Control analysis applied to single enzymes: can an isolated enzyme have a unique rate-limiting step?

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Control analysis is used to analyse and quantify the concept of a rate-limiting step within an enzyme. The extent to which each rate constant within the enzyme limits the steady-state rate of the enzyme and the levels of enzyme intermediate species are quantified as flux and concentration control coefficients. These coefficients are additive and obey summation theorems. The control coefficients of triose phosphate isomerase, carbamate kinase and lactate dehydrogenase are calculated from literature values of the rate constants. It is shown that, contrary to previous assumption, these enzymes do not have a unique rate-limiting step, but rather flux control is shared by several rate constants and varies with substrate, product and effector concentrations, and with the direction of the reaction. Thus the general assumption that an enzyme will have a unique rate-limiting step is unjustified.

INTRODUCTION

Enzyme-catalysed reactions are thought to consist of several component steps or subreactions, characterized by internal rate constants. These different component steps may limit the rate of the overall reaction to different extents. Often the step with the lowest rate constant is considered to be rate-limiting or rate-determining for the overall reaction. However, this qualitative assessment of the dependence of reaction rates on component steps may be misleading and unsuitable where quantitative results and conclusions are required, for example, when relating changes in the rate constant of an individual step (changed by inhibitors, activators, pH, temperature or genetic engineering) to changes in enzyme rate (or vice versa). An analysis relating the kinetic constants of component steps to the net flux of an enzyme is outlined here, using the concepts of metabolic control analysis, which was initiated by Kacser and Burns [1] and Heinrich and Rapoport [2] (see refs. [3] and [4] for more recent developments). This analysis is used to quantify control in three enzymes with known rate constants and to assess whether the concept of a rate-limiting step within an enzyme is valid. Ray [5] has previously proposed a related analysis of rate limitation in enzymes, not derived from control analysis.

DEFINITIONS AND THEOREMS

We will define our system to be an isolated enzyme or transporter (E) with the rate constants (k_1, k_2, ..., k_n) as system parameters, and the steady-state net rate (v), concentration of enzyme intermediates (E_j, for example ES or EP) and kinetic parameters ($V_{max.}$ or K_m) as system variables (V). The rate constants of substrate and product association are second order, and all other rate constants are first order. The substrate [S], product [P] and effector [Ef] concentrations and the total enzyme concentration [E] will be treated as constant parameters, as will conditions such as temperature, pH or electric field. The dependence of the steady-state net values of the system variables on the kinetic constant (k_i) of a component step (i) of the enzyme can be defined as:

$$C^V_{k_i} = \left(\frac{\partial V}{\partial k_i} \times \frac{k_i}{V}\right)_{[S],[P],[Ef],[E],k_j,k_k,\text{etc.}} \quad (1)$$

where C is known as the control coefficient of K_i. For example when V is the net flux of the enzyme (v) we have:

$$C^v_{k_i} = \left(\frac{\partial v}{\partial k_i} \times \frac{k_i}{v}\right)_{[S],[P],[Ef],[E],k_j,k_k,\text{etc.}} \quad (2)$$

where C is called the flux control coefficient of rate constant i over the net rate v. This is equal to the percentage change in enzyme rate divided by the percentage change in the kinetic constant (extrapolated to an infinitesimally small change in rate constant) when no other rate constant is changed and substrate [S], product [P] and other effector [Ef] concentrations are held constant. This control coefficient is a simple and quantitive measure of the extent to which the rate constant of a step limits the overall enzyme rate. The coefficient refers to steady states only and to one state only (of substrates, products and effector levels). If the coefficient is 1, changes in the rate constant cause proportionate changes in the overall rate, and the step could be regarded as rate limiting, but it is not necessarily uniquely rate limiting. If the coefficient is zero, changes in the rate constant cause no change in the overall rate. If the coefficient is negative, increases in the rate constant cause decreases in the overall rate (all the forward rate constants have positive flux control coefficients and all the backward rate constants have negative coefficients). The coefficient normally lies between -1 and $+1$ (see below), but, in enzymes close to equilibrium or enzymes with co-operative kinetics, the rate constants may have coefficients

Abbreviations used: V, system variable; v, steady-state net rate of enzyme; C_k, control coefficient of rate constant k; ϵ_X, elasticity coefficient to X; R_X, response coefficient to X; [X], concentration of particular enzyme effector; [S], concentration of enzyme substrate; [P], concentration of enzyme product; [Ef], concentration of enzyme effector; [E_j], concentration of enzyme intermediate E_j; [E], total concentration of enzyme; [G3P], concentration of D-glyceraldehyde 3-phosphate; [DHAP], concentration of dihydroxyacetone phosphate.

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outside this range. Thus, when assessing the relative control by different rate constants, it is the relative values of control coefficients that matter, rather than their absolute values.

