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Competition for enzymes in metabolic pathways: Implications for optimal distributions of enzyme concentrations and for the distribution of flux control

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Abstract

The structures of biochemical pathways are assumed to be determined by evolutionary optimization processes. In the framework of mathematical models, these structures should be explained by the formulation of optimization principles. In the present work, the principle of minimal total enzyme concentration at fixed steady state fluxes is applied to metabolic networks. According to this principle there exists a competition of the reactions for the available amount of enzymes such that all biological functions are maintained. In states which fulfil these optimization criteria the enzyme concentrations are distributed in a non-uniform manner among the reactions. This result has consequences for the distribution of flux control. It is shown that the flux control matrix $\mathbf{c}$, the elasticity matrix $\boldsymbol{\varepsilon}$, and the vector $\boldsymbol{e}$ of enzyme concentrations fulfil in optimal states the relations $\mathbf{c}^{\mathrm{T}}\boldsymbol{e} = \boldsymbol{e}$ and $\boldsymbol{\varepsilon}^{\mathrm{T}}\boldsymbol{e} = 0$. Starting from a well-balanced distribution of enzymes the minimization of total enzyme concentration leads to a lowering of the SD of the flux control coefficients. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Metabolic control theory; Evolutionary optimization; Competition; Enzyme concentrations; Flux control coefficient; Metabolic pathway; Mathematical modeling

1. Introduction

The structural properties of metabolic systems can be considered as a result of a long-term biological evolution. Therefore, one can assume that these systems have developed towards states where they fulfil their biological function in an optimal manner. On this basis one can draw conclusions about the actual states of metabolic systems using optimization criteria. Mathematically, this means the application of extremum principles to the models of metabolic networks. The following optimization criteria have been formulated among others: (a) maximization of steady state fluxes in metabolic networks (Heinrich et al., 1987; Pettersson, 1993; Heinrich and Klipp, 1996); (b) maximization of the catalytic efficiencies of isolated enzyme reactions (Albery and Knowles,

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1976; Cornish-Bowden, 1976; Mavrovouniotis et al., 1990; Pettersson, 1992; Wilhelm et al., 1994; Heinrich and Klipp, 1996; Pettersson, 1996; Bish and Mavrovouniotis, 1998); (c) minimization of enzyme concentration in unbranched enzymatic chains (Heinrich and Holzhütter, 1985; Brown, 1991); (d) minimization of intermediate concentrations and total osmolarity in biochemical networks (Schuster and Heinrich, 1991; Schuster et al., 1991); (e) improvement of the ATP-yield in ADP-ATP-converting systems (Heinrich et al., 1997; Meléndez-Hevia et al., 1997; Stephani and Heinrich, 1998); (f) economic design of pathways to fulfil the function of metabolite conversion in a minimal number of steps (Meléndez-Hevia and Isidoro, 1985; Meléndez-Hevia et al., 1994).

In this paper we analyze the effects of a minimization of the total enzyme concentration on the distribution of the individual enzyme concentrations and the consequences for the distribution of flux control. There are several reasons to assume that the individual amounts of enzymes within a cellular system should be regulated such that the metabolic fluxes necessary for the maintenance of cell functions can be achieved by low total enzyme amount. Enzymes are osmotically active substances. One strategy to achieve osmotic balance is, therefore, to hold the total amount of enzyme constrained. Furthermore, enzyme synthesis is very expensive for the cell, energetically as well as with respect to the cost of material. It is, therefore, reasonable to assume that various pathways or even individual reactions compete in some sense for the available resources. Recent investigations show that an adjustment of cellular enzyme pattern takes place not only on a long time scale but already within several hours as a consequence of environmental changes. DeRisi et al. (1997) recorded the changes of gene expression in terms of mRNA levels in *Saccharomyces cerevisiae* at changing supply of glucose. Blomberg and coworkers (Blomberg, 1997; Norbeck and Blomberg, 1997) investigated the salt-instigated protein expression of *S. cerevisiae* during growth in media of different salinity. To protect against osmotic stress these cells accumulate glycerol, the production of which is accompanied by overall metabolic changes. For example, an enhanced amount of glycerol 3-phosphate dehydrogenase is produced and also other enzymes involved in the glycerol synthesis were induced to a certain degree. Furthermore, an altered expression (decrease or increase) of glycolytic enzymes was recorded. These investigations indicate that cellular enzyme levels are not only optimized at an evolutionary time scale but can also be rapidly adopted within hours.

Though it is clear that biological systems can be optimized with respect to different criteria in the following the phrase 'optimal state' denotes a state in which the total amount of enzyme is minimized under maintenance of network function which is characterized by the steady state fluxes. This is achieved by proper adjustment of the individual enzyme concentrations. To assess the properties of optimal states one has to consider non-optimized reference states with the same steady state fluxes. In the following we consider as 'reference state' a situation where all individual enzyme concentrations are equal to unity (in suitable, but arbitrary units, e.g. mmol l^{-1}).

Evolutionary pressure or short-term adaptation leading to a special distribution of the available amount of enzyme will also affect flux control in metabolic networks. One may expect that optimization will result in characteristic patterns for flux control coefficients and elasticities. Flux control coefficients are quantities which express for a given steady state of the metabolic system the effect of a small change of a certain reaction rate on the steady state fluxes. Elasticity coefficients reflect the immediate change in the rate of a reaction caused by a change in the concentration of a metabolite.

In this paper the consequences of the minimization of the total enzyme concentration will be analyzed under the assumption that the reaction rates are linear functions of the individual enzyme concentrations. In Section 2, two structurally simple but important examples are investigated, the unbranched pathway of arbitrary length and the branched network consisting of three reactions connected via one common intermediate. These examples indicate that there is a special relation between enzyme concentrations and flux control coefficients in optimal states. In Section 3, it will

be shown that this relation holds true for metabolic systems of arbitrary stoichiometry.

2. Structurally simple systems

2.1. Unbranched pathways

Let us consider an unbranched metabolic pathway consisting of r enzymatic reactions transforming an initial substrate P_1 into a final product P_2 via the intermediates S_1, S_2, . . . , S_n with $n = r-1$. All rates V_i of the individual reactions should be linearly dependent on the corresponding enzyme concentrations but may depend in a nonlinear way on the concentrations of the intermediates, that is:

$$V_i = E_i f_i(S_1, \ldots, S_n) \tag{1}$$

Under steady state conditions:

$$V_i = J \tag{2}$$

for $i = 1, \ldots, r$ the flux J through the chain is a function of the enzyme concentrations E_i (for an explicit expression, see below). The optimal enzyme concentrations $e_i = E_i^{\text{opt}}$ in states of minimal total amount of enzyme $e_{\text{tot}} = E_{\text{tot}}^{\text{opt}}$ at fixed steady state flux $J = J^0$ can be determined by the variational equation:

$$\frac{\partial}{\partial E_i}\left\{\sum_{j=1}^{r} E_j + \lambda(J(E_1, \ldots, E_r) - J^0)\right\} = 0 \tag{3}$$

where λ denotes the Lagrange multiplier. From this equation it follows:

$$\frac{\partial J}{\partial E_i} = -\frac{1}{\lambda} \tag{4}$$

and

$$\frac{e_i}{J}\left(\frac{\partial J}{\partial E_i}\right)_{E_j = e_j} = -\frac{1}{\lambda}\frac{e_i}{J} \tag{5}$$

The left hand term in Eq. (5) represents the flux control coefficient $C_i$of reaction i over the flux J. Taking into account that the sum of these coefficients over all reaction equals unity (summation theorem of metabolic control analysis) Eq. (5) yields $\lambda = -e_{\text{tot}}/J$ and in this way:

$$(C_i)_{E_j = e_j} = \frac{e_i}{e_{\text{tot}}} \tag{6}$$

In the following we denote the control coefficients in optimal states by lower case letters such that $c_i = (C_i)_{E_j = e_j}$. Eq. (6) indicates that minimization of the total enzyme concentration at fixed steady state flux leads to a state where the distribution of flux control coefficients equals the distribution of the individual enzyme concentrations.

