Rate limitation within a single enzyme is directly related to enzyme intermediate levels.

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

DOI
10.1016/0014-5793(94)00617-2

Publication date
1994

Published in
FEBS Letters

FEBS Letters 349 (1994) 131–134

FEBS 14231

Rate limitation within a single enzyme is directly related to enzyme intermediate levels

Boris N. Kholodenko[b,*], Hans V. Westerhoff[a,b], Guy C. Brown

[a]*E.C. Slater Institute, Biocentrum, University of Amsterdam, Plantage Muidergracht 12, Amsterdam, The Netherlands*
[b]*Division of Molecular Biology, Netherlands Cancer Institute, H5, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands*
[c]*Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK*

Received 30 May 1994

Abstract

The extents to which different rate constants limit the steady-state rate of an isolated enzyme can be quantified as the control coefficients of those constants and elemental steps. We have found that the sum of the control coefficients of rate constants characterising unidirectional rates depleting a particular enzyme intermediate is equal to the concentration of that enzyme intermediate as a fraction of the total enzyme concentration. Together with simple measurements this powerful relation may be used (i) to estimate certain enzyme intermediate levels, in particular the free enzyme concentration, and (ii) to estimate the control coefficients of rate constants and steps.

Key words: Rate limitation; Metabolic control analysis; Control coefficient; Enzyme kinetics

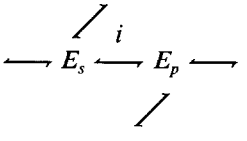
1. Introduction

The steady-state rate of an isolated enzyme or transporter may be limited by one or more rate constants or steps within the protein. The analysis of rate limitation within enzymes, transporters and other functional proteins is central to an understanding of their control, regulation and function. This analysis has been based on the paradigm that an enzyme will have a single rate-limiting step, and has used various ad hoc and non-quantitative rules for identifying such a step. However, it has been shown recently that many enzymes and transporters do not have a single rate-limiting step, but rather the rate is partially limited by several rate constants, and the distribution of control between rate constants and steps changes dramatically with conditions [1–3]. Thus it is necessary to have a quantitative definition of the extent of rate limitation by a rate constant or step, and some method of measuring this extent. A simple, quantitative definition of the extent of rate limitation has been proposed [3–5], and these extents have been called the control coefficients of the rate constants or steps. A number of relations between the kinetic parameters of an enzyme and the control coefficients have been derived [5,6]. However it still remains difficult to measure the control coefficients of steps and rate constants experimentally.

In this paper we show that there is a simple relation between the control coefficients of enzyme rate constants and the level of enzyme intermediates. This powerful relation allows the control coefficients to be estimated from measured enzyme intermediate levels or other simple enzyme manipulations. Alternatively the relation may be used to estimate the free enzyme concentration from simple steady-state rate measurements.

2. Theoretical background

An enzyme may have intermediate forms or states which we will designate as E_i. The same symbol will denote the concentration of i-th enzyme state, and e will designate the total concentration of the enzyme, i.e. the sum of all states, $e = E_1 + E_2 + \ldots + E_m$. Every elemental step ($i$) corresponds to the interconversion of a pair of the enzyme intermediates (E_s and E_p):



(Scheme 1)

with the net rate (v_i):

$$v_i = v_{+i} - v_{-i} = k_{+i} \cdot E_s - k_{-i} \cdot E_p \quad (1)$$

Here v_{+i}, k_{+i} and v_{-i}, k_{-i} are the unidirectional rates and rate constants in the forward and the reverse direction of the i-th step. When ligands are involved in a transition, k_{+i} (and similarly, k_{-i}) equals $k^*_{+i} \cdot L_j$, where k^*_{+i} is the 'true' elemental rate constant of the second order and L_j is the substrate, effector or product concentration which is taken to be constant.

*Corresponding author. Division of Molecular Biology, Netherlands Cancer Institute, H5, Plesmanlaan 121, 1066 CX Amsterdam.
Fax: (31) (20) 5122029.
Boris N. Kholodenko is on leave from the A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russia.

