Metabolic control analysis in drug discovery and disease

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Metabolic control analysis (MCA) provides a quantitative description of substrate flux in response to changes in system parameters of complex enzyme systems. Medical applications of the approach include the following: understanding the threshold effect in the manifestation of metabolic diseases; investigating the gene dose effect of aneuploidy in inducing phenotypic transformation in cancer; correlating the contributions of individual genes and phenotypic characteristics in metabolic disease (e.g., diabetes); identifying candidate enzymes in pathways suitable as targets for cancer therapy; and elucidating the function of "silent" genes by identifying metabolic features shared with genes of known pathways. MCA complements current studies of genomics and proteomics, providing a link between biochemistry and functional genomics that relates the expression of genes and gene products to cellular biochemical and physiological events. Thus, it is an important tool for the study of genotype–phenotype correlations. It allows genes to be ranked according to their importance in controlling and regulating cellular metabolic networks. We can expect that MCA will have an increasing impact on the choice of targets for intervention in drug discovery.

The deterministic view of biology, which holds that the behavior of a biological system ultimately is dictated by its genetic structure, is spawning an explosion of biological information. Genomic analyses have identified hundreds of genes that control an ever-growing network of transcription factors, signal transducer proteins, cell cycle regulators, and metabolic enzymes (e.g., for review, see refs 1–3). The study of the expression of genes and of the effect of mutations on their expression in many organisms forms the basis of functional genomics (see refs 4,5 for reviews). Indeed, when the expression of a sufficiently large array of genes is determined, it is possible to better define the functional state of an organism by the binary outcome of such analyses[6]. More recently, emphasis has shifted to understanding cellular function in terms of the expression of the various coded proteins—so-called proteomics.

Even as new information on DNA, mRNA, and proteins is collated, however, it does not account for the interactions between metabolic substrates and signaling pathways. Because signaling events lead to related metabolic reactions, which in turn modify other metabolic functions or gene expression, it is impossible to understand biological systems merely as a set of components of the genome, transcriptome, proteome, or metabolome. It is extremely difficult to separate signaling events from their related metabolic reactions and to define the precise boundaries of individual gene action within a biological system of complex regulatory mechanisms[7].

With the availability of "complete" genomes, emphasis on individual genes and their direct control over metabolic pathway enzymes must be supplanted by an emphasis on metabolic control in a complex metabolic network. The behavior of metabolic networks in mammalian cells, as indicated by the flow of substrates, is the culmination of regulatory processes at many levels, including the expression of enzymes and regulatory proteins and the transcription of genes into mRNA based on sequence information of genomic components of the DNA. Needless to say, these elements of the regulation of cell functions are interconnected, complex, and regulated at each level by different mechanisms[8]. Such complexity is well known in engineering fields, where sophisticated modeling programs have been developed to simulate the complex behavior of structures to determine interactions among building components that determine ultimate strength. Unlike engineers, biologists are handicapped in their study of complex metabolic behavior in that the structures and the interactions among building components of the system are mostly unknown.

In this review, we summarize progress in the use of metabolic control analysis (MCA) to both characterize system behavior and identify crucial steps in metabolic pathway regulation. Such information is necessary for predicting critical enzymatic target sites for genetic, chemical, or metabolic intervention (through gene therapy, small-molecule drugs, or dietary treatments). We provide examples showing how MCA can be applied to predict the quantitative relationship between aneuploidy and the development of cancer, to understand certain forms of diabetes and enzyme myopathies, and to identify key points of intervention in metabolic pathways linked to cancer. MCA provides a focused approach in the search to identify and characterize influential metabolic reactions in cell behavior, which can be used as targets for effective therapeutic interventions against these still poorly understood disease processes.