Special distributions for e_i and c_i result from Eq. (4) by use of special rate laws. With:

$$V_i = E_i(S_{i-1}k_i - S_i k_{-i}) \tag{7}$$

($S_0 = P_1 = \text{const}$, $S_n = P_2 = \text{const}$) the equation for the steady state flux reads:

$$J = \frac{P_1 \prod_{i=1}^{n} q_i - P_2}{\sum_{j=1}^{n} \frac{1}{E_j k_{-j}} \prod_{m=j+1}^{n} q_m} \text{ with } q_i = \frac{k_i}{k_{-i}} \tag{8}$$

(Heinrich and Klipp, 1996). Introducing Eq. (8) into Eq. (4) leads to:

$$e_i = \frac{J^0}{N}\sqrt{Y_i}\sum_{l=1}^{n}\sqrt{Y_l}, \text{ with } Y_j = \frac{1}{k_{-j}}\prod_{m=j+1}^{n} q_m \tag{9}$$

where N denotes the numerator of Eq. (8).For calculating J^0 we consider a reference state where a given total concentration of enzymes is distributed uniformly such that $E_i = E_{\text{tot}}/n$. In this way one obtains from Eq. (8) and Eq. (9):

$$e_i = \frac{E_{\text{tot}}}{n}\frac{\sqrt{Y_i}\sum_{j=1}^{n}\sqrt{Y_j}}{\sum_{l=1}^{n} Y_l} \tag{10}$$

It is easy to see that this equation implies $e_{\text{tot}}/E_{\text{tot}} \leq 1$. Introducing Eq. (10) into Eq. (6) yields for the control coefficients in optimal states:

$$c_i = \frac{\sqrt{Y_i}}{\sum_{j=1}^{n}\sqrt{Y_j}} \tag{11}$$

In the reference state the control coefficients read:

$$C_i = \frac{Y_i}{\sum_{j=1}^{n} Y_j} \tag{12}$$

To elucidate the effect of optimization on the distribution of flux control we will consider the SDs σ of the flux control coefficients defined as:

$$\sigma^2 = \frac{1}{n}\sum_{i=1}^{n} C_i^2 - \left(\frac{1}{n}\sum_{i=1}^{n} C_i\right)^2 \tag{13}$$

Due to the summation theorem the second term on the right hand side reduces to $-1/n^2$.With Eqs. (10) and (11) one gets in the optimal state:

$$(\sigma^{\mathrm{opt}})^2 = \frac{1}{n^2}\left[\frac{n\sum_{i=1}^{n} Y_i}{\left(\sum_{j=1}^{n}\sqrt{Y_j}\right)^2} - 1\right] = \frac{1}{n^2}\left(\frac{E_{\mathrm{tot}}}{e_{\mathrm{tot}}} - 1\right) \tag{14}$$

Fig. 1 shows the SDs of the flux control coefficients in the reference state and in the optimal state for an unbranched chain of five enzymes in the special case of identical kinetic properties of the enzymes ($q_i = q$, $k_{-i} = k_-$). It is seen that the SD in the optimal case is lower then the one in the reference state for all q-values.

One may prove that the relation $\sigma^{\mathrm{opt}}/\sigma^{\mathrm{ref}} \leq 1$ holds true also for the general case of different kinetic and equilibrium constants (see Appendix A). This means that optimization leads to a state where the distribution of control coefficients becomes more uniform, i.e. the differences between the coefficients decrease. In contrast to that the optimal enzyme concentrations become differentiated compared to the reference state.

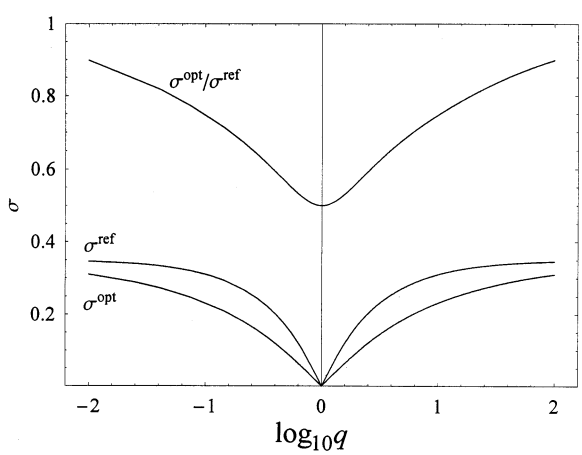


Fig. 1. Standard deviations σ of the flux control coefficients for an unbranched chain of five reaction as functions of the equilibrium constants with $q_i = q$ with $k_{-i} = k_-$. Curve σ^{ref}: reference state; curve σ^{opt}: optimal state; curve $\sigma^{\mathrm{opt}}/\sigma^{\mathrm{ref}}$: ratio of SDs.

It is worth mentioning that the effect of minimization of the total enzyme concentration on the SD of control coefficients depends on the choice of the reference state. This can be illustrated considering the case of minimal SD $\sigma^2 = 0$ where all flux control coefficients are equal to $1/n$. Using Eq. (8) it is easy to see that such a situation can be realized with enzyme concentrations E_i proportional to Y_i. Choosing this state as reference state (instead of $E_i = E_{\mathrm{tot}}/n$), minimization of the total enzyme concentration leads again to Eq. (10) for the optimal enzyme concentrations, to Eq. (11) for the control coefficients in optimal states, and to Eq. (14) for the corresponding SD. Since $e_{\mathrm{tot}}/E_{\mathrm{tot}} \leq 1$ one finds $(\sigma^{\mathrm{opt}})^2 \geq (\sigma^{\mathrm{ref}})^2 = 0$, that is, an increase of the SD of the flux control coefficients compared to the reference state, in contrast to the case with equal enzyme concentrations in the reference state.

The proportionality between enzyme concentration and flux control coefficients expressed in Eq. (6) results also from another optimization principle which is, in some sense, inverse to the considered one, namely the principle of maximization of the steady state flux at fixed total amount of enzymes (Heinrich and Holzhütter, 1985; Heinrich and Klipp, 1996). This result was confirmed by Brown (1991). He stated that this proportionality is valid not only for unbranched chains but for metabolic pathways of any complexity. In the following sections we show that such a conclusion is not correct and that Eq. (6) is only a special case of a more general relation.

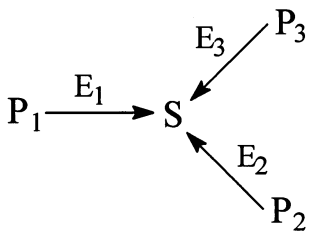
2.2. The branched system

The branched system depicted in Scheme 1 is governed by the differential equation:

$$\frac{\mathrm{d}S}{\mathrm{d}t} = V_1 + V_2 + V_3 \tag{15}$$

Using again linear rate laws which read for the present system:

$$V_i = E_i(P_i k_i - S k_{-i}) \tag{16}$$



Scheme 1.