SSDI 0014-5793(94)00617-5

The extent to which a step (i) within an enzyme or transporter limits the steady-state enzyme rate (flux J) can be quantified by relating simultaneous 1% change in the forward and reverse rate constants of that step to the resulting percent change in the flux. A strict definition extrapolates the percent change to an infinitesimally small change:

$$C_i^J = \frac{k_{+i}}{J} \cdot \frac{\mathrm{d}J}{\mathrm{d}k_{+i}} \Big|_{k_{-i}/k_{+i} = \text{const.}} \quad (2)$$

C_i^J is referred to as the control coefficient of the step i over the flux J. For unbranched mechanisms the control coefficient of any step can assume any value between 0 and 1. For any mechanism the sum of the control coefficients over all the steps is equal to 1 [3,5–7].

If the forward and reverse rate constants are changed independently the corresponding control coefficients will quantify the extent to which each of the rate constants limits the flux:

$$C_{+i}^J = \frac{k_{+i}}{J} \cdot \frac{\mathrm{d}J}{\mathrm{d}k_{+i}}, \quad C_{-i}^J = \frac{k_{-i}}{J} \cdot \frac{\mathrm{d}J}{\mathrm{d}k_{-i}} \quad (3)$$

C_{+i}^J and C_{-i}^J are referred to as the control coefficient of the rate constants. When a step involves the binding of a ligand to the enzyme (e.g. the substrate) the control coefficient of the rate constant involving binding can be measured directly by changing the concentration of the ligand (see below). For unbranched mechanisms of a reaction, forward rate constants have positive control coefficients and reverse rate constants have negative coefficients (i.e. increasing these rate constants decrease the net rate of the enzyme). From Eqs. **(2)** and **(3)** it follows that the control coefficient of the step is equal to the sum of the control coefficients of forward and reverse rate constants:

$$C_i^J = C_{+i}^J + C_{-i}^J \quad (4)$$

3. The relation

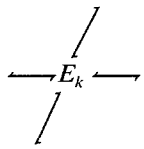
If E_k is not a branch point in the kinetic diagram of the mechanism of a reaction,

$$\xleftarrow{i} E_k \xrightarrow{j} \quad \text{(Scheme 2)}$$

it can be shown that [5] (see Appendix):

$$C_{-i}^J + C_{+j}^J = E_k/e \quad (5)$$

i.e. the sum of the control coefficients of unidirectional rates flowing away from (depleting) any intermediate is equal the concentration of that intermediate as a fraction of the total enzyme concentration (e). More generally when an intermediate is connected to more than two other intermediates [5],



(Scheme 3)

i.e. when E_k is at a branch point of the mechanism, its concentration is given by:

$$\sum_{\substack{\text{all unidirectional} \\ \text{rates flowing away from } E_k}} C_{\pm i}^J = E_k/e \quad (6)$$

where the sum is over the control coefficients of all the rate constants characterising unidirectional fluxes away from the intermediate E_k.

Eqs. **(5)** and **(6)** show that if enzyme intermediate levels can be measured then these measurements can be used to estimate control coefficients, or inversely if control coefficients can be measured independently then enzyme intermediate levels can be estimated. The following sections illustrate how this can be done.

4. Estimation of free enzyme concentration from control coefficients of substrate and product binding

Eq. **(5)** can be applied to the free enzyme (E_1) (when E_1 is not a branch point) to give the following equation:

$$E_1/e = C_{+1}^J + C_{-n}^J \quad (7)$$

C_{+1} is the control coefficient of the rate constant involving binding of the first substrate, and C_{-n} is the control coefficient of the rate constant involving binding of the last product. C_{+1} can be estimated in any condition (as long as substrates bind in a fixed order) by fractionally changing the substrate concentration (S) and measuring the change in the steady-state rate (J) of the enzyme. The control coefficient is given by:

$$C_{+1}^J = (\mathrm{d}J/J)/(\mathrm{d}S/S)_P \quad (8)$$

The subscript P specifies that the product concentration is kept constant. Similarly C_{-n} can be measured by following the dependence of enzyme rate on product concentration (P), as:

$$C_{-n}^J = (\mathrm{d}J/J)/(\mathrm{d}P/P)_S \quad (9)$$

Thus the free enzyme concentration can be estimated as:

$$E_1/e = ((\mathrm{d}J/J)/(\mathrm{d}S/S))_P + ((\mathrm{d}J/J)/(\mathrm{d}P/P))_S \quad (10)$$

This equation can also be applied to initial rate measurements, i.e. when product concentration (P) is zero. In this case the corresponding control coefficient (C_{-n}) is equal to zero.

