,e_j;t^*,\mathbf{x}^*)}{\Delta e_j} \right] \tag{A8}$$

Our next goal is to relate the transient control coefficients, ${}^{tr}C^{x_i}_j(t)$, as defined by eq 4 of the main text, to the periodic

control coefficients, $C^{x_i}_j(t)$, as defined by eq A8. It follows from eqs 4 and A8 that

$$
\begin{aligned}
{}^{\mathrm{tr}}C^{x_i}_j(t) = (e_j/x_i^{\mathrm{per}}(t,e;t^*,\mathbf{x}^*)) \times \\
\lim_{\Delta e_j \to 0} \frac{x^{\mathrm{tr}}{}_i(t,e_j+\Delta e_j;t^*,\mathbf{x}^*) - x_i^{\mathrm{per}}(t,e_j,t^*,\mathbf{x}^*)}{\Delta e_j} = \\
(e_j/x_i^{\mathrm{per}}(t,e_j;t^*,\mathbf{x}^*)) \times \\
\lim_{\Delta e_j \to 0} \left\{ \frac{x_i^{\mathrm{tr}}(t,e_j+\Delta e_j;t^*,\mathbf{x}^*) - x_i^{\mathrm{per}}(t,e_j+\Delta e_j;t^*,\chi(\mathbf{x}^*))}{\Delta e_j} + \right. \\
\left. \frac{x_i^{\mathrm{per}}(t,e_j+\Delta e_j;t^*,\chi(\mathbf{x}^*)) - x_i^{\mathrm{per}}(t,e_j;t^*,\mathbf{x}^*)}{\Delta e_j} \right\} = \\
(e_j/x_i^{\mathrm{per}}(t,e_j;t^*,\mathbf{x}^*))\, \Delta e_j \xrightarrow{\lim} 0 \frac{\Delta_i(t)}{\Delta e_j} + C^{x_i}_j \quad \text{(A9)}
\end{aligned}
$$

with

$$\Delta_i(t) = x_i^{\mathrm{tr}}(t,e_j+\Delta e_j;t^*,\mathbf{x}^*) - x_i^{\mathrm{per}}(t,e_j+\Delta e_j;t^*,\chi(\mathbf{x}^*)) \quad \text{(A10)}$$

Now for any sufficiently small Δe_j, we shall estimate the difference, $\Delta_i(t)$. If the initial condition for $\mathbf{x}^{\mathrm{tr}}$ in eq A10 were $\chi(\mathbf{x}^*)$, this solution would coincide with $\mathbf{x}^{\mathrm{per}}$, since the point $\chi(\mathbf{x}^*)$ belongs to the periodic trajectory that is determined by $[e_j] = e_j + \Delta e_j$. Hence, the difference, $\Delta_i(t)$, can be expressed in terms of the derivative with respect to the initial condition,

$$\Delta_i(t) = \sum_{l=1}^{m} (\partial x_i^{\mathrm{tr}}/\partial x_l^*)(x_l^* - \chi(\mathbf{x}^*)) + \mathrm{o}(|\mathbf{x}^* - \chi(\mathbf{x}^*)|) \quad \text{(A11)}$$

Here o denotes the sum of all terms of higher order with respect to the norm, $|\mathbf{x}^* - \chi(\mathbf{x}^*)|$, of the m-dimensional vector, $\mathbf{x}^* - \chi(\mathbf{x}^*)$. The difference in the initial conditions, $\mathbf{x}^* - \chi(\mathbf{x}^*)$, relates to the distance between the two periodic trajectories, corresponding to enzyme concentrations e_j and $e_j + \Delta e_j$ at time t^*. For every concentration this difference can be expressed as

$$\chi_l(\mathbf{x}^*) - x_l^* = \Gamma_l(\mathrm{x}^*)\Delta e_j + \mathrm{o}(\Delta e_j), \quad l = 1, ..., m \quad \text{(A12)}$$

where $\Gamma_l(\mathbf{x}^*)$ is finite. $\mathrm{o}(\Delta e_j)$ represents the sum of all higher than first-order terms with respect to Δe_j. Applying eqs A10−A12 to eq A9, one obtains

$${}^{\mathrm{tr}}C^{x_i}_j(t) = C^{x_i}_j(t) + (e_j/x_i^{\mathrm{per}}) \sum_{l=1}^{m} (\partial x_i^{\mathrm{tr}}/\partial x_l^*)\Gamma_l(\mathbf{x}^*) \quad \text{(A13)}$$