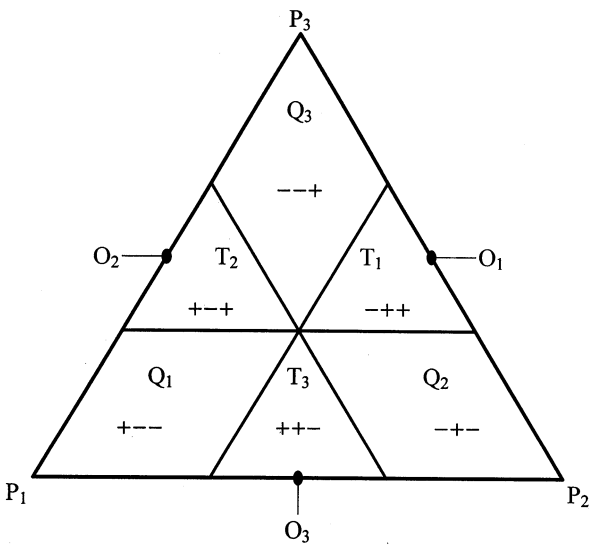


Fig. 2. P-simplex. The concentrations of the outer reactants are defined by the constraint $P_1 + P_2 + P_3 = P$. Each corner P_i is characterized by $P_i = P$ and vanishing values for the other outer reactant concentrations. The points O_i are defined by $P_i = 0$ and $P_j = P/2$ for $j \neq i$. For $k_i = k_{-i} = k$ each flux J_i is positive for $P_i > P/3$, zero at the line $P_i = P/3$ and negative elsewhere. This divides the simplex into six regions, T_i and Q_i, where in each case two fluxes have a different sign than the third one. The signs in the different regions indicate in the given order the signs of the fluxes J_1, J_2, and J_3. '+' means netto-flux directed from P_i towards S and '−' means the opposite direction.

with $i = 1, \ldots, 3$, one obtains for the steady state concentration of the internal metabolite:

$$S = \frac{E_1 P_1 k_1 + E_2 P_2 k_2 + E_3 P_3 k_3}{E_1 k_{-1} + E_2 k_{-2} + E_3 k_{-3}} \tag{17}$$

and for the three steady state fluxes:

$$J_i = \frac{E_i[P_i k_i (E_j k_{-j} + E_m k_{-m}) - k_{-i}(E_j P_j k_j + E_m P_m k_m)]}{E_1 k_{-1} + E_2 k_{-2} + E_3 k_{-3}} \tag{18}$$

with $i, j, m = 1, \ldots, 3$; $i \neq j \neq m$. In this section the indices of fluxes, enzyme concentrations, etc. run, as in Eqs. (16) and (18), from 1 to 3, if not stated otherwise. We denote as reference states the situations where the three enzymes have the same concentration $E_i = E_{\text{tot}}/3$. Without loss of generality the concentration units can be choosen in such a way that in the reference state $E_i = 1$ and $E_{\text{tot}} = 3$. The corresponding reference fluxes are functions of the concentrations P_i of the outer reactants. We will vary the concentrations of the outer reactants within a P-simplex defined by the constraint $P_1 + P_2 + P_3 = P$. For further details of the simplex representation see Fig. 2.

Choosing for example the kinetic parameters as $k_i = k_{-i} = k$ one obtains from Eq. (18)

$$J_i = \frac{E_{\text{tot}} k}{9}(3P_i - P) \tag{19}$$

Each flux J_i is positive for $P_i > P/3$ and negative elsewhere. This divides the simplex into three quadrangular regions Q_i and three triangular regions T_i where in each case two fluxes have a different sign than the third one (Fig. 2).

2.2.1. States of minimal total enzyme concentration

According to Eq. (16) one obtains at given steady state fluxes J_i for the enzyme concentrations:

$$E_i = \frac{J_i}{P_i k_i - S k_{-i}}. \tag{20}$$

States of minimal total enzyme concentrations may be found by variation of the concentration S such that:

$$\frac{\mathrm{d}E_{\text{tot}}}{\mathrm{d}S} = \frac{\mathrm{d}(E_1 + E_2 + E_3)}{\mathrm{d}S} = \sum_{i=1}^{3} \frac{J_i k_{-i}}{(P_i k_i - S k_{-i})^2} = 0 \tag{21}$$

The obtained extremum is a minimum since $\mathrm{d}^2 E_{\text{tot}}/\mathrm{d}S^2 > 0$ which follows from $sign(P_i k_i - S k_{-i}) = sign(J_i)$ (see Eq. (16)). Eq. (21) constitutes a fourth-order equation for the

determination of the optimal intermediate concentration $s = s(J_1, J_2, J_3)$. Introducing its solution into Eq. (20) yields the enzyme concentrations e_i allowing for the minimal total enzyme concentration e_{tot}.

In Fig. 3a and Fig. 4a the optimal concentration of enzyme E_1 and the minimal total enzyme concentration are depicted in the P-simplex representation for $k_i = k_{-i} = k$. Similar representations for e_2 and e_3 can be obtained by a cyclic interchange of the indices. Fig. 3b and Fig. 4b are the corresponding density plots. Each point in these plots represents the solution of the minimization problem for for the corresponding set of reference steady-state fluxes. Similar representations for $E_2$and E_3 can be obtained by a cyclic interchange of the indices.

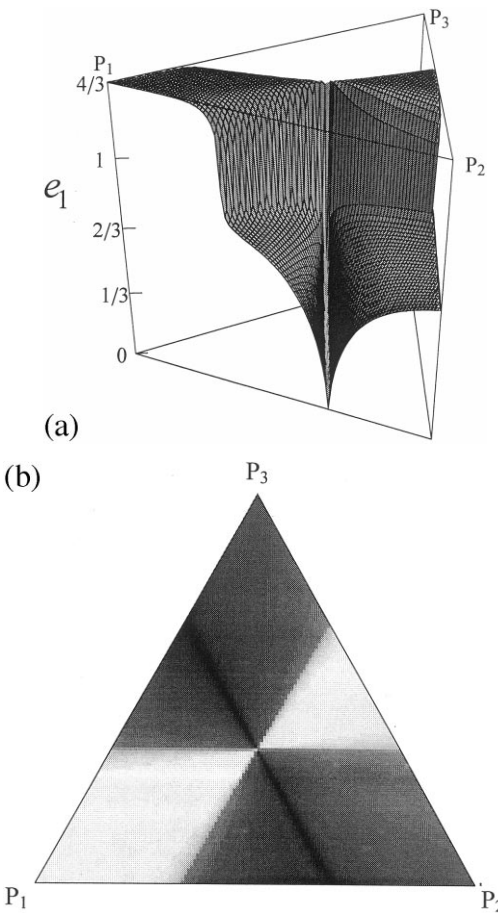


Fig. 3. Optimal concentration e_1. (a) The optimal concentration of enzyme E_1 is depicted in the P-simplex representation for $k_i = k_{-i} = k$. (b) Corresponding density plot. Each point in these plots represents the solution of the minimization problem for for the corresponding set of reference steady-state fluxes. Similar representations for $E_2$and E_3 can be obtained by a cyclic interchange of the indices.