The control coefficients are additive and obey summation theorems (this is obvious because the rate v is a homogeneous function of degree 1 of the kinetic constants [6]):

$$\sum_{i=1}^{n} C^{v}_{k_i} = 1 \tag{3}$$

$$\sum_{i=1}^{n} C^{E_j}_{k_i} = 0 \tag{4}$$

where n is the total number of rate constants. Eqn. (3) is the summation theorem for flux control coefficients. Eqn. (4) is the summation theorem for enzyme intermediate concentration control coefficients where $[E_j]$ is the steady-state concentration of some intermediate such as ES or EP. From these theorems, it is easily derived that the sum of all control coefficients over $V_{max.}$ is equal to 1, the sum of all control coefficients over K_m is equal to zero, and the sum of all control coefficients over $V_{max.}/K_m$ is equal to 1. $V_{max.}/K_m$ is a pseudo-first-order rate constant for the enzyme at low substrate concentrations, and is a measure of the enzyme's substrate specificity [7]. It can also be shown that:

$$C^{V_{max.}/K_m}_{k_i} = C^{V_{max.}}_{k_i} - C^{K_m}_{k_i} \tag{5}$$

We may also define an elasticity coefficient which quantifies the sensitivity of a rate constant to some external effector or influence (X) (e.g. activator, pH, temperature, ionic strength, electric field), thus:

$$\epsilon^{k_i}_{X} = \left(\frac{\partial k_i}{\partial [X]} \times \frac{[X]}{k_i}\right)_{[S],[P],[Ef]} \tag{6}$$

This is just the percentage change in k_i divided by the percentage change in [X] causing it, when the change in [X] is very small and no other parameters changes.

We may also define a response coefficient which quantifies the sensitivity of the steady-state rate of the enzyme to some external effector or influence (X), thus:

$$R^{v}_{X} = \left(\frac{\partial v}{\partial [X]} \times \frac{[X]}{v}\right)_{[S],[P],[Ef]} \tag{7}$$

Now, it follows that:

$$R^{v}_{X} = \sum_{i=1}^{n} \epsilon^{k_i}_{X} \times C^{v}_{k_i} \tag{8}$$

Thus the response of an enzyme to some external effector is dependent on both the sensitivity of the individual rate constants to the effector and the control coefficients of these rate constants over the overall rate. Therefore a change in the distribution of control coefficients in an enzyme will usually cause a change in response to external effectors.

Now for an enzyme it is not possible to change a single rate constant alone. The equilibrium constant (K) of the reaction is a function of the rate constants, give by the Haldane relation:

$$K = \frac{\text{Product of forward rate constants}}{\text{Product of backward rate constants}} \tag{9}$$

Since the equilibrium of a reaction cannot be changed by changing enzyme rate constants, these rate constants can only be changed under the constraint that K remains constant (and if there are cycles in the mechanism there are further constraints). Thus in practice a single rate constant cannot be changed, and the control coefficient defined in eqn. (1) is not realizable. However, the control coefficient of two rate constants changed in proportion is equal to the sum of the individual control coefficients. The individual control coefficients may therefore be extracted experimentally by multiple manipulations of the enzyme where two or more rate constants are changed in proportion. The individual control coefficients of the substrate and product association rate constants can be measured directly simply by measuring the sensitivity of rate to substrate and product concentration (see below). The sum of any undetermined control coefficients is then given by the summation theorem. However, in practice it would be very difficult to determine a significant number of control coefficients experimentally unless the enzyme (and the effect of manipulations of the enzyme) were well characterized. On the other hand, if the enzyme's mechanism and rate constants are known, the individual control coefficients can be directly calculated by differentiating the rate equation with respect to the rate constants (see below).

The control coefficients of individual rate constants cannot be used in the same way as the control coefficients of whole enzymes in metabolic pathways. This is because individual rate constants cannot be changed; thus the control coefficients of individual rate constants are not predictive. Two or more rate constants can be changed in proportion, and these grouped control coefficients (given by the sum of the individual control coefficients) are predictive. Thus in order to predict the effect of changing in proportion any one particular forward rate constant and any other particular backward rate constant, the control coefficients of the rate constants involved should be added. This sum gives the value of the grouped control coefficient for these rate constants, and when multiplied by any (small) fractional change in rate constants gives the predicted change in rate. To predict the effect of changing any two particular forward rate constants or any two particular backward rate constants, the control coefficient of the rate constant that is decreased in value must be substracted from that which is increased in value.

The control coefficients could be defined differently in order to incorporate the Haldane relation. For example, in defining the control coefficient of k_i we could stipulate that when the rate constant is varied some other particular rate constant (e.g. k_j) is also varied in such a way that K is kept constant (see ref. [5]). However, such a definition would have the major drawback that the control coefficient of k_i so defined would always incorporate the control exerted by k_j. In fact, the control coefficient by this new definition (C_i') would be equal to the control coefficient of k_i plus that of k_j {by the simple definition [eqn. (1)]} where k_i and k_j are rate constants in opposite directions (but where k_i and k_j are in the same direction, then $C_i' = C_i - C_j$). Such a definition is restrictive because it assumes which particular sets of rate constants will be involved in any change to the enzyme. By defining the control coefficient for individual rate constants (as we have done), the user of the information may choose to combine the control coefficients as desired, always remembering that the control coefficients are not predictive on their own, but must be combined in such a way that the Haldane relation is obeyed (see above).

DEPENDENCE OF CONTROL COEFFICIENTS ON RATE CONSTANTS AND SUBSTRATE CONCENTRATIONS

We can calculate the control coefficients of any enzyme with known kinetic mechanism and constants, either by differentiating the rate equation with respect to the rate constants to obtain an

$$S+E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E+P$$

Scheme 1 Isomerization reaction

analytical solution or by using a computer to give a numerical estimate of the differential. As an example, we give the analytical solutions for the isomerization reaction shown in Scheme 1. The control coefficients over the initial rate (when [P] = 0), $V_{max.}$, K_m and $V_{max.}/K_m$ are given in terms of the rate constants in Table 1 for the above reaction scheme. The control coefficients over the initial rate in the opposite direction (i.e. when [P] is finite and [S] = 0) are given by substituting k_6 for k_1, k_5 for k_2, k_4 for k_3, k_3 for k_4, k_2 for k_5 and k_1 for k_6 wherever they appear in Table 1 (including the first 'step' column), and replacing [P] for [S]. It is also possible to obtain analytical equations for the control coefficients when both [S] and [P] are finite, by differentiating the reversible rate equation for Scheme 1 (equations not shown).

