METABOLIC ENGINEERING

Metabolic Engineering

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Preface

Strain improvement is an essential part of process development for biotechnological products as a means of reducing costs by developing strains with increased productivity and yield the ability to use cheaper raw materials, or morespeialized desirable characteristics such as improved tolerance to high substrate and/or product concentrations. The procedure for the development of primary and secondary metabolites overproducing microorganisms so far has been mutagenesis and selection. Thus, the development of many highly productive industrial strains with this procedure has been largely an empirical process. The precise genetic and physiological changes resulting in increased overproduction of metabolites in many of these organisms have remained unknown. Success in attempts to further increase the productivities and yields of already highly productive strains will depend on the availability of detailed information on the metabolic pathways.

During the last few years, genetic engineering and amplification of relevant genes have become a fascinating alternative to mutageneses and random screening procedures. Application of recombinant DNA techniques to restructure metabolic networks can improve production of metabolites by redirected metabolite fluxes. However, as the metabolic activities of cell are accomplished by a network of more than 1000 enzymatic reactions and selective membrane transport systems, it is obvious that models and simulations are very important for carrying out effective metabolic design. Thus, in the first chapter techniques for the *in vivo* quantification of carbon fluxes and their control are presented. As the uptake of nutrients and the excretion of products are carrier mediated, transport processes which are also important steps for a complete describition of the metabolic network, structural and functional properties of carrier systems from pro- and eukaryotic organisms are described in the second chapter. A throught understanding of the elements and mechanisms controlling the biosynthesis and transport of a metabolic should make it possible to influence its rate of overproduction in a predictable wav.

Furthermore, in the field of metabolic design, it is essential to combine improved knowledge about substrate uptake, metabolic networks and product excretion with improved biochemical engineering and modeling methods. Up till now, only a limited number of intracellular metabolites can be measured *in vivo*. NMR spectroscopy in principles is ideally suited as a non-invasive technique, however, it is not very sensitive. As described in the third chapter, a membrane-cyclone-reactor placed in the core of the magnet of the NMR spectroscope is quite useful. Thus a lot of intersting ^{13}P-NMR and ^{13}C-NMR data have been obtained from living cells. More

quantitative information about metabolic fluxes can also be obtained from intracellular biopolymers with ^{13}C labeling experiments. Information on the metabolic network of microbial cells for example can be obtained by measuring the ^{13}C labeled amino acids after protein hydrolysis. From this information together with the improved knowledge about biochemical pathways it is possible to quantify a great number of fluxes in microbial cells as described in the fourth chapter. The data which may be obtained in future from the isotopomere signals may be sufficient to quantify hundreds of different fluxes. For new concepts of quantitative bioprocess research and development the interrelation between cell and environment must be studied in more detail. Better fundamental understanding results in process intensification and thus in a more stringent demand for high performance tools. Thus, improved online analysis methods for better bioprocess control and automation are described in the fifth chapter.

We hope that the reader of this book will enjoy some of the fascination we have experienced in a real interdisciplinary work. A biochemist who has to learn about adaptive parameter control in a bioreactor is in a demanding situation as a biochemical engineer who has to learn more about metabolic pathways. A NMR specialist has to learn about sterile technique and a mathematician about the different metabolic pathways. All this has helped us gain a better understanding of the fascinating field of metabolic flux analysis.

Jülich, December 1995

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Quantifying and Directing Metabolite Flux: Application to Amino Acid Overproduction

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The aim of understanding the metabolism of the cell in order to derive how high-level product formation can be achieved has led to astonishing progress with respect to the in vivo quantification of carbon fluxes and the quantification of flux control. This quantification usually holds only for one specific flux situation thus requiring additional approaches in order to obtain indications of how to produce a flux increase of relevance useful for overproduction. This is in part due to the complex responses of cells to high flux increases, including altered gene expression, alteration of energy metabolism, or accumulation of toxic intermediates, thus preventing a quantitative prediction of the targets to be altered. Instead, experimental techniques have to be used to examine the resulting consequences for each individual example. Currently, it is being found that branching points in biosynthesis, precursor supply in fueling reactions, and export of metabolites deserve particular attention in order to increase metabolite flux. By applying molecular techniques to such selected targets of the L-lysine and L-isoleucine biosynthesis of *Corynebacterium glutamicum*, high amino acid overproduction can be obtained with this bacterium.