Asymptotic stability of forced oscillations implies that any difference in the initial conditions vanishes when the time of observation tends to infinity (due to the synchronization by the external force). This implies that

$$\lim_{t \to 0} \frac{\partial x_i^{\mathrm{tr}}(t,\mathbf{e};x^*,t^*)}{\partial x^*} = 0$$

Consequently:

$$\lim_{t \to 0} [{}^{\mathrm{tr}}C^{x_i}_j(t) - C^{x_i}_j(t)] = 0 \quad \text{(A14)}$$

This concludes our proof that for the case of asymptotically stable forced oscillations the transition control coefficients tend to the periodic control coefficients when time tends to infinity.

Appendix B

For the case of forced oscillations we shall derive the variation equation for non-normalized control coefficients. Integration of the differential equations describing the system behavior leads to the periodic and nonperiodic solutions. These specify the periodic and the transient control coefficients, respectively. Analysis of the so-called variation equation identifies intrinsic system properties (i.e., considered a part of periodic external force) that ensure the asymptotic stability of forced oscillations and shed light on the convergence of the transient control coefficients to the periodic control coefficients.

To derive the variation equation, we consider two periodic solutions of the differential equations (see eq A1), i.e., one corresponding to the initial value, e_j, and one corresponding to a perturbed value $e_j + \Delta e_j$:

$$\mathbf{x}^{\mathrm{per}}(t,e_j) = \mathbf{x}^{\mathrm{per}}(t+T,e_j) \quad \text{(B1)}$$

$$\mathbf{x}^{\mathrm{per}}(t,e_j+\Delta e_j) = \mathbf{x}^{\mathrm{per}}(t+T,e_j+\Delta e_j) \quad \text{(B2)}$$

Here t is any moment of time. It follows from eqs A1 and B1 that the difference ($\mathbf{z}$) between these two periodic solutions,

$$\mathbf{z}(t,\Delta e_j) = \mathbf{x}^{\mathrm{per}}(t,e_j+\Delta e_j) - \mathbf{x}^{\mathrm{per}}(t,e_j) \quad \text{(B3)}$$

satisfies the equation

$$\mathrm{d}\mathbf{z}/\mathrm{d}t = \mathbf{N}\cdot[\mathbf{v}(t,\mathbf{x}^{\mathrm{per}}(t,e_j+\Delta e_j),e_j+\Delta e_j) - \mathbf{v}(t,\mathbf{x}^{\mathrm{per}}(t,e_j),e_j)] \quad \text{(B4)}$$

with the following periodicity condition for any t:

$$\mathbf{z}(t,\Delta e_j) = \mathbf{z}(t+T,\Delta e_j) \quad \text{(B5)}$$

For small value sof Δe_j the difference $\mathbf{v}(t,\mathbf{x}^{\mathrm{per}}(t,e_j+\Delta e_j),e_j+\Delta e_j) - \mathbf{v}(t,\mathbf{x}^{\mathrm{per}}(t,e_j),e_j)$ can be expanded into a Taylor series:

$$\mathrm{d}\mathbf{z}/\mathrm{d}t = \mathbf{N}\cdot(\partial\mathbf{v}/\partial\mathbf{x})|_{\mathbf{x}=\mathbf{x}^{\mathrm{per}}(t;\mathbf{e})}\cdot\mathbf{z} + \mathbf{N}\cdot(\partial\mathbf{v}/\partial e_j)|_{\mathbf{x}=\mathbf{x}^{\mathrm{per}}(t;\mathbf{e})}\cdot\Delta e_j + \mathrm{o}(|(\Delta e_j,\mathbf{z})|) \quad \text{(B6)}$$

Notably,

$$(\partial\mathbf{v}/\partial e_j)_k = \delta_{jk}\cdot(\partial v_j/\partial e_j) = \delta_{jk}\cdot w_j(\mathbf{x}^{\mathrm{per}}(t,\mathbf{e}))$$