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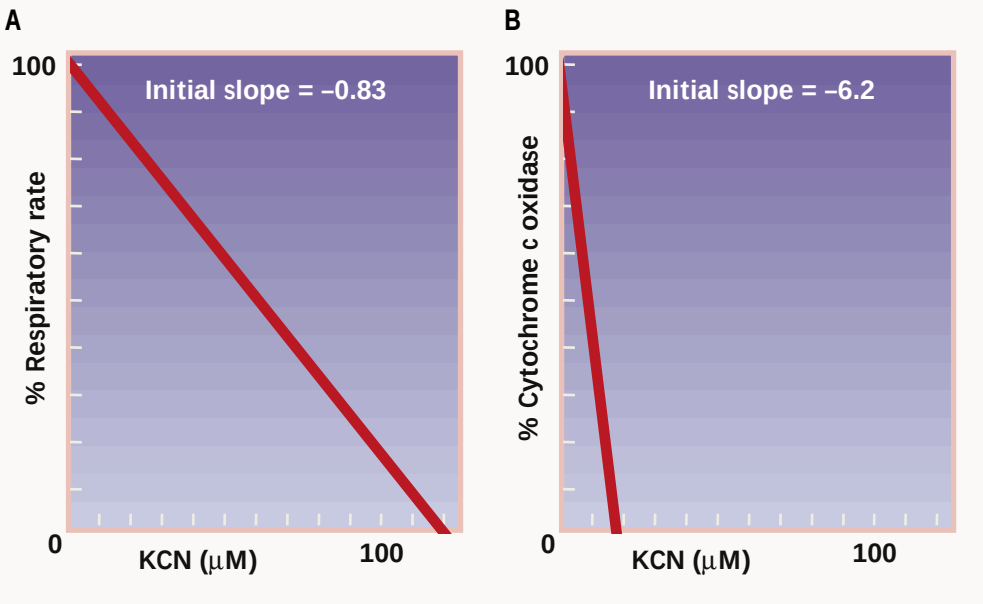

Figure 1. Definition of a control coefficient of an enzymatic step. In this case, the control coefficient corresponds to the fractional change in oxygen consumption in mitochondria over the fractional change in activity of the mitochondrial enzyme cytochrome *c* oxidase. Percentage of oxygen consumption flux (A) and percentage of cytochrome *c* oxidase activity (B) as a function of the concentration of KCN (the specific inhibitor) in rat mitochondria isolated from heart. Adapted from the experiments described by Rossignol *et al.*[20]. Control coefficient is computed as: (initial slope in curve A) /(initial slope in curve B) = –0.83/–6.2 = 0.13.

Modeling metabolic networks

Predicting the behavior of metabolic networks in mammalian cells presents several major challenges. First, our knowledge of the individual components of the network is often inadequate[9,10]. Second, the networks likely involve metabolic steps with nonlinear kinetics that highly increase complexity[9,10]. Third, studying networks at the level of the gene may be difficult, because naturally evolved metabolic networks of mammalian cells inherently are not optimized at the genetic level. Modification of genes can alter only enzyme (protein) synthesis; it has little direct influence on substrate availability or reaction kinetics, which control the behavior of a well-controlled metabolic network.

During the past few decades, a great deal of effort has been devoted to genetic manipulation of cells and microorganisms with the goal of improving the production of biological materials for industrial purposes. However, the targets of such genetic manipulations, which can now be readily done, are still distant from the sites of metabolic control. Similar difficulties have been encountered in the gene therapy of metabolic diseases when single-gene intervention has been used. There are relatively few accounts of successful metabolic flux alterations as a result of gene therapy because of the complex, nonlinear nature of the metabolic control architectures[11–13]. In 1993, this observation led Stephanopoulos and Sinskey[14] to conclude that a rational approach to metabolic engineering that analyzes reaction network characteristics is very much needed in order for the efficient genetic manipulation of metabolic networks to progress.

Actually, as Bailey[10] pointed out, approaching the cell as a complex system has long been an established principle in metabolic engineering and, by facilitating the identification of drug targets in biochemical networks for medical intervention, this should make more efficient the process of validating drug targets and ultimately help redress stagnation in the rate of pharmaceutical discovery. The most crucial challenges are to localize the frequently elusive controlling enzymatic steps that strongly affect metabolic substrate flux and thus serve as adequate targets of further genetic (gene therapy, antisense), chemical (drug), or metabolic (lifestyle/food) interventions.

Principles and applications of MCA

One of the tools frequently used to analyze metabolic networks is stoichiometric models. These models apply mass balances around the network of intracellular metabolites to calculate metabolic fluxes throughout the network. Inputs for this analysis are typically the uptake and secretion rates of sets of metabolites, and often ^{13}C-enriched sources are used. As pointed out by Nielsen[15], however, flux analysis using metabolic stoichiometric models can only study interactions between different pathways and the quantification of flux distributions around branch points, but not evaluate how fluxes are controlled.