A number of general conclusions can be drawn from the analytical solutions for the control coefficients. The control coefficients are not constant but depend on the substrate (and product) concentration, except at very high and very low substrate concentrations (when the control efficients over initial rate are equal to the control coefficients over $V_{max.}$ and $V_{max.}/K_m$ respectively).

When [P] = 0, the control coefficient of substrate association (k_1) over the initial rate is always 1 at very low substrate concentrations [S], 0 at very high [S], and 0.5 when [S] = K_m. In fact, the control coefficient of k_1 is always equal to the response coefficient of the enzyme flux to the substrate concentration (this is a response coefficient according to the above terminology, but is an elasticity coefficient in the normal terminology of metabolic control analysis). This is because a percentage change in substrate concentration always has an equivalent effect on the flux as the same percentage change in k_1 (the substrate association constant), unless the substrate affects some other rate constant. Similarly when the product concentration is zero, the control coefficient of the (second-order) rate constant of product association (C^{k_6} for the enzyme of Scheme 1) is zero, and when the product is present the control coefficient is equal to the response coefficient to product.

The control coefficients of rate constants depend on the relative values of the rate constants (see Table 1 for control over initial rate). However, the fact that one rate constant is very small relative to the others does not make that rate constant uniquely rate limiting. For example, if k_1 is very small relative to the other rate constants, then the control coefficient of k_1 tends towards 1, but the control coefficients of the other rate constants do not approach zero but remain finite, the actual values depending on the relative rate constants. If k_1 is very large compared with the other rate constants, then its control coefficient falls to zero. If k_2 or k_4 are small relative to the others, than their respective control coefficients approach 0. If k_2 or k_4 are large relative to the others, than their control coefficients approach -1 (and the coefficient of k_3 approaches 1 at low substrate concentrations). If k_3 or k_5 are small relative to the others, their respective control coefficients approach 1, but the other control coefficients remain finite (the control coefficient of k_2 tends to -1 at low substrate concentrations). If k_3 or k_5 are large relative to the others, then their control coefficients approach zero.

When [S] is very low and [P] is zero, the control coefficient (and thus control coefficients over $V_{max.}/K_m$) of k_2 is equal and opposite to that of k_3, and that of k_4 equal and opposite to that of k_5.

When rate constants are changed, the control coefficients of

Table 1 Control coefficients expressed in terms of the rate constants for enzyme in Scheme 1 in the forward direction with zero product concentration

Step	C_k over initial rate	C_k over $V_{max.}$	C_k over K_m	C_k over $V_{max.}/K_m$
k_1	$\frac{k_2k_4+k_2k_5+k_3k_5}{(k_2k_4+k_2k_5+k_3k_5)+k_1(k_3+k_4+k_5)[S]}$	0	-1	1
k_2	$\frac{-k_2(k_4+k_5)}{(k_2k_4+k_2k_5+k_3k_5)+k_1(k_3+k_4+k_5)[S]}$	0	$\frac{k_2(k_4+k_5)}{k_2k_4+k_2k_5+k_3k_5}$	$\frac{-k_2(k_4+k_5)}{k_2k_4+k_2k_5+k_3k_5}$
k_3	$\frac{(k_4+k_5)(k_2+k_1[S])}{(k_2k_4+k_2k_5+k_3k_5)+k_1(k_3+k_4+k_5)[S]}$	$\frac{k_4+k_5}{k_3+k_4+k_5}$	$\frac{k_3(k_4+k_5)(k_5-k_2)}{(k_2k_4+k_2k_5+k_3k_5)(k_3+k_4+k_5)}$	$\frac{k_2(k_4+k_5)}{k_2k_4+k_2k_5+k_3k_5}$
k_4	$\frac{-k_4(k_2+k_1[S])}{(k_2k_4+k_2k_5+k_3k_5)+k_1(k_3+k_4+k_5)[S]}$	$\frac{-k_4}{k_3+k_4+k_5}$	$\frac{k_3k_4(k_2-k_5)}{(k_2k_4+k_2k_5+k_3k_5)(k_3+k_4+k_5)}$	$\frac{-k_2k_4}{k_2k_4+k_2k_5+k_3k_5}$
k_5	$\frac{k_2k_4+k_1(k_3+k_4)[S]}{(k_2k_4+k_2k_5+k_3k_5)+k_1(k_3+k_4+k_5)[S]}$	$\frac{k_3+k_4}{k_3+k_4+k_5}$	$\frac{k_3k_5(k_2+k_3+k_4)}{(k_2k_4+k_2k_5+k_3k_5)(k_3+k_4+k_5)}$	$\frac{k_2k_4}{k_2k_4+k_2k_5+k_3k_5}$
k_6	0	0	0	0

Rate equations:

$$V=\frac{k_1k_3k_5[S][E]}{(k_2k_4+k_2k_5+k_3k_5)+k_1(k_3+k_4+k_5)[S]} \qquad V_{max.}=\frac{k_3k_5[E]}{k_3+k_4+k_5} \qquad K_m=\frac{k_2k_4+k_2k_5+k_3k_5}{k_1(k_3+k_4+k_5)} \qquad \frac{V_{max.}}{K_m}=\frac{k_1k_3k_5[E]}{k_2k_4+k_2k_5+k_3k_5}$$

