BIOORGANIC CHEMISTRY 23, 439–449 (1995)

Strategies for Manipulating Metabolic Fluxes in Biotechnology[1]

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Received May 12, 1995

Strategies for biotechnologically manipulating metabolic fluxes are critically examined in relation to a model system. The common idea of first identifying the rate-limiting enzyme in the biosynthetic pathway to a desired end-product, and then increasing its activity, is shown to be completely ineffective; such manipulation typically produces only trivial changes in flux. Manipulating the activities of all of the enzymes in a biosynthetic pathway by amounts calculated to increase a desired flux while leaving all other fluxes and all concentrations unchanged is potentially effective, and can be applied to any system without regard to its regulatory design. however, it requires accurate knowledge of the initial state of the system and the ability to make precise changes to numerous activities. The classical information about the regulatory mechanisms that exist in living organisms suggests that one can make much simpler manipulations, involving only the steps that remove the desired end-product, with almost equally satisfactory results. © 1995 Academic Press, Inc.

INTRODUCTION

The possibility of using genetic manipulation to increase selected enzyme activities, essentially at will, appears at first sight to have opened the door to the application of classical ideas of enzyme regulation to useful industrial objectives. If one can identify the enzyme that catalyzes the rate-limiting step in the normal biosynthetic pathway to a valuable end-product, it would seem straightforward to clone its gene and use the standard techniques of genetic manipulation to produce a greatly increased activity in the organism, with much higher yields of the desired metabolite. In this article we use examples from the literature to show that this approach does not work in practice, we discuss in terms of metabolic control analysis why it cannot be expected to work in the future, and we consider alternative approaches that may have better prospects for success.

A convenient experimental example is provided by a recent study of tryptophan biosynthesis in yeast, for which the biosynthetic part of the pathway (Fig. 1a) consists of five enzymes. In an initial set of experiments the activities of four of these were increased individually by factors of 10 to 50, and in other experiments all of them were similarly increased in various combinations (*1*). These experiments have been

[1] This paper is dedicated to Professor Jeremy Knowles on the occasion of his 60th birthday.

0045-2068/95 $12.00

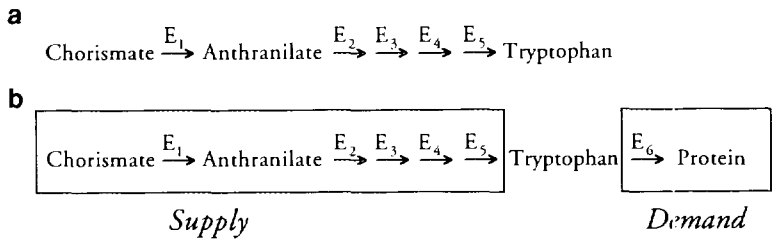

FIG. 1. Biosynthesis of tryptophan in *Saccharomyces cerevisiae*. The identities of the enzymes E_1 to E_5 are as follows: E_1, anthranilate synthase; E_2, anthranilate phosphoribosyl transferase; E_3, phosphoribosylanthranilate isomerase; E_4, indoleglycerol phosphate synthase; E_5, tryptophan synthase. E_6 represents not only tryptophan tRNA synthetase but also all of the subsequent process of protein synthesis. (a) Biosynthetic pathways are usually drawn in textbooks without explicit indication of the reactions that consume the "end-product." (b) However, regulation of such a pathway involves communication via the end-product between a demand block and a supply block. In this example only the most important use of tryptophan is shown as the demand block; other pathways, such as degradation to tryptophanol, constitute competing demand blocks.

discussed elsewhere from the same point of view as the present article (*2*), and only a summary need be given here: increasing any single enzyme activity, by factors in the range of 10- to 50-fold, had no significant effect on the flux to tryptophan; however, when all five activities were increased by factors of about 20 (or all five apart from phosphoribosylanthranilate isomerase, which appears to have little influence on the flux) there was a substantial increase in flux, albeit considerably less than the increase in the activities of the pathway enzymes. This study is the most detailed that can be found in the literature, but others have led to equally disappointing results. For example, similar efforts to improve the yield of ethanol in alcoholic fermentation of yeast gave results essentially the same as those for tryptophan biosynthesis (*3*).