During the past two decades, two main theoretical frameworks have been developed for studying the genetic, enzymatic, and substrate level control mechanisms in metabolic networks. These are the biochemical systems theory (BST), developed by Savageau (see reviews[16,17]), and MCA (see reviews[18,19]). Although these two theories share many elements and are to a large extent equivalent, MCA has become the preferred method for the study of metabolic control properties of metabolic steps, probably because non-mathematicians find its language easier to understand.

In MCA, the control exerted by each and every enzyme in a metabolic network over substrate flux or any other systemic parameter (i.e., metabolite concentration, hormone secretion, or cell proliferation) can be described quantitatively as a control coefficient. Control coefficients of enzymatic steps are defined as the fractional change in the systemic property over the fractional change in enzyme activity. Control coefficients are frequently determined by measuring these fractional changes after applying specific enzyme inhibitors. An enzymatic step is considered to have the highest control coefficient when fractional change of enzyme activity and fractional change of the system parameter are parallel to each other. This is illustrated in Figure 1, where the determination of control coefficient of cytochrome *c* oxidase on mitochondrial oxygen consumption is plotted according to the method of Rossignol *et al.*[20] In this case, the control coeffiecient is low and altering the levels of the oxidase would have relatively little effect on oxygen consumption of the system, mitochondria.

In metabolic networks where the individual enzyme–substrate kinetics are known, MCA can be used to derive the individual control coefficients that predict the response of the system to perturbations[18] as, for instance, changes in substrate availability or "genetic" perturbation of the network. On the other hand, in poorly defined systems where enzyme–substrate kinetics are unknown, control coefficients can be experimentally determined by varying the parameter of interest and measuring the changes in the relevant substrate flux. For example, when determining the control coefficient of a metabolic enzyme (enzyme A) on tumor cell proliferation, one would administer the specific inhibitor of enzyme A to cultures of tumor cells and subsequently measure the parallel decrease in enzyme A activity and cell proliferation. The slope of the log–log plot of tumor cell proliferation versus enzyme activity gives the control coefficient of enzyme A on cell proliferation (C_A). In the particular case where the activity

decreases by the same relative amount as the cell proliferation, the value of the control coefficient is unity. We assume that the enzyme inhibitor we used in this example is a specific inhibitor of enzyme A and does not affect other enzymes in tumor cells.

Figure 2 shows the double log plots of the dependence of tumor cell proliferation on the activity of an hypothetical enzyme A having a low (Fig. 2A) and high (Fig. 2B) control coefficient. An enzyme is considered to have a high control coefficient when changes in its activity are reflected totally in changes of tumor cell proliferation. In such a (hypothetical) case, the control coefficient value for the enzyme would be unity. An enzyme is considered to have a low control coefficient when changes in its activity have small or negligible effect on the tumor cell proliferation. Thus, by means of control coefficients, MCA provides an easily understandable quantitative description of how metabolic enzymes or transport proteins control biological functions in complex mammalian cells.

Control coefficient distribution among various enzymatic steps in a metabolic pathway gives us the initial assessment of where to intervene within a metabolic network. By either direct enzyme inhibitors or genetic manipulations of these steps, one hopes to achieve the desired values of metabolic substrate flux that affect systemic parameters such as cell proliferation or cell-cycle progression in studies of cancer cells. In spite of the fact that the application of MCA in biotechnology is still a young field, some promising examples have captured the attention of metabolic engineers, drug developers, and basic scientists (see refs 18,19 for reviews).

Drug target identification

An important puzzle in drug discovery is how to identify key targets in disease pathways. Differential expression of genes between diseased and healthy tissue is commonly taken as an indication of the specific targets of disease pathways. Such an approach ignores the obvious fact that the robust metabolic network of feedback loops and regulatory mechanisms within cells has evolved to maintain homeostasis and to withstand a variety of genetic and environmental insults. The interlinking of disease pathways through such a metabolic network increases the difficulty of identifying therapeutic targets using information from gene expression analysis. On the other hand, MCA approaches the problem of drug targeting by examining the contribution of individual components within a metabolic network, providing a theoretical framework for describing metabolic/signaling/genetic systems of any complexity.