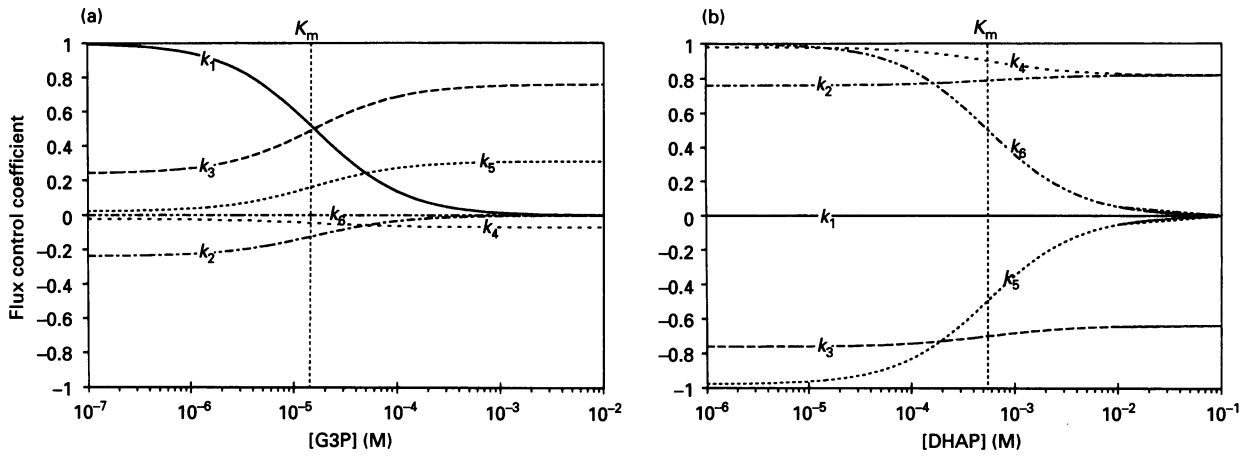

Figure 1 Control coefficients of rate constants over the initial rates for the forward and reverse reactions of triose phosphate isomerase

Control coefficients were calculated as for Scheme 1 with the rate constants of triose phosphate isomerase (see Table 2). The rate constants were named for the reaction occurring in the direction G3P → DHAP, i.e. k_1 is the association rate constant for G3P. The control coefficients were calculated analytically using Table 1 for (**a**) and the equivalent Table with the rate constants transposed for (**b**), i.e. k_1 becomes k_6, k_2 becomes k_5, k_3 becomes k_4, k_4 becomes k_3, k_5 becomes k_2 and k_6 becomes k_1. The same results were obtained when numerical differentiation was used (fractionally increasing the rate constant and calculating the fractional change in velocity, reducing the step size until it had no significant effect on the value for the control coefficient). In (**a**) [DHAP] was set to zero and [G3P] was varied. In (**b**) [GP3] was set to zero and [DHAP] was varied. The vertical line shows the K_m for G3P (**a**) and DHAP (**b**).

not only those rate constants but also the control coefficients of other rate constants are changed. This has the implication that, when rate constants are experimentally titrated (by pH, temperature or inhibitors), the extent of rate limitation by different steps changes continuously.

For reversible reactions with [S] and [P] finite, as the reaction approaches equilibrium (i.e. [P]/[S] approaches the equilibrium constant, K) the control coefficients of individual forward and reverse rate constants approach positive and negative infinity. This is a consequence of the Haldane relation, as the variation of the rate constants affects the equilibrium constant. However, the grouped control coefficients of the forward and reverse rate constants for a step remain less than 1 as the reaction approaches equilibrium (see Figure 2).

APPLICATION OF CONTROL ANALYSIS TO REAL ENZYMES

We have calculated the control coefficients for three real enzymes from literature values of the rate constants. The coefficients were calculated either from the analytical solutions generated by differentiating the rate equations or by using a computer to give a numerical estimate of the differential. Which method was used is given in the legends to the Figures and Tables. Where both methods were used, they gave identical results.

Triose phosphate isomerase catalyses the isomerization of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P), and the estimated rate constants are given in refs. [8] and [9]. To a first approximation [11], this reaction can be studied using Scheme 1. In order to calculate the control coefficients over the initial rate, two methods were used. Either the equations of Table 1 were used with the rate constants of refs. [8] and [9] substituted in, or the fractional change in rate for a fractional change in a given rate constant was calculated (using the rate equation of Table 1 and the rate constants of refs. [8] and [9]), the control coefficient being estimated as the ratio of these fractions when the change in rate constant tended to zero. These two methods gave identical values for the coefficients. Figure 1(a) shows the variation with substrate concentration of the calculated flux control coefficients for the forward reaction (G3P → DHAP) when [DHAP] = 0. Figure 1(b) shows the variation of flux control coefficient for the reverse reaction when [DHAP] is varied and [G3P] = 0 (the analytical solutions were analogous with those in Table 1, see above). In both directions, control can be seen to be shared between different rate constants