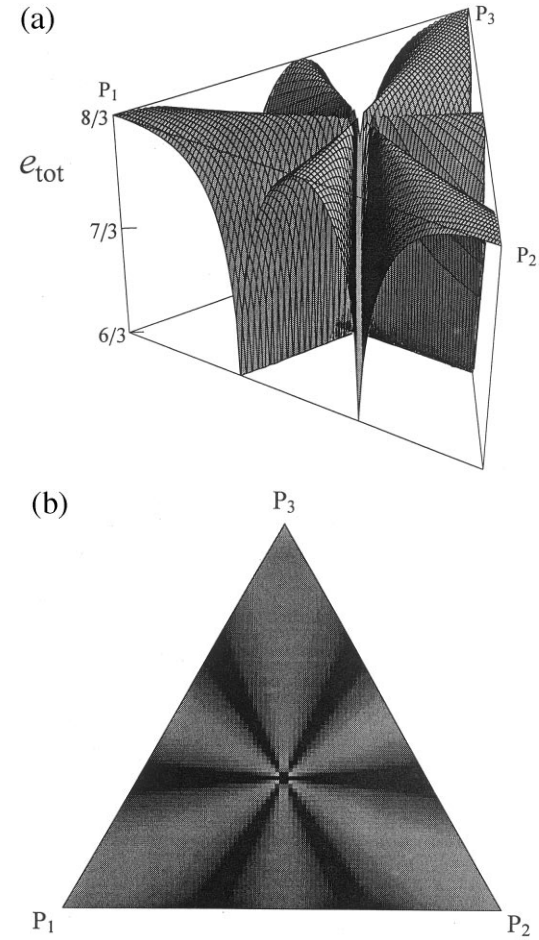


Fig. 4. The minimal total enzyme concentration e_{tot} is depicted in the P-simplex representation (a) and as a density plot (b) for $k_i = k_{-i} = k$. Each point in these plots represents the solution of the minimization problem for the corresponding set of reference steady-state fluxes.

The heights of the surfaces for optimized e_i vary between three levels with $e_i = 0$, $e_i \cong 2/3$, and $e_i \cong 4/3$. At the corner P_1 as well as at the point O_1, for example, one obtains:

$$\begin{pmatrix} e_1 \\ e_2 \\ e_3 \end{pmatrix} = \begin{pmatrix} 4/3 \\ 2/3 \\ 2/3 \end{pmatrix} \tag{22a}$$

$$e_{tot} = (8/9)E_{tot} \tag{22b}$$

Inspection of Fig. 3A and B together with Fig. 2 shows that e_i is, in general, close to the highest level, if the corresponding flux J_i has the opposite sign as the other two fluxes. This is the case, for example, in the region Q_1 where $J_1 > 0$ and $J_2,\ J_3 < 0$, and in the region T_1 where $J_1 < 0$ and $J_2,\ J_3 > 0$. In the interior of these two regions the solution (Eq. (22a)) for e_1 is also approximately fulfilled. The fluxes in these two regions represent different situations. In the first case (region Q_1) one substrate (P_1) is transformed into two products (P_2,P_3), and in the second case (region T_1) two substrates (P_2,P_3) are transformed into one product (P_1). In both cases that reaction which has to balance the other two gets the higher enzyme concentration in the optimal state. Close to the boundaries of these regions rather sharp changes in the optimal enzyme concentrations occur. The concentration e_1 is maximal along the line $P_2 = P_3$ where $J_1 = (2/3)k(P_1 - P_2)$, $J_2 = J_3 = -(1/3)k(P_1 - P_2)$, and $s = (1/2)(P_1 + P_2)$ and where e_1 is the same as given in Eq. (22a). The enzyme concentration e_1 is lowest at the line $P_1 = P/3$, where the fluxes are $J_1 = 0$ and $J_2 = -J_3 = (1/2)k(P_2 - P_3)$. Here, the analytical solution of Eq. (21) yields $s = (1/2)(P_2 + P_3)$ and the corresponding optimal enzyme concentrations are $e_1 = 0$ and $e_2 = e_3 = 1$. As expected, no enzyme (e_1) is necessary to maintain a zero net flux J_1. Accordingly, the case $P_i = P/3$ (centre of the simplex) where all fluxes vanish is characterised by vanishing concentrations of all three enzymes.

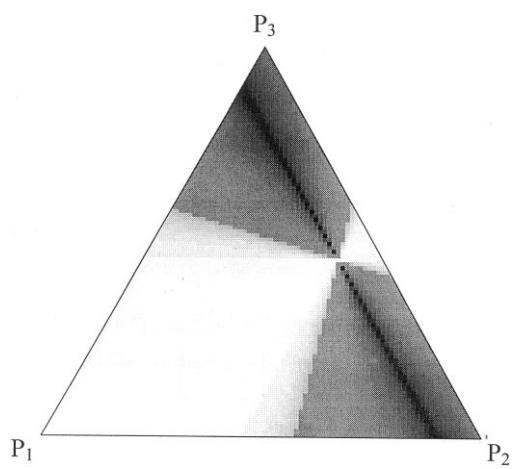


Fig. 5. Optimal enzyme concentration e_1 in the nonsymmetrical case: representation of optimal enzyme concentrations for equilibrium constants deviating from unity ($q_1 = 2$, $q_2 = q_3 = 1/2$) in a density plot. The flux in the direction from P_1 to P_2 and P_3 is favoured thermodynamically. Accordingly, the region Q_1 with $J_1 > 0$ and $J_{2,3} < 0$ in the P-simplex increases compared to the corresponding region for $q_i = 1$ (Fig. 2), whereas the region T_1 shrinks. The corresponding regions T_2, T_3, and Q_2, Q_3 for increased e_2 and increased e_3 also change their shape and shift towards the line of $P_1 = 0$.

The optimal concentration e_1 depicted in Fig. 3A and B and similar results for e_2 and e_3 yield the minimal total enzyme concentration represented in Fig. 4A and B. Since $k_i = k_{-i} = k$ this plot is fully symmetrically with respect to an exchange of the indices of the external metabolites P_i. The total enzyme concentration varies in the range $2/3E_{\text{tot}} \le e_{\text{tot}} \le 8/9E_{\text{tot}}$ except for the point $P_i = P/3$ where $e_{\text{tot}} = 0$. It is worth mentioning that in optimal states the minimal total enzyme concentration is always lower than in the reference state, whereas the individual enzyme concentrations may be lower or higher than in the reference state depending on the external metabolite concentrations.

In the general case the equilibrium constants may deviate from unity. In Fig. 5 optimized concentrations e_1 are depicted for $q_1 = 2$, $q_2 = q_3 = 1/2$ as a density plot. This distribution of equilibrium constants favours thermodynamically the flux in the direction from P_1 to P_2 and P_3. Accordingly, the region Q_1 with $J_1 > 0$ and $J_{2,3} < 0$ in the P-simplex increases compared to the corresponding region for $q_i = 1$ (Fig. 2). As a consequence, the region Q_1 where e_1 is higher then e_2 and e_3 also extends towards higher values of P_2 and P_3, whereas the region T_1 with raised e_1 at $J_1 < 0$ and $J_{2,3} > 0$ shrinks. The corresponding regions for increased e_2 and increased e_3 also change their shape and shift towards the line of $P_1 = 0$.