An attractive feature of MCA is that it does not require all system components to be characterized *a priori*[18,19]. Moreover, control coefficients can be estimated for different components of the network and for pertinent environmental factors. The control coefficients give a first approximation of which proteins or pathways may exert more control on the system properties to be modified. Targeting the steps with higher control coefficients on relevant system properties, such as tumor growth or obesity, could be a good strategy to design effective therapeutic agents.

An extension of MCA is the systems approach to drug discovery, currently advocated by Entelos (Menlo Park, CA). In this "top-down" strategy, computer models are constructed for specific diseases (e.g., human obesity, asthma, and AIDS) that integrate the major physiological systems involved in human metabolism. Such a systems model permits the simulation of therapeutic interventions. It is claimed that the approach has provided insights into the reasons why many anti-obesity agents have not yet yielded the expected weight loss in clinical trials. Such analysis identified drug targets that are not the gene products identified as being differentially expressed, but others lying up- or downstream of these genes within the metabolic network (Michelson, S., personal communication).

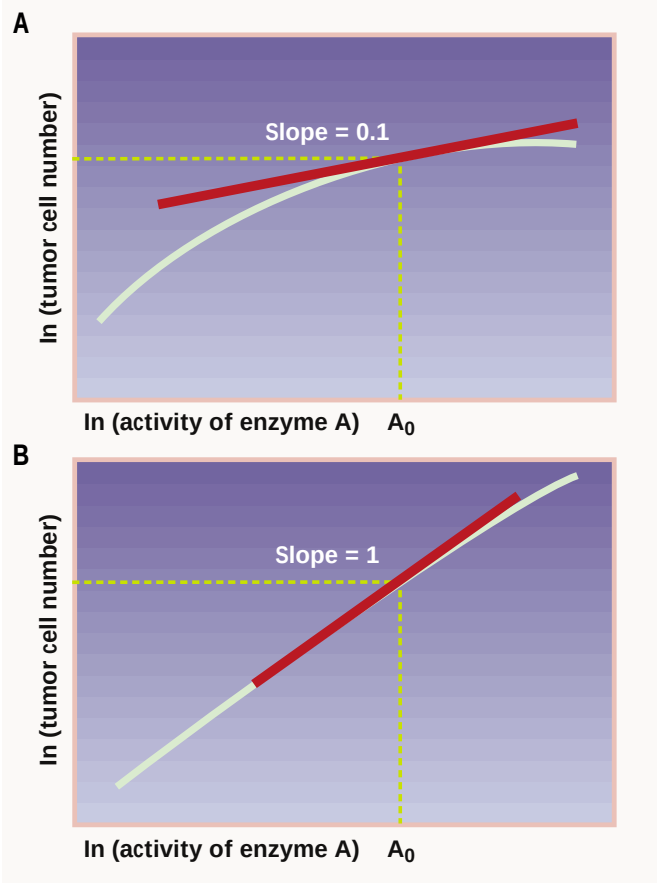

Figure 2. A double logarithmic plot of the dependence of tumor cell proliferation, versus an hypothetical enzyme A activity. The tumor cell proliferation control coefficient is computed as the slope at the initial point A_0: (A) Enzyme A shows a control coefficient of 0.1 on the tumor cell proliferation; (B) Enzyme A shows a control coefficient of 1 on the tumor cell proliferation.

In a manner similar to differential expression profiling, the analysis and comparison of control coefficient profile between host and parasite or between tumor cells and their normal counterpart allows the identification of differences in metabolic adaptation that can be exploited to identify selective drug targets against a parasite or a cancer with minimal effect on the host.