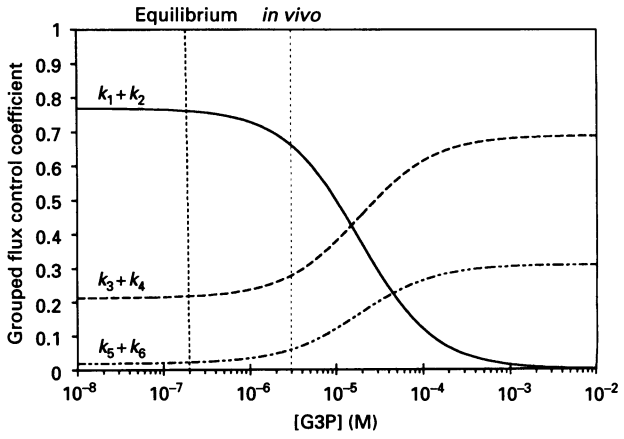

Figure 2 Flux control coefficients of rate constants for triose phosphate isomerase in the presence of non-zero substrate and product concentrations

[DHAP] was set to the *in vivo* level of 95 μM and [G3P] varied. Flux control coefficients were calculated for the grouped kinetic constants: k_1+k_2, k_3+k_4 and k_5+k_6. The equation for the steady-state mechanism of Scheme 1 was used in the reversible form to find the effect of rate constants on velocity with non-zero substrate and product concentrations. The control coefficients were calculated either numerically by calculating the increase in flux for a small increase in the rate constants or analytically by differentiating the rate equation with respect to the relevant rate constant. Both methods gave identical traces. Vertical lines represent the amount of G3P present at equilibrium and that present *in vivo*. The *in vivo* triose phosphate concentrations (in muscle) used were those of the reactive free carbonyl forms calculated from ref. [10] by correcting for the equilibrium constants of the dehydration equilibria given in ref. [8].

Table 2 Parameter control coefficients for triose phosphate isomerase

Kinetic Scheme 1. Rate constants are taken from ref. [11]. $C^{V_{max.}}$, control coefficient of rate constant over $V_{max.}$; C^{K_m} control coefficient of rate constant over K_m; $C^{V_{max.}/K_m}$, control coefficient of rate constant over $V_{max.}/K_m$. f (forward) and r (reverse) subscripts represent values calculated from the steady-state equations for the initial rates of the forward (G3P → DHAP) and reverse (DHAP → G3P) reactions, i.e. when [DHAP] = 0 and [G3P] = 0 respectively. The control coefficients were calculated analytically using Table 1 for the forward reaction and the equivalent Table with the rate constants transposed for the reverse reaction, i.e. k_1 becomes k_6, k_2 becomes k_5, k_3 becomes k_4, k_4 becomes k_3, k_5 becomes k_2 and k_6 becomes k_1. The same results were obtained if numerical differentiation was used (fractionally increasing the rate constant and calculating the fractional change in the parameter, reducing the step size until it had no significant effect). Calculated kinetic constants: $V_{max._f} = 4827\ s^{-1}$; $K_{m(G3P)} = 16\ \mu M$; $V_{max._f}/K_{m(G3P)} = 3.0 \times 10^8\ M^{-1} \cdot s^{-1}$; $V_{max._r} = 364\ s^{-1}$; $K_{m(DHAP)} = 560\ \mu M$; $V_{max._r}/K_{m(DHAP)} = 6.5 \times 10^5\ M^{-1} \cdot s^{-1}$.

Step	Rate constant	$C^{V_{max._f}}$	$C^{K_{m(G3P)}}$	$C^{V_{max._f}/K_{m(G3P)}}$	$C^{V_{max._r}}$	$C^{K_{m(DHAP)}}$	$C^{V_{max._r}/K_{m(DHAP)}}$
k_1	$4 \times 10^8\ M^{-1} \cdot s^{-1}$	0.00	−1.00	1.00	0.00	0.00	0.00
k_2	$2000\ s^{-1}$	0.00	0.24	−0.24	0.82	0.057	0.761
k_3	$7000\ s^{-1}$	0.789	0.52	0.24	−0.64	0.125	−0.761
k_4	$2000\ s^{-1}$	−0.069	−0.047	−0.022	0.82	−0.16	0.978
k_5	$20000\ s^{-1}$	0.310	0.289	0.022	0.00	0.978	−0.978
k_6	$3 \times 10^7\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	0.00	−1.00	1.00

throughout the range of [S], i.e. there is no single rate-limiting step in this enzyme.

Table 2 shows the rate constants used for these plots and the control coefficients over $V_{max.}$, K_m and $V_{max.}/K_m$ for the different rate constants. The shared control is not unique to the simplified version of the reaction mechanism [11], as similar results are seen when the more complex mechanism analysed by Albery and Knowles [8] is used. In this case, control by the internal steps over $V_{max.}/K_m$ is 0.36.

Control coefficients were also calculated for conditions in which both substrate and product were present, using a reversible kinetic equation for Scheme 1. The partial differentials of the kinetic equation were solved analytically (not shown), and also estimated numerically (by computer); both methods gave the same values for the coefficients. The calculated control coefficients of the individual rate constants approached plus or minus infinity when the reaction approached equilibrium (see above), thus we prefer to present the results as grouped control coefficients for the forward and backward rate constants of each step. The grouped control coefficients were calculated by summing the individual control coefficients.

Figure 2 shows the grouped control coefficients of k_1+k_2, k_3+k_4 and k_5+k_6 close to *in vivo* concentrations of substrate and product (measured in muscle [10]). [DHAP] was fixed at 95 μM and [G3P] was varied around equilibrium. Although the majority of the control is in the association/dissociation step for G3P, as suggested by Albery and Knowles [9], significant control still lies in the internal steps. The pairing of the control coefficients to give grouped control coefficients is somewhat arbitrary; other groups of coefficients could have been chosen for presentation, although the pairing chosen here gives the control coefficient of individual enzyme steps.