Here the derivatives $\partial\mathbf{v}/\partial\mathbf{x}$ and $\partial\mathbf{v}/\partial e_j$ are taken at the periodic solution (B1) at time t. The symbol o denotes the sum of all higher order terms of the Taylor expansion. w_j is the turnover rate v_j/e_j of enzyme j. Dividing both sides of eq B6 by Δe_j, taking the limit to infinitesimal Δe_j, and switching the order of differentiation, one arrives at

$$\mathrm{d}\Gamma^{\mathbf{x}}_{e_j}/\mathrm{d}t = \mathbf{N}\cdot(\partial\mathbf{v}/\partial\mathbf{x})\cdot\Gamma^{\mathbf{x}}_{e_j} + \mathbf{N}\cdot(\partial\mathbf{v}/\partial e_j) \quad \text{(B7)}$$

Here $\Gamma^{\mathbf{x}}_{e_j}$ is the jth column of the matrix, $\Gamma^{\mathbf{x}}_{\mathbf{e}}$, of non-normalized periodic control coefficients. The latter matrix is connected to the matrix of normalized control coefficients through

$$\Gamma^{\mathbf{x}}_{\mathbf{e}} = (\mathrm{diag}\ \mathbf{x})\cdot\mathbf{C}^{\mathbf{x}}_{\mathbf{e}}\cdot(\mathrm{diag}\ \mathbf{e})^{-1} \quad \text{(B8)}$$

The periodic control matrix, $\Gamma^{\mathbf{x}}_{\mathbf{e}}$, can be found as the solution of eq B7 that satisfies the following boundary condition (cf. eq B5):

$$\Gamma^{\mathbf{x}}_{e_j}(t^*) = \Gamma^{\mathbf{x}}_{e_j}(t^*+T) \quad \text{(B9)}$$

The (linear) equation (B7) is called the variation equation. It is a generalization of the equation used by Heinrich and Reder (eq 4 in ref 38). Its unique periodic solution determined by the boundary condition (B9) is presented as (this can be verified

by the direct substitution)

$$\Gamma^{\mathbf{x}}_{\mathbf{e}} = \mathbf{F}(t)\cdot[(\mathbf{I} - \mathbf{F}(T))^{-1}\cdot\mathbf{F}(T)\int_0^T \mathbf{F}(\tau)^{-1}\cdot\mathbf{N}\cdot(\partial\mathbf{v}(\tau)/\partial\mathbf{e})\,d\tau + \int_0^t \mathbf{F}(\tau)^{-1}\cdot\mathbf{N}\cdot(\partial\mathbf{v}(\tau)/\partial\mathbf{e})\,d\tau \quad \text{(B10)}$$

Here $\mathbf{F}(t)$ is the fundamental matrix of the homogeneous equation

$$d\mathbf{F}/dt = \mathbf{N}\cdot(\partial\mathbf{v}/\partial\mathbf{x})\cdot\mathbf{F} \quad \text{(B11)}$$

$\mathbf{I}$ is the m by m identity matrix. Explicitly, $\mathbf{F}$ is expressed as follows:

$$\mathbf{F}(t) = \exp(\int_0^t \mathbf{N}\cdot(\partial\mathbf{v}/\partial\mathbf{x}(\tau))\,d\tau) \quad \text{(B12)}$$

For simplicity it is assumed here that the m by n stoichiometry matrix, $\mathbf{N}$, has maximum rank, m, i.e., that all the metabolite concentrations are linearly independent.

If the metabolite concentrations are constrained by the moiety conservations, only the control coefficients with respect to linearly independent metabolites must be considered. In this case the stoichiometry matrix, $\mathbf{N}$, can be presented as the product of the link matrix, $\mathbf{L}$, and the reduced matrix $\mathbf{N^R}$ of the maximal row rank of $\mathbf{N}$.[13] Reducing to the independent concentration ($\mathbf{x^R}$), the variation equation (B7) takes the form

$$d\Gamma^{\mathbf{x^R}}_{\mathbf{e}}/dt = \mathbf{N^R}\cdot(\partial\mathbf{v}/\partial\mathbf{x})\cdot\mathbf{L}\cdot\Gamma^{\mathbf{x^R}}_{\mathbf{e}} + \mathbf{N^R}\cdot(\partial\mathbf{v}/\partial\mathbf{e}) \quad \text{(B13)}$$

Its solution is given by eq B10 after substituting the matrix $\mathbf{N^R}$ for $\mathbf{N}$ and the corresponding substitution of the matrix $\mathbf{N^R}\cdot(\partial\mathbf{v}/\partial\mathbf{x})\cdot\mathbf{L}$ for $\mathbf{N}\cdot(\partial\mathbf{v}/\partial\mathbf{x})$ in eqs B11 and B12.