The framework of MCA in drug design also provides insight into the possible advantages and disadvantages of irreversible and noncompetitive inhibitors with respect to competitive inhibitors[21]. While specific irreversible inhibitors are the best option to permanently inactivate a particular enzyme, small-molecule drugs are generally competitive inhibitors. Cornish-Bowden and Eisenthal[21] have pointed out that competition works both ways. Any molecule that can compete with the substrate for binding to the active site of an enzyme is a molecule that the substrate can compete with. In practice, this means that in many cases a modest increase in the substrate can overcome the inhibitory effect of the drug. On the other hand, though noncompetitive inhibitors overcome this problem, they can cause a catastrophic increase in an intermediate metabolite of the pathway and the metabolic system can quickly become unstable. Thus, Cornish-Bowden and Eisenthal[21] emphasized that one must consider not just the therapeutic value of a small-molecule drug in terms of its effectiveness in competition with its natural substrate analogue for binding to the enzyme, but also the possibility that effective competition by noncompetitive

inhibitor can cause a catastrophic increase in pathway intermediates and result in a metabolic collapse.

Another promising area of MCA application is the rational design of combined drug therapy for metabolic disorders. Although the presence of every enzyme in a sequence is essential to a metabolic process, the overall stimulatory or inhibitory effect of a drug is likely achieved with lower concentrations for those enzymes with high flux-control coefficients than for enzymes with low coefficients[18,22]. MCA can be used to identify where particular metabolic enzymes with high substrate flux-control coefficients reside along the network of enzyme pathways as potential targets for intervention.

Functional genomics

One of the major challenges of functional genomics is to determine the roles of genes newly discovered by sequencing the genome of an organism[23]. The functional significance of a gene can be studied at the level of the genome, transcriptome, proteome, and metabolome[24,25]. However, as many genes do not seem to be required for survival, energy production, metabolic fluxes, or cell growth[23,24], the task of determining gene function is much more than simple determination of gene expression.

Recently, Raamsdonk and colleagues[23] have shown that metabolome analysis can be used in combination with MCA to elucidate the function of "silent" genes in an approach called functional analysis of co-responses in yeast (FANCY)[23,26]. The FANCY approach requires knowledge of the metabolome (metabolite concentrations under a specific nutrient condition) of a known mutant and the wild type. Clustering of these metabolites allows the separation of the mutant and the wild-type. Similar metabolic study is then carried out for the silent gene mutant. When a silent gene encoding an unknown function or product is deleted, the resulting metabolite profile and its response to change in the study condition (i.e., the co-response coefficient, as defined by Hofmeyr and Cornish-Bowden[27,28]) will be similar to that obtained for the deletion of a (known) gene that acts on the same functional domain of the cell[23,29,30].

Using *Saccharomyces cerevisiae* strains, Raamsdonk *et al.*[23] showed experimentally that the measurement of co-response coefficient profiles can reveal the function of a silent gene, even when deletion of the gene has no measurable effect on yeast growth rate. On the basis of these results, Cornish-Bowden and Cárdenas[31] predict that co-response coefficient profile analysis combined with multivariate statistical techniques, such as principal-components and discriminant-function analysis[32], will in the near future enable the classification of even completely unknown genes into related groups.

Genetic disorders

MCA is also useful for understanding genotype–phenotype correlation in genetic disorders. Clinical manifestations of genetic disorders are known to be highly variable, as sometimes affected individuals with low levels of a given enzyme or protein may not show clinical symptoms, whereas others fall severely ill with just minor changes in the expression of some other metabolic enzymes. The variability is often explained as the "threshold effect" in genetic diseases. This phenomenon can be understood using MCA. The threshold effect depends primarily on the control coefficient of the affected enzyme or protein on the system parameter responsible for the disease symptoms. The dominant or recessive characteristics of mutant alleles can thus be examined using MCA.

Mazat and coworkers[20, 33] have used MCA to explain an important feature of mitochondrial disease: the existence of a threshold in the expression of oxidative phosphorylation complex deficiencies on the respiratory flux or on ATP synthesis. Large decreases in the complex activity initially only result in small decrease in flux. It is only after reaching a certain inhibition level (in most cases higher than 50%) that respiratory flux abruptly falls toward zero. Mazat's group showed that these threshold curves are inescapable consequence of the fact that most of the individual control coefficients of the different oxidative phosphorylation complexes on these fluxes are small.