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1 Introduction

It is a fact that knowledge of individual enzymes and pathways is insufficient to understand the entire metabolism. This view arises from such extreme poles as the theoretical considerations of the biochemical system theory [1, 2] and the practical example of overproduction of metabolites, where manipulating one reaction or even a set of reactions usually does not result in overproducers of industrial interest. Therefore, a comprehensive view of the entire cellular metabolism is mandatory. This is a current focus of research in biotechnology. In this field experts from a variety of disciplines, e.g. engineers, molecular biologists, biophysicists, biochemists, or physiologists, are busy using a variety of concepts and techniques. The aim is simply to quantify and direct the metabolite flux. The inherent aim is of course to predict in quantitative terms how to tailor the metabolism of the cell for maximal production of metabolites of industrial interest. As mentioned, the research to reach this goal comes from very different directions. For instance, the engineering approach originates from process engineering where control of the overall process is at the centre of attention and the cell is regarded merely as a catalytic component. This mechanistic view, focusing on extracellular parameters and using them as regulatory devices, has been extremely successful in improving and controlling production processes. The physiological approach is located at the other pole of research where the catalytic component itself, namely the cell, is at the centre. Here, for example, individual enzymes are deregulated, competing reactions reduced, or limiting activities increased. This approach is directed by and large towards qualitative physiological information. It has been most successfully applied to strain development. In fact, most of the classical strains producing amino acids, nucleotides, vitamins or antibiotics have been successfully bred without a complete understanding of the entire metabolic network. At this time, attempts are being made to combine these extreme poles and the diverse disciplines for a rational design of cellular metabolism with the aim of achieving maximum output of the desired metabolite.

It is the purpose of this review to present an overview of the different methodologies and concepts developed for flux quantification and their major areas of application. In particular, it will consider what kind of questions relevant for practical purposes can be answered by these approaches. Furthermore, it will describe how individual reactions or pathways structures in the cell can be identified. This appears trivial since the basic network of carbon flux seems to be known already from textbooks. However, there are exciting recent discoveries for organisms of long-standing use in industry. We then will outline an example of intracellular flux quantification using the most advanced methodology. Finally, the use and development of the practical means to direct intracellular fluxes will be described, together with their successful application.

2 Principles of Flux Estimations

Approaches giving access to cellular metabolism are shown in Table 1. Short general comments on their application and limitations are also given. Methods 1 and 2 are analytical tools based on theories and models. Methods 3–5 are experimental techniques for quantifying intracellular fluxes, and methods 6 and 7 are experimental techniques directed towards individual reactions.

2.1 Kinetic Based Models

The kinetic-based models for describing the metabolism rely on the kinetic parameters of each individual enzyme. The most detailed is the description of an enzyme reaction when the rate constants for the interconversion of the intermediate complexes (enzyme-substrate, enzyme-product) are also included in the respective formalism [3]. These reactions are simplified when specific rate constants are set to zero, for instance when the dissociation of the enzyme-substrate complex is considered to be irreversible. Then the description of that specific enzyme reaction becomes reduced and kinetic parameters can be obtained more easily from Lineweaver-Burk plots, Hill plots etc. [4]. Such kinetic parameters are the affinity constants for substrate(s), the affinity constants for activating or inhibiting effectors for the allosterically controlled enzymes, and the maximal velocity of the in vivo reaction. This information, together with that on concentrations of substrates and effectors, as well as fluxes through reactions, can be cast together in a model of the metabolism. Since, in vivo, the net fluxes through reactions are significantly smaller than the maximal velocities of enzymes, while the kinetic parameters hold for the entire activity range of the enzymes, this kind of analysis is in principle predictive. Thus, the advantage of such a description is that it follows a mathematical analysis for rate-limiting steps.