In Fig. 6 variations of the optimal enzyme concentrations with changing equilibrium constants are shown for the case $q_2 = q_3 = 1/q_1$ at the point where $P_1 = P$ and $P_2 = P_3 = 0$. Hence, the second and third reaction degrade metabolite S irreversibly. For details of these dependencies see the legend to Fig. 6. Note, that the tendencies at

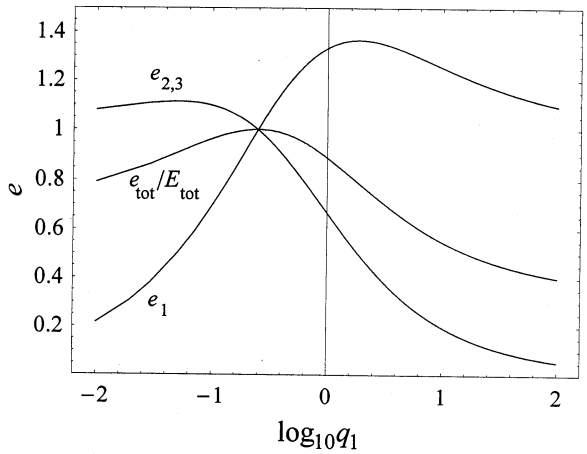


Fig. 6. The dependence of the optimal enzyme concentrations on the equilibrium constants is shown for $q_1 = 1/q_2 = 1/q_3$ at the point where $P_1 = P$ and $P_2 = P_3 = 0$. In the case $q_1 \gg 1$, $q_{2,3} \ll 1$, where the fluxes from P_1 towards P_2 and P_3 are thermodynamically favoured, e_1 tends to its reference value $E_1 = 1$ whereas e_2 and e_3 tend to zero. In the opposite case ($q_1 \ll 1$) e_1 tends zero where as e_2 and e_3 tend to their reference values. The curves display maxima, for e_1 at $q_1 = 1 + \sqrt{3}/2$, for e_2 and e_3 at $q_1 = 5/2 - \sqrt{6}$, and for e_{tot} at $q_1 = 1/4$. The maximum of the latter curve is characterised by $e_{tot} = E_{tot}$, which means that the reference state is already the optimal one.

low and high q_1 resemble the situation in the unbranched chain, where in the irreversible case only the reactions at the very beginning of the pathway are characterized by high enzyme concentration in optimal states.

2.2.2. Flux control coefficients in the reference state and in the optimal state

The flux control coefficients C_j^i of reactions j over the fluxes J_i for the branched system depicted in Scheme 1 can be calculated using the summation theorem of metabolic control analysis:

$$\sum_{j=1}^{3} C_j^i = 1 \tag{23}$$

the connectivity theorem:

$$\sum_{j=1}^{3} C_j^i \varepsilon_S^j = 0 \tag{24}$$

and the three branch-point relationships (Fell and Sauro, 1985), which read for the present system:

$$J_i C_j^m = J_j C_i^m \tag{25}$$

with $i \neq j \neq m$. Eq. (24) contains the normalized elasticity coefficients:

$$\varepsilon_S^i = \frac{S}{V_i}\frac{\partial V_i}{\partial S} = -\frac{S}{V_i} E_i k_{-i} \tag{26}$$

which, in the following, we will abbreviate with ε_i. Eqs. (23)–(25) represent nine equations for the nine flux control coefficients which are the elements of the flux control matrix **C**. One obtains:

$$\mathbf{C} = \mathbf{I}_3 - \frac{1}{\varepsilon_1 J_1 + \varepsilon_2 J_2 + \varepsilon_3 J_3} \begin{pmatrix} \varepsilon_1 J_1 & \varepsilon_1 J_2 & \varepsilon_1 J_3 \\ \varepsilon_2 J_1 & \varepsilon_2 J_2 & \varepsilon_2 J_3 \\ \varepsilon_3 J_1 & \varepsilon_3 J_2 & \varepsilon_3 J_3 \end{pmatrix} \tag{27}$$

where $\mathbf{I}_3$ denotes the 3×3- identity matrix. Using Eq. (26) we can rewrite the flux control matrix **C** as:

$$\mathbf{C} = \mathbf{I}_3 - \frac{1}{E_1 k_{-1} + E_2 k_{-2} + E_3 k_{-3}} \begin{pmatrix} E_1 k_{-1} & \frac{J_2}{J_1} E_1 k_{-1} & \frac{J_3}{J_1} E_1 k_{-1} \\ \frac{J_1}{J_2} E_2 k_{-2} & E_2 k_{-2} & \frac{J_3}{J_2} E_2 k_{-2} \\ \frac{J_1}{J_3} E_3 k_{-3} & \frac{J_2}{J_3} E_3 k_{-3} & E_3 k_{-3} \end{pmatrix} \tag{28}$$

2.2.2.1. Reference states. Eq. (28) indicates that the control coefficients C_i^i of all reactions on their own fluxes are independent of the flux distribution and in this way also independent of the concentrations of the external metabolites. Since $E_i = E_{tot}/3$ it follows that $C_i^i = 1 - k_{-i}/(k_{-1} + k_{-2} + k_{-3})$. In the special case $k_{-i} = k$ one finds $C_i^i = 2/3$. The coefficients C_j^i for $i \neq j$ depend on the flux ratios and may be positive or negative. In particular, one finds that $C_j^i = 0$ if $J_j = 0$ and that they tend towards infinity for $J_i \to 0$, where the sign depends on the signs of the fluxes such that $C_j^i > 0$ if $sign(J_i) \neq sign(J_j)$ and $C_j^i < 0$ if $sign(J_i) = sign(J_j)$ (Fig. 7).

At corner P_1 as well as at the point O_1 the matrix of control coefficients reads for $k_i = k_{-i} = k$:

$$\mathbf{C} = \begin{pmatrix} 2/3 & 1/6 & 1/6 \\ 2/3 & 2/3 & -1/3 \\ 2/3 & -1/3 & 2/3 \end{pmatrix}. \quad (29)$$

The control matrices for the points P_2, P_3 and O_2, O_3 follow from Eq. (29) by appropriate interchange of the indices.

2.2.2.2. Optimal states. Due to the changes of the enzyme concentrations the flux control coefficients c_j^i in optimal states will deviate from their reference values. For $k_i = k_{-i} = k$ one obtains from Eqs. (22a) and (28) at the corner P_1 as well as at the point O_1:

$$\mathbf{c} = \begin{pmatrix} 1/2 & 1/4 & 1/4 \\ 1/2 & 3/4 & -1/4 \\ 1/2 & -1/4 & 3/4 \end{pmatrix} \quad (30)$$

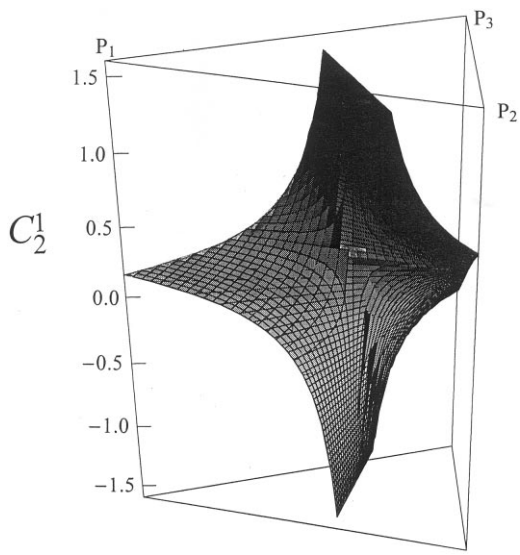


Fig. 7. Flux control coefficients in the reference state. The control coefficient C_2^1 is depicted in the P-simplex representation for $k_i = k_{-i} = k$. C_2^1 is zero for $J_2 = 0$ and tends to infinity for $J_1 \to 0$ (see Eq. (26)). In the various regions (T, Q) the sign of the control coefficient depends on the sign of the corresponding fluxes: $sign\,(C_j^i) = sign\,(-J_j/J_i)$. In the corners C_2^1 assumes the values $1/6(P_1)$, $2/3(P_2)$, and $-1/3(P_3)$, resp. The corresponding representations for the other control coefficients C_j^i, $i \neq j$ can be obtained by appropriate interchange of indices. The values of the control coefficients C_i^i are independent of the reactant concentrations in the reference state ($=2/3$ for the choosen kinetic parameters) and, therefore, are not represented.