Agius[34] has also demonstrated a strong association between glucokinase activity, an enzyme with a high control coefficient, and maturity-onset diabetes of the young type 2 (MODY-2) in individuals with a single mutant allele of the glucokinase gene. MODY-2 is caused by mutations in one allele of the glucokinase gene and is characterized by an autosomal dominant inheritance. Affected individuals are heterozygotes with one normal and one mutant allele[34–36]. Agius *et al.*[37] have reported a control coefficient of glucokinase on glycogen synthesis close to one, indicating a truly rate-limiting step. On the basis of MCA applications, the high control coefficient of glucokinase on hepatic glycogen synthesis is sufficient to induce abnormality of hepatic glycogen synthesis in individuals with MODY-2 who have just a single mutant allele[35]. Kacser and Burns[38] have shown that dominance is dependent on allelic differences, as dominance and recessivity of the mutant allele are related to the control coefficient values of the enzyme that it encodes. The finding by Agius *et al.* supports this explanation: MODY-2 presents with a dominant inheritance that correlates with the high control coefficient of glucokinase on glycogen synthesis.

Another example of the genetic dominance concept derived from MCA analyses is enzyme erythropathies associated with deficiencies of triose phosphate isomerase (TPI)[39,40]. In this case, the concentrations of dihydroxyacetone phosphate and the glycolytic flux are not affected, even at low concentrations of TPI[41]. Individuals heterozygous for the mutated alleles do not show clinical symptoms, and those who are homozygous suffer only mild clinical symptoms and rarely death, despite low TPI activities. The recessive inheritance of this inborn metabolic error corresponds to a deficiency in TPI activity, an enzyme with a very low control coefficient in the glycolytic pathway carbon flux. The relatively benign clinical presentations (see ref. 39 for review) are expected for defects of an enzyme with low control coefficient in an otherwise vital metabolic process. On the basis of this theory, genetic changes that determine enzymes with high control coefficients will show dominant inheritance, whereas genetic changes affecting metabolic enzymes with low control coefficients will show recessive inheritance.

Aneuploidy and cancer pathogenesis

Aneuploidy—an abnormal number of chromosomes with either normal or abnormal morphology—has been detected in practically all of the >20,000 solid human cancers for which genotypes have been reported to date[42–44]. It was proposed over 100 years ago[45,46] as one of the major pathogenic factors in cancer development. The aneuploidy hypothesis of cancer is problematic, however, in that it does not provide a specific explanation of how the diversity of cancer-specific phenotypes is produced through gene mutations[44,47].

The role of aneuploidy in determining the phenotypes of cancer cells has recently been re-examined by Rasnick and Duesberg[48] using a modified form of MCA. Their results indicate that the control of phenotypic transformation is determined by the fraction of the genome undergoing changes that affect chromosome number, not by the magnitude of differential expression of individual genes. In this model, the transformation of a robust normal phenotype into cancer requires the alteration of a massive number of genes, a process that can only be accomplished by aneuploid changes in the genome. As a result, cancerous phenotype is associated with cells that have progressed beyond a certain threshold of aneuploidy, which amplifies a massive number of genes (e.g., those involved in glucose intake, cell proliferation, and growth promotion) effectively in rapid periods. Thus, according to these investigators, the initial step in car-

cinogenesis is likely to be the process of aneuploidy. Mechanisms resulting in the increase of aneuploidy in the promotion phase of malignant transformation include either genetic instability of aneuploid cells or tetraploidization followed by a gradual suppression and/or loss of genetic information exerting growth control[48].

The work of Rasnick and Duesberg[48] based on MCA analysis, suggests the hypothesis that certain cancer phenotypes are associated with aneuploidy exceeding a certain threshold, which determines the normal and proliferative metabolic activities. Further studies are required to validate the aneuploidy hypothesis against the mutation hypothesis in determining the major pathogenic factors in cancer cell development. How the expression of many metabolic enzymes through aneuploid changes in the genome results in the development of tumor specific metabolic profile remains to be investigated. As stated below, however, it is likely that aneuploidy significantly increases the expression of certain metabolic enzymes that direct the selective use of glucose toward nucleic acid synthesis and cell proliferation. Such increases in metabolic enzyme activities in the proliferative state are a common final pathway for tumor growth–promoting factors, such as cell-surface hormones, cytokines, transforming growth factors, environmental pollutants, and aging-related specific signal-transducer pathways.