Now we shall compare the periodic solution corresponding to the value, e_j, to the transient solution that occurs when this enzyme concentration (e_j) is perturbed by Δe_j at the moment t^*. Either solution satisfies eq A1 (with different values of enzyme concentrations) and assumes the same initial value ($\mathbf{x}^*$) at the initial moment t^*. Their difference ($\mathbf{y}$),

$$\mathbf{y}(t,\Delta e_j) = \mathbf{x}^{\mathrm{tr}}(t,e_j+\Delta e_j;t^*,\mathbf{x}^*) - \mathbf{x}^{\mathrm{per}}(t,e_j;t^*,\mathbf{x}^*)$$

will satisfy eq B4. However, the periodic boundary condition (B5) does not apply to the function $\mathbf{y}$, and instead of eq B5 the initial condition

$$\mathbf{y}(t^*,\Delta e_j) = 0 \quad \text{(B14)}$$

must be considered. Similarly as above, one can show that the matrix (${}^{\mathrm{tr}}\Gamma^{\mathbf{x}}_{\mathbf{e}}$) of non-normalized transient control coefficients satisfies the same variation equation (eq B7), that of the matrix ($\Gamma^{\mathbf{x}}_{\mathbf{e}}$) of the periodic control coefficients. Most importantly, however, the unique periodic solution (eq B10) of the variation equation corresponding to the matrix $\Gamma^{\mathbf{x}}_{\mathbf{e}}$ is determined by the boundary condition (B9), whereas the solution corresponding to the matrix ${}^{\mathrm{tr}}\Gamma^{\mathbf{x}}_{\mathbf{e}}$ is determined by the zero initial condition (B15) (see eq B14):

$${}^{\mathrm{tr}}\Gamma^{\mathbf{x}}_{\mathbf{e}}(t^*) = 0 \quad \text{(B15)}$$

The solution of eq B7 with the initial condition (B15) can be presented in the following form:

$${}^{\mathrm{tr}}\Gamma^{\mathbf{x}}_{\mathbf{e}}(t) = \mathbf{F}(t)\int_0^t \mathbf{F}^{-1}(\xi)\cdot\mathbf{N}\cdot(\partial\mathbf{v}(\xi)/\partial\mathbf{e})\,d\xi \quad \text{(B16)}$$

Here $\mathbf{F}(t)$ is the same matrix as the $\mathbf{F}(t)$ of eq B10, i.e., the fundamental matrix of the homogeneous equation given by eq B12. Due to the dependence of $\partial\mathbf{v}/\partial\mathbf{x}$ on t, it follows from eqs B8, B12, and B16 that in the case of forced oscillations transient control coefficients do not obey the summation theorems that they would satisfy in the case of relaxation processes near the steady states (cf. ref 38).