However, the obvious disadvantage is that for most systems the required detailed information will not be readily available. Another disadvantage is that there is no guarantee that all the expressions are comprehensive, simply because molecular research may not yet have identified all the specific reactions or regulatory properties that occur in vivo. The validity of the model depends, moreover, on the applicability of the enzyme constants that are derived from in vitro studies and therefore might be significantly different from the in vivo situation. Thus, in vivo, the polyelectrolytic conditions and protein concentrations are very different from the diluted in vitro conditions [5]. An additional problem is that an influence of concentrations on gene expression must be included, if known at all. Therefore, the requirements which have to be fulfilled usually prohibit the application of this approach for quantification of the entire cellular metabolism. Nevertheless, such a model has been made, for instance, for

Table 1. Means of analysing and quantifying fluxes

Type of analysis	Requirements	Advantage/Use	Disadvantage
(1) Kinetic-based models	Knowledge of the kinetic parameters of all reactions involved	Analysis of control architecture, and in theory prediction of limitations	The required information will usually be severely limited
(2) Control theories	Flux measurements at slightly altered enzyme activities	Numerical value for the importance of an enzyme. Values valid for one specific flux situation only	Experimentally difficult to achieve a set of minutely altered individual enzyme activities
(3) Tracer experiments	Specifically labeled substrates. Identification of label in products	Identification of pathway structure. Gives flux ratios at branch points	Yields relative fluxes
(4) Magnetization transfer	NMR-visibility Instrument necessary	The only direct in vivo quantification of reaction rates	Low sensitivity of NMR spectroscopy. Typically only rate constants between 0.05 and 5 s^{-1} are accessible
(5) Metabolite balancing	Establishment of metabolite and of carbon balance	Readily accessible, yields absolute extracellular fluxes	Requires extreme simplification of intracellular network
(6) Enzyme analysis	Establishment of assay	Identification of pathways. Identification of allosteric control	No information on the contribution to in vivo use and flux
(7) Genetic analysis	Applicability of directed mutagenesis (reverse genetics)	Assay for the necessity of a reaction, for the presence of isoenzymes	No information on in vivo use and flux. Gene deletion disturbs metabolism

the highly specialized red blood cell metabolism [6]. The model accounts for the pentose phosphate pathway, glycolysis, nuelcotide synthesis, transmembrane transport of key ions, pH dependence, magnesium complexation, electroneutrality, and osmotic balance. However, it has not been used as a predictive tool, nor have practical experiments been made to assay the validity of the model. For *Corynebacterium glutamicum* a kinetic-based model of the phosphoenolpyruvate-converting reactions was made [7] within the framework of a metabolite balance study. The kinetic representation used published kinetic constants for phosphoenolpyruvate carboxylase, pyruvate kinase, and five move enzymes, as well as estimates for intracellular metabolite concentrations and fluxes. Based on simulations of the kinetic representation, it was established that phosphoenolpyruvate carboxylase is of the utmost importance for high lysine yield. However, this conclusion was not experimentally verified. Instead, a sub-

sequently constructed mutant devoid of phosphoenolpyruvate carboxylase was influenced neither in lysine overproduction for growth [8], which unambiguously negates the conclusion drawn from this kind of analysis.