Targets for cancer therapies

Tumor cells are characterized by a high rate of glucose turnover and rapid proliferation, which must be a consequence of changes in the expression of numerous metabolic enzymes involved in glucose oxidation or anabolic glucose use[49–52]. Indeed, the dependence of tumors on glucose turnover is routinely exploited in the diagnosis and classification of human malignancies. Positron-emission tomography studies using the radioactive analog glucose tracer [^{18}F]fluorodeoxyglucose have shown that the increased rate of glucose accumulation in various cancer cells strongly correlates with increased malignancy and invasiveness[53–56]. Besides its diagnostic significance, the dependence of human tumors upon glucose for the *de novo* synthesis of ribose, purines, and pyrimidines can also be exploited for therapeutic intervention. Glucose metabolic enzymes with high control coefficients in nucleic acid synthesis and proliferation are potential targets for new drug development. MCA studies can be used to identify these target enzymes with high control coefficients, and specific tumor growth–modulating agents that impact on these metabolic pathways.

Unlike normal cells, which metabolize glucose for energy, tumor cells use glucose primarily for specific intracellular anabolic processes, mainly the synthesis of nucleotides. Other anabolic reactions, such as lipid- and protein-synthesis pathways, are depleted of glucose carbons[57]. As stated above, such metabolic differences between normal and tumor cells may be the result of significant aneuploidy in undifferentiated cells. The increased expression of anabolic metabolic enzymes probably requires aneuploidy above the threshold for supplying optimal substrate levels in rapidly and continuously dividing malignant cells.

Cell-transforming agents, such as transforming growth factor-β (TGF-β) and organophosphate pesticides, induce a severe imbalance in glucose carbon redistribution between structural and regulatory macromolecules associated with cell proliferation and those associated with cell differentiation[58]. Cell transformation induced by TGF-β is accompanied by increased glucose carbon deposition into nucleic acid and amino acids through non-oxidative metabolic reactions[58]. Such evidence suggests that cell transformation and tumor growth are associated with two processes: first, the activation of metabolic enzymes that divert glucose carbon use from metabolism to nucleic acid synthesis; and second, the phosphorylation, allosteric regulation, and transcriptional regulation of intermediary metabolic enzymes and their substrates.

The most effective existing cancer treatments (e.g., chemotherapy drugs or radiation) interrupt these processes by directly inhibiting DNA and RNA synthesis. The main disadvantage of these therapies is the narrow therapeutic margin of limited selectivity and high toxicity. Using insights from MCA of tumor-cell metabolic abnormalities, it should be possible to alter the malignant phenotype by altering the metabolic functional capabilities of cancer cells. This principle could be applied to the development either of new drugs or of prevention strategies. It could identify regulatory enzymes in metabolic processes that mediate the anabolic use of glucose in cancer cells, even without knowledge of the signal transducer pathways. When chemical inhibitors are used to reverse the anabolic glucose-metabolic process that allows unregulated cell growth, the malignant phenotype of the cells may also be reversed, as predicted by MCA.

One specific metabolic target for such an approach is the non-oxidative part of the pentose cycle. Transketolase, which catalyzes non-oxidative ribose synthesis in tumor cells, has been identified as the key enzyme in the regulation of glucose carbon recruitment for the *de novo* synthesis of nucleic acid ribose[59]. Transketolase has an exceptionally high growth control coefficient in *in vivo* tumor proliferation[60], as determined by MCA. Accordingly, the chemically modified transketolase cofactor oxythiamine has been shown to be an effective treatment in experimental cancer in animals[59]. Specifically, oxythiamine induces a dose-dependent arrest in the progression of the cell cycle in Ehrlich's tumors in mice[61]. Moreover, from the high tumor growth control coefficient of transketolase, it is possible to predict that in advanced tumors, which are commonly thiamine deficient, supplementation of thiamine could significantly increase tumor growth through transketolase activation[57,60]. This has been validated *in vivo* using mice with ascites tumors[60].

Other examples of natural and synthetic tumor-growth inhibitors are genistein and the wheat germ extract Avemar, both of which strongly inhibit the use of glucose for nucleic acid synthesis as a central mechanism of their anti-proliferative action[62,63]. As non-oxidative ribose synthesis is almost unique to most types of tumors, enzyme inhibitors with high control coefficients in both glucose use and non-oxidative anabolic glucose use for *de novo* nucleic acid synthesis are likely to be of great value in future cancer treatment protocols.