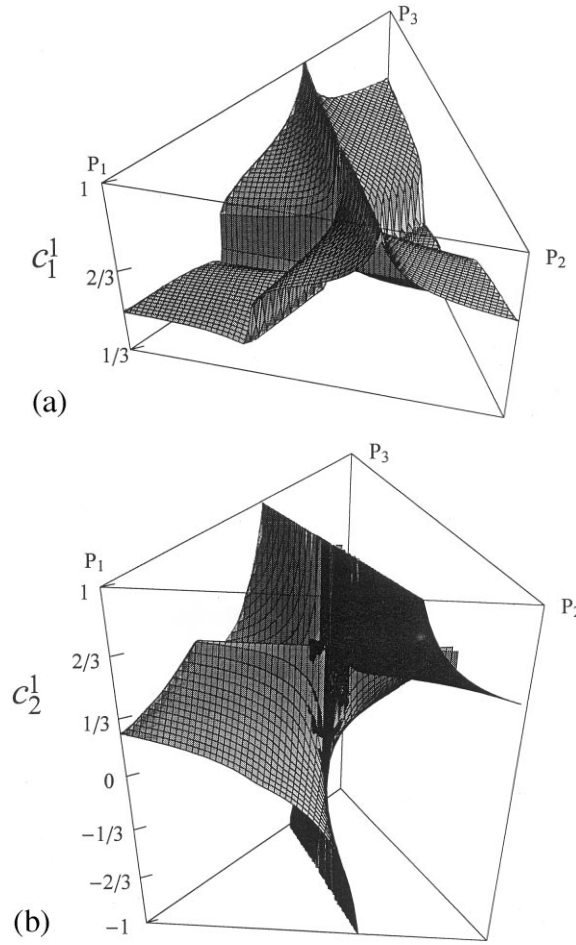


Fig. 8. Flux control coefficients in the optimal state. (a) The control coefficient c_1^1 in P-simplex representation for $k_{\pm i} = k$. Values at the corners:$1/2(P_1)$ and $3/4(P_{2,3})$. In the regions Q_1 and T_1, where $e_1 \cong (4/3)E_1$, the coefficient c_1^1 exhibits decreased values compared to the reference values ($c_1^1 \cong 1/2$, $C_1^1 \cong 2/3$). In the other regions ($T_{2,3}$, $Q_{2,3}$) with $e_1 < E_1$ the coefficient c_1^1 is increased. These regions regions contain the line $P_1 = 1/3$ where $J_1 = 0$. Approaching this line the concentration e_1 vanishes, while the control coefficient tends to unity. (b) The control coefficient c_2^1. It has the same signs, nulls and singularities in the regions of the P-simplex as in the reference state. The values at the corners are $1/4(P_1)$, $1/2(P_2)$ and $-1/4(P_3)$ (compare Fig. 7 for reference values). The other control coefficients in optimal states can be obtained by appropriate interchange of indices.

and corresponding equations at the other points P_i and O_i ($i \neq 1$). Fig. 8a shows the flux control coefficient c_1^1 which is obtained by introducing the optimal enzyme concentrations into Eq. (28). In

the region Q_1 as well as in the region T_1 characterized by increased concentration e_1 ($e_1 \cong 4/3$) the coefficient c_1^1 is decreased compared to the reference value. In the other regions of the P-simplex where the optimal concentration e_1 is lower than in the reference state the coefficient c_1^1 is increased. These regions contain the line $P_1 = 1/3$ where $J_1 = 0$. Approaching this line the concentration e_1 vanishes, while the control coefficient tends to unity.

The control coefficient c_2^1 has in regions of the P-simplex the same signs as in the reference state (Fig. 8B). It becomes also zero if $J_2 = 0$, i.e. for $P_2 = 1/3$ where $e_2 = 0$, and it has a singularity for $J_1 = 0$. On the corners it assumes the values $c_2^1 = 1/4 > C_2^1 = 1/6$ (P_1) and $c_2^1 = 1/2 < C_2^1 = 2/3$ (P_2) and $c_2^1 = -1/4 > C_2^1 = -1/3$ (P_3). The other control coefficients in optimal states can be obtained by appropriate interchange of indices.

2.2.3. General conclusions

2.2.3.1. Standard deviations. It may be questioned which general conclusions concerning the change of the control coefficients due to minimization of total enzyme concentration can be drawn. In the previous section we have considered the SD of the control coefficients in the optimal compared to the reference state for an unbranched chain. A similar consideration can be made for the branched system. Using for example the control matrices given in Eqs. (29) and (30) one obtains for the ratio of the SDs of all control coefficients for the corners P_i and the points O_i:

$$\frac{\sigma^{\mathrm{opt}}}{\sigma^{\mathrm{ref}}} = \frac{\sqrt{3}}{2} < 1 \tag{31}$$

A general equation for the ratio of the SDs of all control coefficients of the branched system reads:

$$\frac{\sigma^{\mathrm{opt}}}{\sigma^{\mathrm{ref}}} = \frac{(k_{-1} + k_{-2} + k_{-3})}{(e_1 k_{-1} + e_2 k_{-2} + e_3 k_{-3})} \sqrt{\frac{\left(\frac{e_1 k_{-1}}{J_1}\right)^2 + \left(\frac{e_2 k_{-2}}{J_2}\right)^2 + \left(\frac{e_3 k_{-3}}{J_3}\right)^2}{\left(\frac{k_{-1}}{J_1}\right)^2 + \left(\frac{k_{-2}}{J_2}\right)^2 + \left(\frac{k_{-3}}{J_3}\right)^2}}. \tag{32}$$

For $k_i = k_{-i} = k$ numerical calculations show that this ratio is always smaller than or equal to $\sqrt{3}/2$. The fact that this ratio is smaller than unity gives a further support to the hypothesis that the optimization of the enzyme concentrations leads to states where control coefficients are distributed more uniformly than in the reference state.

2.2.3.2. Relations between control coefficients and enzyme concentrations in optimal states. In the optimized state Eq. (26) can be re-arranged to give:

$$e_i = -\frac{\varepsilon_i^{\mathrm{opt}} J_i}{s k_{-i}} \tag{33}$$

The condition (Eq. (21)) of minimized total enzyme concentration can be rewritten under consideration of Eq. (20) to give:

$$\sum_{i=1}^{3} \frac{e_i^2 k_{-i}}{J_i} = 0 \tag{34}$$

and with the use of Eq. (33) it follows:

$$\sum_{i=1}^{3} \varepsilon_i^{\mathrm{opt}} e_i = 0 \tag{35}$$

Hence, in optimized states we have a relation between the elasticities and the enzyme concentrations which not necessarily holds in non-optimized states.

Eq. (35) may be used to derive a simple relation between the control coefficients $c_i^j$and the optimal enzyme concentrations which is independent of the elasticties. Introducing $\varepsilon_i^{\mathrm{opt}}$ into Eq. (27) yields c_i^j. Taking into consideration Eqs. (28) and (35), one derives:

$$\sum_{k=1}^{3} c_i^k e_k = e_i \tag{36}$$

The validity of this relation can be easily checked for special cases, for example, by introducing the enzyme concentrations from Eq. (22a) and the control coefficients from Eq. (30). Using instead of e_i the concentrations $E_i = 1$ and for the control coefficients the values from Eq. (29) one verifies that Eq. (36) is not valid for the reference state.