2.2 *Control Theories*

Several extensive theories have been developed, not relying on enzyme kinetics, with the aim of quantifying the control strength of a particular reaction on the overall flux in a metabolic pathway. The theories are metabolic control analysis [1, 2], the biochemical systems theory [9] and a flux-oriented theory [10] combining aspects of the first two theories. All the theories include a form of sensitivity analysis, where the response of the whole system (usually the flux) to a small change (in the range of a few per cent) in a parameter (usually an enzyme activity) is quantified to derive specific control coefficients. Although inhibitors can be used to achieve the necessary small enzyme activity variations [11], or genetic instruments applied [12], there are severe practical problems in obtaining the sets of data required. Therefore dynamic approaches with in vitro systems are also used [13]. The current rapid sampling techniques developed to quantify intracellular metabolite concentrations [14, and see the contribution to this volume by Weuster-Botz et al.] will probably enable an easier data acquisition for flux analysis in the future. In contrast to the small changes required to derive control coefficients eventually, large enzyme activity variations can be obtained rather easily by genetic engineering. As a consequence, a theoretical framework has recently been presented by one of the originators of metabolic control analysis to analyze the response of metabolic systems to such large perturbations [15, 16]. Another recent development within metabolic control analysis is a "top-down approach", where the entire metabolism is divided into pathways which are connected by individual specific metabolites [17]. If this specific metabolite can be varied in its concentration, and the resulting flux changes quantified, information about the control exercised by complete sections of metabolism can be obtained, instead of for individual reactions only. Of major interest is, of course, how the different mathematical treatments of often abstract situations capture the relevant aspects of metabolism and control. In a "consumer test" [18] four different theories were compared to derive control coefficients for in vitro gluconeogenesis. Considerable difficulties were encountered in applying the theory to the practical situation. Slightly different answers were obtained to the question of which reactions exert significant control over pathway flux, but no answer could be obtained to the question of which regulatory mechanism is most important for pathway control under physiological conditions.

The important virtue of metabolic control analysis is that it has revealed that control of the overall flux of a metabolic pathway is distributed among several reaction steps instead of being localized at a single rate-determining reaction [19]. Apart from the practical difficulties mentioned before, a basic disadvan-

tage of control analysis is that it only gives coefficients for one specific flux situation and that it has no predictive power for significantly different situations. Moreover, the assessment of control strength for allosterically controlled enzymes would pose even more severe practical problems for acquiring the necessary experimental data, although such allosterically controlled enzymes are of major importance when metabolite overproduction is attempted. Thus it seems in general more pracicable to oversynthesize the feedback-resistant enzyme in question by r-DNA techniques, thereby making an extreme change in enzyme activity, and simply evaluate the result. As an example, oversynthesis of feedback-resistant prephenate dehydratase within aromatic amino acid biosynthesis results in increased flux towards phenylalanine [21]. The goal of merely deriving a number for the control strength of the dehydratase reaction would have required knowledge of the actual in vivo activity of the enzyme, together with that of cellular effector concentrations at various fluxes. However, the success of flux increase can be judged directly by metabolite accumulation, and then new reactions have to be considered directly in the next step of flux increase. This could be, in the simplest case, a new "limiting" enzyme in the pathway, or even an entirely different kind of reaction. One such documented consequence of enzyme overexpression is the accumulation of high levels of pathway-related intermediates disturbing the cellular metabolism. This is presumably the case with many strains derived for metabolite overproduction, since impaired growth is often reported upon oversynthesis of controlling enzymes. As an example, the accumulation of toxic intermediates has actually been shown to occur in strains overproducing tryptophan [21], and in strains overproducing threonine [22].

2.3 Tracer Experiments

An important experimental technique for flux quantification is the use of tracers that can be either radioactive or stable isotopes. They are used on the assumption that they are biologically indistinguishable from their normal analogues. Although the main use of tracers is to analyze the structure of pathways, with a recent example of citrate metabolism in anaerobes [23], determination of the fluxes through pathways is also possible. Extensive use has been made of ^{14}C labeled precursors [24]. Specifically, [1-^{14}C]glucose and [6-^{14}C]glucose were used to quantify the use of the pentose phosphate pathway and glycolysis [25]. In an attempt to quantify cellular fluxes in *E. coli*, accumulation ratios of ^{14}C label into several cellular fractions were quantified. Together with a detailed analysis of ^{13}C label incorporated into glutamate, and a series of mass conservation equations, the flux rates through the tricarboxylic acid and dicarboxylic acid cycle were quantified [26, 27].

Of special benefit is the use of the ^{13}C isotope instead of the ^{14}C isotope. There are many reasons for this. First of all, incorporation of label in the individual carbons of one metabolite is directly accessible with ^{13}C NMR