Targets for protozoal disease therapy

MCA has also been used to identify the enzymatic steps that control glycolysis in *Trypanosoma brucei*, a protozoal parasite responsible for African sleeping sickness in humans and for the related disease, *nagana*, in livestock, which has important economic consequences[21,64–67]. As *T. brucei* infecting the bloodstream rely entirely on rapid glycolysis for energy production—and because there are significant differences in the glycolytic reactions of the parasite and the host—the glycolytic pathway has been investigated as a source of targets for antiprotozoal agents[21,64–67].

Early work focused on selecting from the protozoan's glycolytic pathway a target enzyme that exerts stringent control on glycolysis in the parasite, while only moderately affecting glycolysis in the host[64,66,68]. In the course of these studies, two features of *T. brucei* glycolysis became apparent. First, the parasite lacks a functional citric acid cycle and thus many of the glycolytic reactions occur in a special organelle, the glycosome. Second, glucose transport in *T. brucei* depends primarily on glucose concentration in the host's plasma, which controls glucose flux only at low extracellular glucose concentrations[66].

At high glucose concentrations, the control of glycolytic flux is taken over by aldolase (ALD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), and glycerol-3-phosphate dehydrogenase (GDH). Therefore, when normal glucose control is present in the host, the parasite's glucose transporter would

seem to be the most promising target for anti-trypanosomal drug development, followed by ALD, GDH, GAPDH, and PGK. However, individuals with a 95% deficiency in ALD, GAPDH, or PGK show negligible clinical symptoms of hemolysis or erythrocyte dysfunctions[41], and MCA reveals that this is because ALD, GAPDH, and PGK have low control coefficients. Therefore, drugs directed against ALD, GAPDH, or PGK would also have high selectivity against trypanosome glycolysis and survival, with minimal host toxicity.

An alternative approach has been suggested by Eisenthal and Cornish-Bowden[21,65,69], who focused on increasing glycolytic metabolite concentrations in parasite cells to toxic levels. As these authors point out[65], this strategy is the most effective when applying a non-competitive inhibitor of an enzyme with a small flux-control coefficient. Three potential candidate steps in parasite glycolysis were first identified that could be blocked with an noncompetitive inhibitor: glycerol transport and its components (phosphoglycerate mutase and enolase); phosphoenolpyruvate transport; and pyruvate transport.

Glycerol transport was subsequently ruled out as a potential target because it carries very low flux under aerobic conditions in this parasite. The fact that the parasite's mammalian host oxidizes pyruvate, rather than excretes it, distinguishes pyruvate transport as the prime candidate for targeting with a non-competitive inhibitor, with the intent of inducing a toxic level of pyruvate in *T. brucei*. The task now is to characterize specific inhibitors of the pyruvate transporter in *T. brucei* identified by MCA as a promising target.

Conclusions

It is clear that large-scale approaches such as genomics, proteomics, and metabolomics have been very successful in establishing the components comprising metabolic and signaling networks. However, the identification of key target enzymes that translate the effect of individual single steps into perceptible effects on the entire network remains a central problem in biological research. The methods of determining substrate flux and the analysis of such measurement using MCA provide an understanding of the contribution of the individual components to the biological behavior of the whole system. Such knowledge can be exploited to provide a rational approach to select the best candidate targets for drug design, as illustrated by the examples in this review.

It is increasingly accepted that knowledge of a given gene sequence cannot be easily translated into a defined phenotype in a particular environment. As reviewed by Kacser and Small[70], certain pathway patterns allow a metabolic system to exist in two alternative stable steady states, depending on the environment. Such dual stability implies that environmental perturbation can switch the system from one state to the other. Thus, a single genotype may show two alternative phenotypes without any change in gene expression. Moreover, the instability resulting from gene–environment interactions is a systemic property that cannot be assigned to any single element.

MCA is thus an important tool in the study of genotype–phenotype correlation. It allows genes to be ranked according to their importance in controlling and regulating cellular metabolic networks. We can expect that MCA will have an increasing impact on the choice of targets for selective modification by genetic, chemical, or other means.

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