We limit this discussion to systems that in the absence of a periodic external force exhibit a unique asymptotically stable steady state. Then, all the eigenvalues of the matrix ($\mathbf{N}\cdot\partial\mathbf{v}/\partial\mathbf{x}$) of the system linearized at the steady state, eq B7, have negative real parts. Due to the continuity of the derivatives, $\partial\mathbf{v}/\partial\mathbf{x}$, the eigenvalues of the matrix $\mathbf{N}\cdot\partial\mathbf{v}/\partial\mathbf{x}$ continue to have negative real parts in some vicinity of the steady state. It follows from eq B12 that if the periodic trajectory is located inside the vicinity, the norms of the eigenvalues of the matrix $\mathbf{F}(T)$ (i.e., the characteristic numbers of eq B11) are smaller than 1. This ensures the existence of the solution of eq B7, hence, of the transient control coefficients, ${}^{\mathrm{tr}}C^{x_i}_j(t)$, at any time. The convergence of ${}^{\mathrm{tr}}C^{x_i}_j(t)$ to the periodic control coefficient, $C^{x_i}_j(t)$, is justified by eq A13 (see Appendix A), provided that the derivatives, $dx_i^{\mathrm{tr}}/dx_l^*$, with respect to the initial conditions tend to zero as time tends to infinity. These derivatives satisfy the homogeneous variation equation (B11), and they can be expressed as linear combinations of the columns of the matrix $\mathbf{F}(t)$. It follows from eq B12 that any such combination vanishes when time tends to infinity (since all the eigenvalues of $\mathbf{N}\cdot\partial\mathbf{v}/\partial\mathbf{x}$ have negative real parts). In the case when the amplitudes of forced oscillations are large, the premise that the eigenvalues of the matrix $\mathbf{N}\cdot\partial\mathbf{v}/\partial\mathbf{x}$ have negative real parts at all the points of the periodic trajectory (see section A1 of the main text) ensures the existence of both periodic and transient control coefficients (expressed explicitly by eqs B10 and B16) and the convergence of the latter to the former when time tends to infinity.

Appendix C

The kinetic equation for the metabolic system depicted in Scheme 1 of the main text can be written as follows:

$$dx/dt = -(k_{-1}e_1 + k_2e_2)x + k_1e_1S \quad \text{(C1)}$$

When the substrate concentration (S) is time invariant, the system has an asymptotically stable steady state, x_0:

$$x_0(\mathbf{e}) = \frac{k_1Se_1}{k_{-1}e_1 + k_2e_2} \quad \text{(C2)}$$

When S changes periodically,

$$S(t) = S_0(1 + a\sin(\omega_0 t)) \quad \text{(C3)}$$

Equation C1 can be written as

$$dx/dt = -(k_{-1}e_1 + k_2e_2)x + k_1e_1S_0a\sin(\omega_0 t) + k_1e_1S_0 \quad \text{(C4)}$$

We will seek a periodic solution, x^{per}, in the following form:

$$x^{\mathrm{per}}(t) = x_0(e_1,e_2) + B_1(e_1,e_2)\sin(\omega_0 t) + B_2(e_1,e_2)\cos(\omega_0 t) \quad \text{(C5)}$$

Substituting eq C5 into eq C4 and equating the terms of the left-hand side, placing at functions $\sin(\omega_0 t)$ and $\cos(\omega_0 t)$, 1, to

those of right-hand side, one finds the periodic solution (C5) as the following:

$$x^{\mathrm{per}}(t) = x_0(e) + \frac{k_1 s_0 a e_1}{[(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2]^{1/2}} \sin(\omega_0 t - \varphi) \tag{C6}$$

Here, $-\varphi$ is the initial (at $t = 0$) phase of oscillations,

$$\varphi = \arcsin \frac{\omega_0}{[(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2]^{1/2}} \tag{C7}$$

Substituting eq C6 into the rate equations (see eq 9 of the main text), one derives expressions for the fluxes through the first (J_1) and second (J_2) reactions:

$$J_1(t) = J_0 + A \sin(\omega_0 t + \chi)$$
$$J_2(t) = J_0 + A_2 \sin(\omega_0 t - \varphi) \tag{C8}$$

$$J_0 = \frac{e_1 e_2 k_1 k_2 S_0}{k_{-1}e_1 + k_2e_2}$$

$$A_1 = S_0 a k_1 e_1 \left\{ \frac{(k_2e_2)^2 + {\omega_0}^2}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2} \right\}^{1/2} \tag{C9}$$

$$A_2 = \frac{S_0 a k_1 k_2 e_1 e_2}{[(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2]^{1/2}} \tag{C10}$$

$$\chi = \arcsin \frac{k_{-1}e_1\omega_0}{\{[k_2e_2(k_{-1}e_1 + k_2e_2) + {\omega_0}^2]^2 + (k_{-1}e_1\omega_0)^2\}^{1/2}}$$