3. The general case

We consider a metabolic system consisting of r reactions and n metabolites. Again we assume that each reaction rate V_i is linearly related to the enzyme concentration E_i (and only to E_i) as given in Eq. (1). No restrictions are made with respect to the topology of the network except that, for sake of simplicity, conservation relations for metabolites are excluded (for extensions to systems with conservation relations see below). The dynamics of the system is described by

$$\frac{d\boldsymbol{S}}{dt} = \mathbf{N}\boldsymbol{V} \tag{37}$$

where $\boldsymbol{V} = (V_1, V_2, \ldots, V_r)^T$ denotes the vector of reaction rates, $\boldsymbol{S} = (S_1, S_2, \ldots, S_n)^T$ the vector of metabolite concentrations, and $\mathbf{N}$ the stoichiometric matrix. Under steady-state conditions the equation system:

$$\mathbf{N}\boldsymbol{V}(\boldsymbol{S},\boldsymbol{E}) = 0 \tag{38}$$

describes in an implicit manner the dependence $\boldsymbol{S} = \boldsymbol{S}(\boldsymbol{E})$ of the metabolite concentrations on the enzyme concentrations $\boldsymbol{E} = (E_1, E_2, \ldots, E_r)^T$. Using these functions the steady state fluxes $\boldsymbol{J} = (J_1, J_2, \ldots, J_r)^T$ may be expressed as $\boldsymbol{J} = \boldsymbol{V}(\boldsymbol{S}(\boldsymbol{E}),\boldsymbol{E})$.

The control coefficients may be obtained by implicit differentiation of Eq. (38) with respect to the enzyme concentrations:

$$\mathbf{N}\frac{\partial \boldsymbol{V}}{\partial \boldsymbol{S}}\frac{\partial \boldsymbol{S}}{\partial \boldsymbol{E}} + \mathbf{N}\frac{\partial \boldsymbol{V}}{\partial \boldsymbol{E}} = 0 \tag{39}$$

By taking into account:

$$\frac{\partial \boldsymbol{J}}{\partial \boldsymbol{E}} = \frac{\partial \boldsymbol{V}}{\partial \boldsymbol{E}} + \frac{\partial \boldsymbol{V}}{\partial \boldsymbol{S}}\frac{\partial \boldsymbol{S}}{\partial \boldsymbol{E}} \tag{40}$$

one obtains:

$$\frac{\partial \boldsymbol{J}}{\partial \boldsymbol{E}} = \left(\mathbf{I}_r - \frac{\partial \boldsymbol{V}}{\partial \boldsymbol{S}}\left(\mathbf{N}\frac{\partial \boldsymbol{V}}{\partial \boldsymbol{S}}\right)^{-1}\mathbf{N}\right)\frac{\partial \boldsymbol{V}}{\partial \boldsymbol{E}} \tag{41}$$

$\mathbf{I}_r$ denotes the $r \times r$ identity matrix. The scaled flux control coefficients:

$$C_j^i = \frac{J_j}{J_i}\frac{\partial J_i/\partial E_j}{\partial V_j/\partial E_j} \tag{42}$$

are obtained from Eq. (41) and can be expressed in matrix form as follows:

$$\mathbf{C} = \mathbf{I}_r - (\mathrm{dg}\boldsymbol{J})^{-1}\frac{\partial \boldsymbol{V}}{\partial \boldsymbol{S}}\left(\mathbf{N}\frac{\partial \boldsymbol{V}}{\partial \boldsymbol{S}}\right)^{-1}\mathbf{N}(\mathrm{dg}\boldsymbol{J}) \tag{43}$$

$(\mathrm{dg}\boldsymbol{J})$ denotes a square matrix containing in the main diagonal the elements of the vector $\boldsymbol{J}$. [For more details of the derivation of control coefficients see Heinrich and Schuster (1996)]. In the following we have to consider the transpose $\mathbf{C}^T$of the flux control matrix $\mathbf{C}$ which reads:

$$\mathbf{C}^T = \mathbf{I}_r - (\mathrm{dg}\boldsymbol{J})\mathbf{N}^T\left[\left(\mathbf{N}\frac{\partial \boldsymbol{V}}{\partial \boldsymbol{S}}\right)^{-1}\right]^T\left(\frac{\partial \boldsymbol{V}}{\partial \boldsymbol{S}}\right)^T(\mathrm{dg}\boldsymbol{J})^{-1} \tag{44}$$

As in the previous sections we compare systems which are characterized by the same steady state fluxes, but have different distributions of enzyme concentrations. In particular we are interested in states were the fluxes may be produced by a minimal amount of total enzyme concentration. Let us compare first all states which are characterized by the same reaction rates

$$V_i = V_i^0 \tag{45}$$

for $i = 1, \ldots, r$. We assume that the vector $\boldsymbol{V}^0$ fulfils the steady state relation $\mathbf{N}\boldsymbol{V}^0 = 0$ such that $\boldsymbol{V}^0 = \boldsymbol{J}$. Fixation of the fluxes leads by virtue of Eq. (1) to a relation between the enzyme concentrations and the metabolite concentrations such that:

$$E_i = E_i(S_1, S_2, \ldots, S_n) = \frac{V_i^0}{f_i} \tag{46}$$

Combining this equation with the principle of minimal total enzyme concentration:

$$E_{\mathrm{tot}} = \sum_{i=1}^{r} E_i \rightarrow \min \tag{47}$$

leads to the variational equation:

$$\frac{\partial E_{\mathrm{tot}}}{\partial S_j} = -\sum_{i=1}^{r}\frac{V_i^0}{f_i^2}\frac{\partial f_i}{\partial S_j} = 0 \tag{48}$$

which determines the metabolite concentrations s_j in the optimal state. Since $f_i(s_1, s_2, \ldots, s_n) = V_i^0/e_i$ (compare Eq. (46)) it follows:

$$\sum_{i=1}^{r} \frac{e_i \partial V_i}{V_i^0 \partial S_j}\bigg|_{S_j = s_j} = 0 \tag{49}$$

This relation can be rewritten in matrix form as:

$$\left(\frac{\partial \boldsymbol{V}}{\partial \boldsymbol{S}}\right)^{\mathrm{T}} (dg\boldsymbol{J})^{-1}\boldsymbol{e} = 0 \tag{50}$$

where the derivatives have to be taken at the concentrations in the optimal state. Postmultiplication of Eq. (44) with the vector $\boldsymbol{e}$ containing the optimized enzyme concentrations leads by virtue of Eq. (50) to:

$$\mathrm{c}^{\mathrm{T}}\boldsymbol{e} = \boldsymbol{e} \tag{51}$$

Eq. (51) expresses the functional relation between enzyme concentrations and flux control coefficients in states of minimal total enzyme concentration. It represents the general form of Eq. (36) for enzymatic networks.

Premultiplication of Eq. (50) with the diagonal matrix containing the optimal intermediate concentrations, (dg$\boldsymbol{s}$), yields:

$$\boldsymbol{\varepsilon}^{\mathrm{T}}\boldsymbol{e} = 0 \tag{52}$$

where $\boldsymbol{\varepsilon} = (\mathrm{dg}\boldsymbol{J})^{-1}(\partial \boldsymbol{V}/\partial \boldsymbol{S})|_{\boldsymbol{S}=\boldsymbol{s}}(\mathrm{dg}\boldsymbol{s})$ denotes the matrix of elasticity coefficients in optimal states.

It is easy to see that Eqs. (51) and (52) hold true also for systems which contain conservation relations. In this case Eq. (44) for the transpose of the matrix of control coefficients is modified by a link matrix **L** such that the term $(\partial \boldsymbol{V}/\partial \boldsymbol{S})^{\mathrm{T}}(\mathrm{dg}\boldsymbol{J})^{-1}$ remains unaffected (Reder, 1988). Since the validity of Eq. (50) does not depend on the existence of conservation relations Eqs. (51) and (52) remain valid.