Triose phosphate isomerase is thought to be 'diffusion-controlled', although the concept of diffusion control is somewhat ambiguous. Generally for any enzyme, $k_{cat.}/K_m$ may have a maximal value equal to approximately $10^9\ M^{-1} \cdot s^{-1}$ [7]. This is because the substrate association rate constant has an upper limit set by diffusion equal to approximately $10^9\ M^{-1} \cdot s^{-1}$ [7]. An enzyme-catalysed reaction cannot go faster than the rate at which enzyme and substrate collide, and this collision rate has a physical upper limit. An enzyme with a $k_{cat.}/K_m$ close to the maximum is said to be diffusion-controlled, and must be limited by substrate association alone (i.e. the flux control coefficient of k_1 is 1 and all others are zero). However, if such an enzyme is partially or wholly saturated with substrate, control must change away from substrate association to other steps. So the term diffusion-controlled is only appropriate at low substrate concentrations.

Albery and Knowles [9] have suggested that triose phosphate isomerase is a catalytically 'perfect' enzyme and diffusion-controlled with respect to the reaction with G3P. Thus at substrate concentrations below K_m they state that the internal steps must have little or no effect on flux. This is clearly not the case (see Figure 1a, 1b and Table 2). Despite varying the rate constants within the error values given by Albery and Knowles [8], we found that the control by internal steps remained significant (not shown). Thus possession of a $V_{max.}/K_m$ of between 10^8 and $10^9\ M^{-1} \cdot s^{-1}$ is not sufficient to make an enzyme solely diffusion-controlled. Could triose phosphate isomerase further evolve to become diffusion-controlled? We found that increasing k_3 (and k_2 in proportion to maintain the overall equilibrium constant) had very little effect on the control distribution, in agreement with the calculations of Ellington and Benner [12] on the control of turnover by internal equilibrium constants. However, we found that either increasing k_3 or decreasing k_2 by a factor of 10 (while keeping the equilibrium constant the same by varying k_4 and k_5 respectively) was sufficient to produce a diffusion-controlled enzyme, with virtually all control over $V_{max.}/K_m$ located in k_1 (not shown). Thus, the enzyme could further evolve to become diffusion-controlled. Note, however, that Pettersson [13] and Pettersson and Pettersson [14] have pointed out that diffusion-controlled enzymes are not necessarily 'catalytically perfect', if we take into account the fact that enzyme activities *in vivo* may change substrate and product concentrations.

Albery and Knowles [8,9] also state that, for a diffusion-controlled enzyme, the rate of the reverse reaction must be limited by the separation of product from enzyme. Although there is control in this step, there is also significant control in other steps (Table 2).

We may also analyse control in multisubstrate enzymes, for example carbamate kinase, the rate constants of which have been estimated [11,15] using the minimal mechanism in Scheme 2,

$$E+A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA+B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} [EAB \rightleftharpoons EPQ] \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EP+Q \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E+P$$

Scheme 2 Carbamate kinase reaction

Table 3 Parameter control coefficients for carbamate kinase

$C^{parameter}$, control coefficient over parameter. The effect of changing the rate constants on the initial velocity was calculated from the steady-state rate equation for Scheme 2 using the rate constants from ref. [11]. The control coefficients were calculated using numerical differentiation of the equations defining the enzyme parameter ($V_{max.}$, K_m etc.). The forward coefficients (f) for the reaction MgATP + carbamate → MgADP + carbamoyl phosphate are therefore calculated in the absence of MgADP and carbamoyl phosphate and the reverse coefficients (r) for the reaction MgADP + carbamoyl phosphate → MgATP + carbamate are calculated in the absence of MgATP and carbamate. Calculated kinetic constants: $V_{max.f} = 91.7\ s^{-1}$; $K_{MgATP} = 8.3\ \mu M$; $K_{carbamate} = 76\ \mu M$; $K_{MgATP.carbamate} = 9\ nM$; $V_{max.r} = 736.7\ s^{-1}$; $K_{MgADP} = 53\ \mu M$; $K_{carbamoylP} = 103\ \mu M$; $K_{MgADP.carbamoylP} = 0.8\ nM$.

Step	Rate constant	$C^{V_{max.f}}$	$C^{K_{MgATP}}$	$C^{V_{max.f}/K_{MgATP}}$	$C^{K_{carbamate}}$	$C^{V_{max.f}/K_{carbamate}}$	$C^{K_{MgATP.carbamate}}$
k_1	$1.1 \times 10^7\ M^{-1} \cdot s^{-1}$	0.00	−1.00	1.00	0.00	0.00	−1.00
k_2	$1300\ s^{-1}$	0.00	0.00	0.00	0.00	0.00	1.00
k_3	$4.2 \times 10^6\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	−1.00	1.00	−1.00
k_4	$1700\ s^{-1}$	0.00	0.00	0.00	0.715	−0.715	0.715
k_5	$680\ s^{-1}$	0.135	0.135	0.00	−0.58	0.715	−0.58
k_6	$1.0 \times 10^7\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	0.00	0.00	0.00
k_7	$106\ s^{-1}$	0.865	0.865	0.00	0.865	0.00	0.865
k_8	$1.4 \times 10^7\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	0.00	0.00	0.00