Here J_0 is the steady-state flux. A_1 and A_2 are the amplitudes of the oscillations of the fluxes, and χ is the initial phase of oscillations of the flux through the first reaction. Using eqs C8−C10, the derivation of the periodic control coefficients is straightforward:

$$C_{e_1}^{J_1}(t) = \left[J_0 C_{e_1}^{J_0} + \frac{S_0 a k_1 e_1 [(k_2e_2)^2 + {\omega_0}^2]}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2} \sin(\omega_0 t + \zeta_3) \right] / J_1(t)$$

$$C_{e_2}^{J_1}(t) = \left\{ J_0 C_{e_2}^{J_0} + \frac{S_0 a k_1 k_2 k_{-1} e_1^2 e_2}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2} \sin(\omega_0 t - \zeta_4) \right\} / J_1(t)$$

$$C_{e_1}^{J_2}(t) = \left\{ J_0 C_{e_1}^{J_0} + \frac{S_0 a k_1 k_2 e_1 e_2}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2} [(k_2e_2)^2 + {\omega_0}^2]^{1/2} \times \sin(\omega_0 t + \zeta_1) \right\} / J_2(t)$$

$$C_{e_2}^{J_2}(t) = \left\{ J_0 C_{e_2}^{J_0} + \frac{S_0 a k_1 k_2 e_1 e_2}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2} [(k_{-1}e_1)^2 + {\omega_0}^2]^{1/2} \times \sin(\omega_0 t + \zeta_2) \right\} / J_2(t)$$

Here, $C_{e_1}^{J_0}$ and $C_{e_2}^{J_0}$ are steady-state flux control coefficients.

$$C_{e_1}^{J_0} = \frac{k_2e_2}{k_{-1}e_1 + k_2e_2} \qquad C_{e_2}^{J_0} = \frac{k_{-1}e_1}{k_{-1}e_1 + k_2e_2}$$

and ζ_i, $i = 1, 2, 3, 4$, are the initial phases of oscillations of periodic control coefficients:

$$\zeta_1 = \arcsin \frac{\omega_0[(k_{-1}e_1)^2 - (k_2e_2)^2 - {\omega_0}^2]}{\{[k_2e_2(k_{-1}e_1 + k_2e_2) + {\omega_0}^2]^2 + (k_{-1}e_1\omega_0)^2\}^{1/2}} \times [(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2]^{-1/2}$$

$$\zeta_2 = \arcsin \frac{\omega_0[(k_2e_2)^2 - (k_{-1}e_1)^2 - {\omega_0}^2]}{\{[k_{-1}e_1(k_{-1}e_1 + k_2e_2) + {\omega_0}^2]^2 + (k_2e_2\omega_0)^2\}^{1/2}} \times [(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2]^{-1/2}$$

$$\zeta_3 = \arcsin \frac{2k_{-1}e_1\omega_0[k_2e_2(k_{-1}e_1 + k_2e_2) + {\omega_0}^2]}{[(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2][(k_2e_2) + {\omega_0}^2]}$$

$$\zeta_4 = \arcsin \frac{2\omega_0(k_{-1}e_1 + k_2e_2)}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2}$$

Differentiating eqs C9 and C10 with respect to ln e_1, ln e_2, and ω_0, one obtains the control coefficients over the amplitudes of oscillations of fluxes, A_1 and A_2, with respect to the enzyme concentrations, e_1 and e_2, and the frequency, ω_0, of a periodic force,

$$C_{e_1}^{A_1} = \frac{k_2e_2(k_{-1}e_1 + k_2e_2) + {\omega_0}^2}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2}$$

$$C_{e_2}^{A_1} = \frac{k_2k_{-1}e_1e_2[k_2e_2(k_{-1}e_1 + k_2e_2) - {\omega_0}^2]}{[(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2][(k_2e_2)^2 + {\omega_0}^2]}$$

$$C_{\omega_0}^{A_1} = \frac{\omega_0^2 k_{-1}e_1(k_{-1}e_1 + 2k_2e_2)}{[(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2]^2}$$

$$C_{e_1}^{A_2} = \frac{k_2e_2(k_{-1}e_1 + k_2e_2) + {\omega_0}^2}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2}$$

$$C_{e_2}^{A_2} = \frac{k_{-1}e_1(k_{-1}e_1 + k_2e_2) + {\omega_0}^2}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2}$$

$$C_{\omega_0}^{A_2} = -\frac{{\omega_0}^2}{(k_{-1}e_1 + k_2e_2)^2 + {\omega_0}^2}$$

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