Interestingly, the same results can be achieved more simply by diluting the suspension and measuring the response of the steady-state reaction rate (J) to volume (Vl) change (cf. [8]):

$$E_1/e = -(\mathrm{d}J/J)/(\mathrm{d}Vl/Vl) + \rho \qquad \textbf{(11)}$$

the factor ρ is equal to −1 or 0 depending on whether the flux is calculated per volume unit or per mg of protein, respectively.

The same logic can be used to estimate the steady state concentration of the second free intermediate (E^*) of a two substrate two product ping-pong mechanism (Scheme 4):

$$E \underset{k_{-1}}{\overset{k^*_{+1}S_1}{\rightleftharpoons}} ES_1 \underset{k^*_{-2}P_1}{\overset{k_{+2}}{\rightleftharpoons}} E^* \underset{k_{-3}}{\overset{k^*_{+3}S_2}{\rightleftharpoons}} ES_2 \underset{k_{-4}P_2}{\overset{k_{+4}}{\rightleftharpoons}} E \quad \text{(Scheme 4)}$$

Then,

$$E^*/e = C^J_{-2} + C^J_{+3}$$

C^J_{-2} and C^J_{+3} are estimated by measuring the dependence of enzyme rate on P_1 and S_2.

5. Estimation of control coefficients from enzyme intermediate concentrations

Eqs. **(5)** and **(6)** relate the control coefficients of the rate constants involved in depleting an enzyme intermediate to the concentration of that intermediate. If the fractional concentration of some intermediate (E_j) is measured to be α then it follows from Eq. **(5)** (for a non-branch point intermediate) that the control coefficient of the rate constant forward from E_j must be greater than α. This follows because the control coefficient of backward rate constants are necessarily negative. In unbranched mechanisms control coefficients of the steps can not exceed +1 [5]. Consequently, if E_j is measured to be virtually equal to 1 (i.e. almost all the enzyme is in the one form E_j) then C_{+j} must be equal to 1 and $C_{-(j-1)}$ must be equal to 0. Since the concentration of the next intermediate (E_{j+1}) must be virtually 0 and the ratio $-C_{-j}/C_{+j}$ is proportional to E_{j+1}/E_j it follows that C_{-j} must be 0 [6]. By the same logic the control coefficients of rate constants of all other steps must almost equal 0.

6. Illustration

We shall first illustrate the use of the method for the simplest two step mechanism of an enzyme (Scheme 5):

$$E \underset{k_{-1}}{\overset{k^*_{+1}S}{\rightleftharpoons}} ES \underset{k^*_{-2}P}{\overset{k_{+2}}{\rightleftharpoons}} E \quad \text{(Scheme 5)}$$

The control coefficients, C_{+1} and C_{-2}, can be estimated by measuring the dependence of enzyme rate on S and P and these control coefficients can be used to measure the concentration of the free enzyme at the operational steady state (see Eqs. **(8–10)**). Then the concentration of ES can be estimated. The ratio of the control coefficients of reverse and forward rate constants of any particular step is equal to minus the disequilibrium ratio of that step [5,6]:

$$C^J_{+1}/C^J_{-1} = -(E/ES)_{\text{steady state}}/(E/ES)_{\text{eq}} = -K_1(E/ES)_{\text{steady state}} \qquad \textbf{(12)}$$

where $K_1 = k^*_{+1}S/k_{-1}$ is the (apparent) equilibrium constant of step 1. K_1 can be estimated from C_{+1} and C_{-2} measured in the operational steady state, together with the knowledge of the free-energy difference ($\Delta G = \mu_S - \mu_P$) of the total reaction S to P:

$$K_1 = -(C^J_{+1} + C^J_{-2}\cdot\exp(\Delta G/RT))/(C^J_{+1} + C^J_{-2}) \qquad \textbf{(13)}$$

From Eqs. **(12)** and **(13)** we can estimate C_{-1} and, then C_{+2} (using that the sum of all the control coefficients is equal to 1). Importantly, knowledge of all the control coefficients together with the steady-state reaction rate allows us to estimate all the rate constants.