Step	Rate constant	$C^{V_{max.r}}$	$C^{K_{MgADP}}$	$C^{V_{max.r}/K_{MgADP}}$	$C^{K_{carbamoylP}}$	$C^{V_{max.r}/K_{carbamoylP}}$	$C^{K_{MgADP.carbamoylP}}$
k_1	$1.1 \times 10^7\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	0.00	0.00	0.00
k_2	$1300\ s^{-1}$	0.57	0.57	0.00	0.57	0.00	0.57
k_3	$4.2 \times 10^6\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	0.00	0.00	0.00
k_4	$1700\ s^{-1}$	0.43	0.43	0.00	0.15	0.29	0.15
k_5	$680\ s^{-1}$	0.00	0.00	0.00	0.29	−0.29	0.29
k_6	$1.0 \times 10^7\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	−1.00	1.00	−1.00
k_7	$106\ s^{-1}$	0.00	0.00	0.00	0.00	0.00	1.00
k_8	$1.4 \times 10^7\ M^{-1}\ s^{-1}$	0.00	−1.00	1.00	0.00	0.00	−1.00

Table 4 Control coefficients for lactate dehydrogenase

Lactate + NAD^+ → Pyruvate + NADH

$C^{parameter}$, control coefficient of rate constant over the parameter. Control coefficients were calculated as in Table 3 using the rate equation for the ordered Bi Bi mechanism of kinetic Scheme 3 and the rate constants from [16]. The forward reaction was lactate + NAD^+ → pyruvate + NADH and the reverse reaction pyruvate + NADH → lactate + NAD^+. C^v *in vivo* was calculated using the full reversible rate equation for kinetic Scheme 3 and the *in vivo* muscle substrate and metabolite concentrations (from ref. [10]): [lactate] = 3700 μM, [NAD^+] = 541 μM, [NADH] = 50 μM, [pyruvate] = 380 μM. Numerical differentiation of the reversible rate equation with respect to the relevant rate constant was used to calculate the flux control coefficients.

Calculated kinetic constants: $V_{max.f} = 14.3\ s^{-1}$, $K_{NAD^+} = 1.6\ \mu M$, $K_{lactate} = 900\ \mu M$, $K_{NAD^+.lactate} = 53\ nM$; $V_{max.r} = 98.1\ s^{-1}$, $K_{NADH} = 2.7\ \mu M$, $K_{pyruvate} = 200\ \mu M$, $K_{NADH.pyruvate} = 0.09\ nM$.

Step	Rate constant	$C^{V_{max.f}}$	$C^{K_{NAD^+}}$	$C^{V_{max.f}/K_{NAD^+}}$	$C^{K_{Lactate}}$	$C^{V_{max.f}/K_{Lactate}}$	$C^{K_{NADH.pyruvate}}$
k_1	$8.74 \times 10^6\ M^{-1} \cdot s^{-1}$	0.00	−1.00	1.00	0.00	0.00	−1.00
k_2	$526\ s^{-1}$	0.00	0.00	0.00	0.00	0.00	1.00
k_3	$6.07 \times 10^4\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	−1.00	1.00	−1.00
k_4	$1200\ s^{-1}$	0.00	0.00	0.00	0.73	−0.73	0.73
k_5	$1002\ s^{-1}$	0.033	0.033	0.00	−0.70	0.73	−0.70
k_6	$246\ s^{-1}$	−0.018	−0.018	0.00	0.39	−0.41	0.39
k_7	$190\ s^{-1}$	0.094	0.094	0.00	−0.32	0.41	−0.32
k_8	$1.21 \times 10^6\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	0.00	0.00	0.00
k_9	$16\ s^{-1}$	0.892	0.892	0.00	0.89	0.00	0.89
k_{10}	$3.63 \times 10^7\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	0.00	0.00	0.00

Step	Rate constant	$C^{V_{max.r}}$ (reverse)	$C^{K_{NADH}}$	$C^{V_{max.r}/K_{NADH}}$	$C^{K_{pyruvate}}$	$C^{V_{max.r}/K_{pyruvate}}$	$C^{K_{NAD^+.Lactate}}$	C^v (*in vivo*)
k_1	$8.74 \times 10^6\ M^{-1} \cdot^{-1}$	0.00	0.00	0.00	0.00	0.00	0.00	−0.42
k_2	$526\ s^{-1}$	0.19	0.19	0.00	0.19	0.00	0.19	0.56
k_3	$6.07 \times 10^4\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	0.00	0.00	0.00	−0.32
k_4	$1200\ s^{-1}$	0.41	0.41	0.00	0.15	0.26	0.15	0.51
k_5	$1002\ s^{-1}$	−0.33	−0.33	0.00	−0.07	−0.26	−0.07	−0.44
k_6	$246\ s^{-1}$	0.73	0.73	0.00	0.14	0.59	0.15	0.63
k_7	$190\ s^{-1}$	0.00	0.00	0.00	0.59	−0.59	0.59	−0.21
k_8	$1.21 \times 10^6\ M^{-1} \cdot s^{-1}$	0.00	0.00	0.00	−1.00	1.00	−1.00	0.29
k_9	$16\ s^{-1}$	0.00	0.00	0.00	0.00	0.00	1.00	−0.05
k_{10}	$3.63 \times 10^7\ M^{-1} \cdot s^{-1}$	0.00	−1.00	1.00	0.00	0.00	−1.00	0.44

where A is MgATP, B is carbamate, P is MgADP and Q is carbamoyl phosphate. The initial rate of this reaction has been analysed using the following kinetic equation [11]:

$$v = \frac{V_{max.}[A][B]}{K_{AB} + K_A[B] + K_B[A] + [A][B]} \quad (10)$$

where $V_{max.}$ is the rate at saturating [A] and [B], K_A is the K_m of A at saturating [B], K_B is the K_M of B at saturating [A], and K_{AB} is a constant. Table 3 gives the control coefficients for carbamate kinase calculated using the rate constants in ref. [11] derived from Marshall and Cohen [15]. Flux control is shared between rate constants in all conditions except where the nucleotide concentration is very low and the other substrate is saturating. Marshall and Cohen [15] have asserted that 'the rate-limiting step in both directions is the dissociation of the nucleotide from the enzyme'. If we assume that these authors were referring to control over $V_{max.}$, then they were correct in that nucleotide association/dissociation partially limits the maximum rates, but they were wrong in that these rate constants are not uniquely rate-limiting.