Metabolic control analysis originated in articles written more than 20 years ago (*4*, *5*), but it remains relatively unknown to many biochemists and nearly all biotechnologists. Yet it readily explains the disappointing results of attempts to improve yields by increasing activities of rate-limiting enzymes. The essential point is that the classical conception of a rate-limiting enzyme that completely determines the flux through a pathway is an oversimplification; instead, the control of flux in any pathway is shared among all the enzymes of the organism, in proportions that cannot be predicted *a priori*. Moreover, even if one enzyme happens to have a large share of the total flux control, this share always decreases if its activity is increased. Clear experimental examples of this sort of behavior can be found in studies of arginine biosynthesis in *Neurospora crassa* (*6*).

Westerhoff and Kell (*7*) have described how metabolic control analysis can be applied to biotechnological questions, but it has yet to have a major impact on the practice of biotechnology; efforts to use genetic engineering to realize commercial objectives have been guided more by a vague optimism than by application of the principles of metabolic control.

Metabolic control analysis offers a powerful means of examining the general properties of metabolic systems, but its very generality means that it may overlook some of the specific properties of real systems. In particular, it makes no assumptions about the existence of the regulatory properties of real enzymes, and thus does not take advantage of the knowledge of the regulatory design of biological systems that has accumulated during the past 40 years—feedback inhibition, allosteric and cooperative interactions, etc. (*8–11*). Consequently, although techniques based on control analysis for altering fluxes and metabolite concentrations may well be effective they are likely to be more complicated, and hence experimentally more difficult, than necessary. In this paper we examine a model system to illustrate this point.

In a previous work (*12*) we argued that the classical mechanisms are essential for achieving satisfactory regulation, but to be properly understood they need to be considered in the framework of a systemic theory of control such as metabolic control analysis. Most pathways can be considered to consist of a *supply block*, consisting of a series of reactions leading to a particular end-product, and a *demand block*, consisting of the reactions that consume the end-product. (In reality an "end-product" is not the end of metabolism but the link metabolite between the two blocks. The term is thus misleading, but as it is universally used in this sense in biochemistry we shall retain it here.)

The demand block is commonly omitted from textbook illustrations of metabolic pathways (which are thus shown as in Fig. 1a rather than as in Fig. 1b). Inclusion of the demand block is necessary, however, for understanding metabolic regulation, because the usual effect of feedback inhibition of the first committed step in a pathway by its end-product is to transfer much of the control into the demand block from the enzymes of the supply block. In this way they allow precursors for other pathways to be provided as they are required. This point was made in one of the original articles on metabolic control analysis (*4*), but it was given little emphasis and has largely been ignored by later workers. In a recent article Kacser (*13*) even describes the concept of a regulatory enzyme as one of the "victims" of metabolic control analysis, implying that it is a concept that could usefully be discarded.

Nonetheless, simulations of various metabolic systems have shown that the classical regulation mechanisms do have an important role in metabolic regulation (*2, 12, 14*). This role is essentially as it was proposed 20 years ago (*4*), i.e., the transfer of control from supply to demand, but there has been an unexpected result, namely that cooperativity and feedback inhibition are far more important for the concentration of the feedback inhibitor than for the flux through the pathway. In other words fluxes can be regulated reasonably effectively without either cooperativity or feedback inhibition, but only at the expense of very large changes in metabolite concentrations.

METABOLIC CONTROL ANALYSIS

The terminology and principal ideas of metabolic control analysis are thoroughly discussed in various recent reviews (*15, 16*), and only a very brief summary will be

given here. The initial objective was to move away from the traditional mechanism-based view of metabolism toward a *systemic* view, in which kinetic behavior is discussed in terms of how the whole system responds to a change in the parameters that define its environment. If a change in some parameter p changes the rate v_i of the ith enzyme (E_i) when considered in isolation from the pathway (i.e., in the presence of all metabolites that influence it, at the concentrations that occur in the complete system, but in the absence of all other enzymes), then the sensitivity of a flux J through the system can be expressed in terms of a *flux control coefficient* (*17*):

$$C_i^J = \frac{\partial \ln J}{\partial p} \Big/ \frac{\partial \ln v_i}{\partial p}. \qquad [1]$$

The identity of the parameter p does not have to be specified, because the value of C_i^J does not depend on it. If, as is often but not always the case, the rate v_i is directly proportional to the total concentration e_i of E_i, then it may be convenient to take p as identical to e_i, in which case C_i^J may be defined more simply. However, although this type of definition was used originally (*4*), it led to a widespread misunderstanding that metabolic control analysis deals only with effects brought about by changes in enzyme concentrations or changes in limiting rates of enzyme reactions. The present trend in metabolic control analysis is to use the more general definition given in Eq. [1].