For a particular 3 step mechanism (Scheme 6):

$$E \underset{k_{-1}}{\overset{k^*_{+1}S}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EP \underset{k^*_{-3}P}{\overset{k_{+3}}{\rightleftharpoons}} E \quad \text{(Scheme 6)}$$

the ability to measure ES or EP would be sufficient to determine all the control coefficients. The concentration of the free enzyme can be estimated at the operational steady state by measuring C_{+1} and C_{-3}. Because there are only three intermediates, the ability to estimate E and measure one other intermediate (e.g. ES) fixes the concentration of the other intermediate (EP). Measuring the concentration of ES at the operational steady state and at equilibrium allows us to estimate the disequilibrium ratios over the elemental steps. Using these ratios we can estimate C_{-1} and C_{+3} and, then all other control coefficients.

Alternatively, one may use the data on the concentrations of enzyme intermediates and reaction rates measured at least at two different steady states to determine

the rate constants directly from Eq. 1. Then, all the control coefficients can also be determined.

7. Discussion

We have shown that there is a simple relationship between enzyme intermediate levels and the control coefficients of rate constants. We have illustrated the use of this relation to estimate the control coefficients in very simple enzyme mechanisms. However, this relationship can also be used to estimate the control coefficients in more complex mechanisms when combined either with knowledge of the rate constants of substrate and product binding or with the use of non-metabolized analogs of substrates and/or products (in order to estimate the concentration of the enzyme intermediate to which the analog binds). Importantly, using these approaches even in the case when none of the enzyme intermediates can be directly measured the control coefficients can still be estimated.

Acknowledgements: This work was supported by the Netherlands Organization for Scientific Research (NWO) and the Royal Society (London).

References

[1] Haynes, D.H. and Mandveno, A. (1987) Physiol. Rev. 67, 244–284.
[2] Christensen, H., Martin, M.T. and Waley, S.G. (1990) Biochem. J. 266, 853–861.
[3] Brown, G.C. and Cooper, C.E. (1993) Biochem. J. 294, 87–94.
[4] Ray Jr., W.J. (1983) Biochemistry 22, 4625–4637.
[5] Kholodenko, B.N. and Westerhoff, H.V. (1994) Biochim. Biophys. Acta (in press).
[6] Brown, G.C. and Cooper, C.E. (1994) Biochem. J. 300, 159–164.
[7] Van Dam, K., Van der Vlag, J., Kholodenko, B.N. and Westerhoff, H.V. (1993) Eur. J. Biochem. 212, 791–799.
[8] Kholodenko, B.N. (1990/1991) J. Nonlinear Biol. 1, 107–126.

Appendix

We shall use an approach developed in [5]. Let in the initial steady state of the reaction the concentration of the intermediate E_k be increased by a factor λ. Simultaneously we decrease by the same factor (λ) all the rate constants characterising the unidirectional rates flowing away from the intermediate E_k (Schemes 2 and 3). Then, the new steady state will be attained with the same values of all the rates and the same concentrations of all other intermediates (E_j, $j \neq k$) as in the initial steady state. The new values of parameters (rate constants and the total enzyme concentration) are related through:

$$k_{+i}(\lambda) = k_{+i}/\lambda,\ k_{-i}(\lambda) = k_{-i}/\lambda$$

$$e(\lambda) = \sum_{j \neq k} E_j + E_k(\lambda) = e + (\lambda - 1)\cdot E_{k'} \qquad \textbf{(A1)}$$

The enzyme rate (J) is a unique function of these parameters. Using Eq. **(A1)** and the definitions **(3)** of the main text, one may express the derivative of the flux with respect to λ (at $\lambda = 1$) through the responses to the corresponding parameter changes:

$$0 = d\ln J/d\ln\lambda = -\sum_{\substack{\text{all unidirectional} \\ \text{rates flowing away from } E_k}} C^J_{\pm i} + E_k/e \qquad \textbf{(A2)}$$

Using this equation one arrives at Eqs. **(5)** and **(6)** of the main text. These equations can be used to estimate the enzyme intermediate levels in any condition except thermodynamic equilibrium (see [5]). At thermodynamic equilibrium additional information is required for such estimations (see Eq. **(13)** of the main text and Kholodenko et al., in preparation).