We have also analysed control in lactate dehydrogenase, using the mechanism in Scheme 3, where A is NAD^+, B is lactate, P is

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EAB \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EPQ \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EP \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E$$

Scheme 3 Lactate dehydrogenase reaction

NADH and Q is pyruvate. Table 4 shows the control coefficients calculated using the rate constants estimated by Sudi [16]. Control is again spread among a number of rate constants, except in certain extreme conditions. The control coefficients for the reversible reaction are calculated using the substrate and product concentrations measured in muscle [10]. Again in these conditions control is widely spread.

DISCUSSION

The relative sizes of the control coefficients of the individual steps of an enzyme (i.e. the control distribution) are not constant but vary with the substrate, product and effector concentrations, the relative kinetic constants, the substrate type and direction of the enzyme, and conditions such as temperature, pH and ionic strength. There are few conditions in which all control is located in one step only. Therefore the concept of a rate-limiting or rate-determining step is of limited use. The term must be used with caution, the conditions strictly defined, and it should be made explicit whether the term implies that the step is uniquely rate-limiting.

Rate constants may be changed by effector metabolites and ions, pH, ionic strength, electric field, temperature, covalent modification or genetic engineering. The response of enzyme flux to a change in effector level depends on both the sensitivity of the rate constants to the effector (the elasticity coefficients to the effector) and the sensitivity of the enzyme flux to the rate constants (the flux control coefficients of the rate constants). Changing conditions will result in a change in control distribution and thus a change in response to effectors or conditions. Thus, for example, the response of a transporter to changes in the transmembrane electric field should depend on the concentration of metabolite or ion transported. pH titrations of the maximum rate of an enzyme have been used to assign pK values of essential ionization groups in enzymes, but such titrations can only give true equilibrium pK values (rather than 'kinetic' pK values) if the affected group remains fully rate-determining over the whole range of rates [17,18]. Genetic engineering of enzymes designed to change the rate or affinity of enzymes must take into account the control coefficients of the individual steps, and what these may be in the actual conditions designed for use, and how they may change as the relative rate constants are changed.

If a rate constant has a particular control coefficient over the flux of the isolated enzyme in a particular set of conditions, that rate constant has a different (lower) control coefficient over the flux when the enzyme is located within a metabolic pathway or cell. In fact, the control by the rate constant over the pathway flux is equal to the control by the rate constant over the isolated enzyme rate multiplied by the control by the whole enzyme over the pathway flux.

Several previous attempts have been made to quantify the 'importance' of component steps to the overall rate of an enzyme reaction. Free-energy profiles have been used to illustrate the relative stability of enzyme-bound intermediates and transition states (e.g. [8]). However, the use of these profiles has been criticized, and the alternative use of 'kinetic barrier' diagrams recommended instead [16,19]. In the diagrams of Burbaum et al. [19], the classical free-energy profiles are modified such that all the second-order rate constants are written as pseudo-first-order rate constants by including *in vivo* substrate concentrations. In Sudi [16], the diagrams are further modified to show the relative concentrations of enzyme-bound intermediates and the one-way fluxes through each component step, when the enzyme is at equilibrium. The kinetic barrier has been defined as the reciprocal of the one-way flux through each step when at equilibrium, and when this parameter is normalized (so that the sum for all steps adds up to 1) has been called the 'fractional resistance' [16]. This provides a useful measure of the kinetic barrier at each step, but unfortunately the diagram and fractional resistance only refer to the enzyme at equilibrium, and do not give a measure of the extent to which each component step controls the overall rate of the enzyme.

Northrop [20] considered the use of isotope effects on enzyme rates to detect rate-limiting steps. He pointed out that, even when a step has the lowest rate constant, it is not necessarily rate-limiting and concluded that the concept of a rate-limiting step was outmoded. In reaction to this, Ray [5] defended the concept of a rate-limiting step, and proposed a new definition, involving a parameter called the 'sensitivity index'. This index was defined for the forward steps only as the fractional change in reciprocal $V_{max.}$ (or $V_{max.}/K_m$) with a fractional change in the reciprocal rate constant (of the forward step) when the equilibrium constant involving that step is kept constant. This parameter is equivalent to a $V_{max.}$ control coefficient grouped for the forward and reverse kinetic constants of a step. However, this sensitivity index is not as simple and generally useful as the control coefficients defined here (see the Definitions and theorems section). Ray [5] shows how the sensitivity index is related to parameters quantifying an isotope effect on an enzyme, and this may be one approach to estimating control coefficients experimentally. However, Ray [5] was concerned to defend the concept of a single rate-limiting step, which we can now see to be misleading and generally inapplicable. Instead of searching for one rate-limiting step, we should accept that different steps will limit the overall rate to different extents, and these extents (quantified by the flux control coefficients) will vary in different conditions.

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