Control coefficients are defined similarly for other kinds of variables; for example, a *concentration control coefficient* refers to effects on the concentration s_j of an intermediate S_j:

$$C_i^{s_j} = \frac{\partial \ln s_j}{\partial p} \Big/ \frac{\partial \ln v_i}{\partial p}. \qquad [2]$$

In the standard mechanistic approach to enzymology the kinetic properties of enzymes are expressed in terms of Michaelis constants, inhibition constants, limiting rates, etc. For purposes of metabolic control analysis, however, it is more convenient to decrease the emphasis on mechanism by using quantities that relate effects to causes in a more phenomenological way, similar in definition to control coefficients:

$$\varepsilon_{s_j}^{v_i} = \frac{\partial \ln v_i}{\partial \ln s_j}. \qquad [3]$$

This quantity $\varepsilon_{s_j}^{v_i}$ is called the *elasticity* of v_i with respect to s_j. As this term can seem rather obscure it is helpful to realize that it is the same as the more familiar concept of *order of reaction,* which for enzymes is never a constant, as it depends on the concentrations of substrates, products, and other effectors.

It is obvious that the properties of a system must depend on the properties of its components, but the form of the dependence is less obvious. The important relationships for an introductory understanding of control analysis are the *summation relationships,* which are as follows,

$$\sum_{\text{all steps}} C_i^J = 1, \qquad [4]$$

for any flux J, and

$$\sum_{\text{all steps}} C_i^{s_j} = 0, \qquad [5]$$

for any metabolite concentration s_j. In a simple system, summation over all steps is the same as summation over all enzymes, but in more complex cases it also includes transport steps and allows for the possibility that one enzyme may catalyze more than one step. Provided that one accepts that a control coefficient does in some way measure control, i.e., that it is not just a name, Eq. [4] immediately establishes the crucial fact about flux control, that it is shared among all the enzymes in a system, and in a system of n enzymes the mean flux control coefficient is $1/n$, though as control is not shared evenly individual values can be far from the mean. The existence of negative flux control coefficients in branched pathways implies that the mean of the absolute values may be substantially larger than $1/n$. Nonetheless, in experimental and model systems that have been studied it is rare to find enzymes with flux control coefficients that approach or exceed unity.

It is important to realize that the properties of a system expressed by Eqs. [4] and [5] and similar relationships are independent of any specific regulatory properties of the component enzymes, and although such properties may well exist and affect the distribution of control among the enzymes, striking regulatory properties do not guarantee high flux control coefficients for the enzymes that possess them. For example, despite the well-known sensitivity of phosphofructokinase to various effectors, it is quite misleading to regard it as the primary controlling enzyme in glycolysis: in studies of glycolysis in the human erythrocyte, under various conditions of pH, temperature, and phosphate concentration, it always had flux control coefficients in the range 0.1–0.3, compared with values in the range 0.7–0.9 for hexokinase (*18*).

MODEL

We shall examine the possible ways of manipulating metabolic fluxes on an industrial scale in relation to the model pathway shown in Fig. 2, which is more complicated (and hence, we hope, closer to reality) than those studied in previous works (*2, 12, 14*). It shows the biosynthesis of two end-products, S_{4a} and S_{4b}, from a precursor, X_0, via a branch-point metabolite, S_2. A different type of symbol is used for the precursor to emphasize that it is considered external to the system, i.e., its concentration is fixed and does not depend on the properties of the eight enzymes in the system, whereas all of the other metabolite concentrations are set by the system itself. Although inevitably the numerical values assumed for the eight kinetic equations are arbitrary, the essential features of the model are not arbitrary, and correspond to a typical case of sequential feedback regulation, as discussed in Stadtman's classic article on metabolic regulation (*19*): each of the two end-products inhibits the first committed step of its formation, and the branch-point metabolite inhibits the first step of the whole pathway. Other regulatory designs are, of course, possible (*19*), but similar types of behavior result, in that the normal tendency of