4. Discussion

In the present paper we have shown that minimization of the total enzyme concentration in metabolic networks at fixed steady state fluxes lead generally to states where the enzyme concentrations differ from those in a reference state. Starting from an even distribution some enzyme concentrations increase and others decrease during optimization whereas the total enzyme concentration decreases. The optimal individual concentrations are, therefore, better adapted to the functional requirements of the network. This differentiation of enzyme concentrations can be viewed as a competition for the limited resources for the synthesis of enzymes within cells. For example, in the case of an unbranched enzymatic chain those enzymes which have high control coefficients in the reference state should be expressed in higher concentrations in the optimal state, while the concentrations of the enzymes with lower control coefficients will decrease. A similar result is obtained for the branched pathway.

Resulting from changes in the enzyme concentrations the distribution of flux control coefficients in states of minimal total enzyme concentration will deviate from the reference distribution. The flux control coefficients of those enzymes with increased concentrations will decrease during optimization. In the case of an unbranched chain one arrives eventually at a state where the enzyme concentrations and the flux control coefficients show the same distribution. At first sight it may be surprising that enzymes with high concentrations, which implies a high rate of their reactions, exert high flux control, although fast steps are usually considered to have low flux control. Our analysis demonstrates once more that high flux control is not only related to the rate of the enzyme but also to its location in the chain.

For branched networks as studied in Section 2.2 there is in optimal states no longer a proportionality of concentrations of certain enzyme and its flux control coefficients. Instead, one arrives at the more complex but also linear Eq. (36) which relate all control coefficients of a certain enzyme E_i to the concentrations of all enzymes. Rewriting Eq. (36) in matrix form yields Eq. (51). This equation is shown to be of general validity for all metabolic networks provided that the enzyme concentrations enter the rate equations in a linear manner. The proportionality of enzyme concentrations and flux control coefficients found for the unbranched chain is a special case of this general relation. According to Eq. (51) the vector of the optimal enzyme concentrations can be viewed as an eigenvector of the transpose of the flux control matrix to the eigenvalue 1. In that respect this

relation resembles the summation theorem $\mathbf{C}\boldsymbol{k} = \boldsymbol{k}$, where $\boldsymbol{k}$ denotes any vector of the nullspace of the stoichiometric matrix (Reder, 1988; Heinrich and Schuster, 1996). The latter equation indicates that $\boldsymbol{k}$ is an eigenvector of $\mathbf{C}$ to the eigenvalue 1.

Minimization of total enzyme concentration may contribute to a more uniform distribution of the flux control among the enzymes. For the two structurally simple systems considered this tendency is expressed by a lowering of the SDs of these coefficients compared to a reference state with non-differentiated values of enzyme concentrations. This effect can be a part of an answer to the question raised by Mazat et al. (1996): 'Why are most flux control coefficients so small?', at least for unbranched chains where all flux control coefficients are positive and their sum is constrained to unity. Whether or not the SD is decreased during optimization depends on the choice of the reference state as demonstrated in Section 2.1.

As shown in Section 3 minimization of the total enzyme concentration leads not only to a special relation between control coefficients and enzyme concentrations (Eq. (51)) but also to a special relation between elasticity coefficients and enzyme concentrations (Eq. (52)). It is worth mentioning that there is a direct relation between these two equations. Eq. (52) results from Eq. (51) if the latter equation is premultiplied by the transpose of the elasticity matrix and by taking into account the connectivity theorem in the form $\boldsymbol{\varepsilon}^{\mathrm{T}}\mathbf{C}^{\mathrm{T}} = 0$ which is fulfilled also for the optimal case.

In the case that the system does not contain conservation relations the optimal enzyme concentrations for a system with r reactions and n metabolites are determined by n steady state conditions for the metabolite concentrations, $r - n$ constraints for the independent steady state fluxes and n conditions for the elasticities given Eq. (52). The latter equation results from the minimization of the total enzyme concentration by variation of the metabolite concentrations which are the only quantities which may be changed at fixed fluxes and unknown enzyme concentrations. This procedure for determining e_i may fail in the case that the system contains irreversible reactions. The reason is that some metabolite concentrations drop out from Eq. (48) and remain undetermined. Minimal total enzyme concentrations may then be obtained by vanishing values of the individual enzyme concentrations and the flux constraints are fulfilled by infinite values of the metabolite concentrations. Unique solutions could be found by introducing upper limits for the metabolite concentrations, for example due to osmotic constraints.

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Appendix A. Proof for $(\sigma^2)^{\mathrm{ref}} \geq (\sigma^2)^{\mathrm{opt}}$ for an unbranched chain

With the notations $x_i = \sqrt{Y_i}$ assertion $(\sigma^2)^{\mathrm{ref}} \geq (\sigma^2)^{\mathrm{opt}}$ holds true if:

$$\left(\sum_i x_i^2\right)^3 \leq \left(\sum_i x_i^4\right)\left(\sum_j x_j\right)^2 \tag{A1}$$

for $x_i \geq 0$. The validity Eq. (A1) may be proved as follows. One derives easily:

$$A = \left(\sum_i x_i^2\right)^3 = \sum_i x_i^6 + 3\sum_{i\neq j} x_i^4 x_j^2 + \sum_{i\neq j\neq k} x_i^2 x_j^2 x_k^2, \tag{A2}$$

and:

$$B = \left(\sum_i x_i^4\right)\left(\sum_j x_j\right)^2 = \sum_i x_i^6 + \sum_{i\neq j} x_i^4 x_j^2 + 2\sum_{i\neq j} x_i^5 x_j + \sum_{i\neq j\neq k} x_i^4 x_j x_k, \tag{A3}$$

and in this way:

$$B - A = \underbrace{2\sum_{i\neq j}(x_i^5 x_j - x_i^4 x_j^2)}_{T_1} + \underbrace{\sum_{i\neq j\neq k}(x_i^4 x_j x_k - x_i^2 x_j^2 x_k^2)}_{T_2}. \tag{A4}$$

For the first sum T_1 to be nonnegative it is sufficient to show that the individual terms:

$$T_1(i,j) = x_i^5 x_j - x_i^4 x_j^2 + x_j^5 x_i - x_j^4 x_i^2 \tag{A5}$$

are nonnegative. Since:

$$T_1(i,j) = x_i x_j (x_i^2 + x_i x_j + x_j^2)(x_i - x_j)^2 \quad \text{(A6)}$$

one obtains $T_1(i,j) \geq 0$ and, therefore, $T_1 \geq 0$.For the sum T_2 to be nonnegative it is sufficient to show that the individual terms:

$$T_2(i,j,k) = x_i^4 x_j x_k + x_j^4 x_i x_k + x_k^4 x_i x_j - 3x_i^2 x_j^2 x_k^2 \quad \text{(A7)}$$

are nonnegative. One obtains:

$$T_2(i,j,k) = x_i x_j x_k (x_i^3 + x_j^3 + x_k^3 - 3x_i x_j x_k) \quad \text{(A8)}$$

Without loss of generality one may assume that $x_i \leq x_j, x_k$ such that $x_j - x_i = d_1 \geq 0$ and $x_k - x_i = d_2 \geq 0$. In this way one obtains:

$$T_2(i,j,k) = x_i x_j x_k [d_1^3 + d_2^3 + 3x_i (d_1^2 + d_2^2 - d_1 d_2)]$$
$$\geq x_i x_j x_k (d_1^3 + d_2^3 + 3x_i (d_1 - d_2)^2) \quad \text{(A9)}$$

such that $T_2(i,j,k) \geq 0$ and, therefore, $T_2 \geq 0$.

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