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Systems Biology of Metabolism

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Abstract

Metabolism is highly complex and involves thousands of different connected reactions; it is therefore necessary to use mathematical models for holistic studies. The use of mathematical models in biology is referred to as systems biology. In this review, the principles of systems biology are described, and two different types of mathematical models used for studying metabolism are discussed: kinetic models and genome-scale metabolic models. The use of different omics technologies, including transcriptomics, proteomics, metabolomics, and fluxomics, for studying metabolism is presented. Finally, the application of systems biology for analyzing global regulatory structures, engineering the metabolism of cell factories, and analyzing human diseases is discussed.

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1. INTRODUCTION

All life forms depend on the ability to convert nutrients into chemical species that can be used as building blocks for macromolecules such as proteins, lipids, deoxyribonucleic acids (DNAs), ribonucleic acids (RNAs), and carbohydrates. Many cells are also producing so-called secondary metabolites that are secreted by the cell and serve important functions such as defense and communication. The set of these chemical reactions is referred to as metabolism, and most cells perform thousands of different reactions, most of which are catalyzed by specific enzymes. Metabolism is typically divided into catabolism, the breakdown of carbon and energy sources to 12 precursor metabolites, and anabolism, the biosynthesis of building blocks and their further assembly into macromolecules (**Figure 1a**). Catabolism is associated with the generation of Gibbs free energy, which is primarily captured in the high-energy phosphate bonds of adenosine triphosphate (ATP), and redox power, which is stored in reduced nicotinamide adenine dinucleotidephosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH). NADPH is used for the biosynthesis of macromolecules, and NADH is oxidized either through the conversion of precursor metabolites to chemicals that can be secreted by the cells, for example, by the conversion of pyruvate to lactate, or through oxidative phosphorylation (**Figure 1a**).

The 1920s and 1930s were the golden age of metabolism studies when many cellular metabolites were identified and the enzymes of all key catabolic pathways were identified. The two most prominent examples of pathways identified during this period are the Embden–Meyerhof–Parnas (EMP) pathway, which after identification of the pathway intermediates by Otto Fritz Meyerhof between 1912 and 1918 was described as a complete pathway by Gustav Georg Embden in the 1930s, and the Krebs cycle, also referred to as the tricarboxylic acid (TCA) cycle or the citric acid cycle, which was discovered by Hans Krebs in 1937. The EMP pathway, often referred to as glycolysis, is the most highly conserved metabolic pathway. It is present in practically all living cells, and it most likely evolved as a series of spontaneous reactions in the prebiotic world for the conversion of glucose to pyruvate (1). In the 1950s and 1960s, many other pathways were identified, including the Leloir pathway (1951), in which galactose is converted to glucose-6-phosphate, and many amino acid biosynthetic pathways.

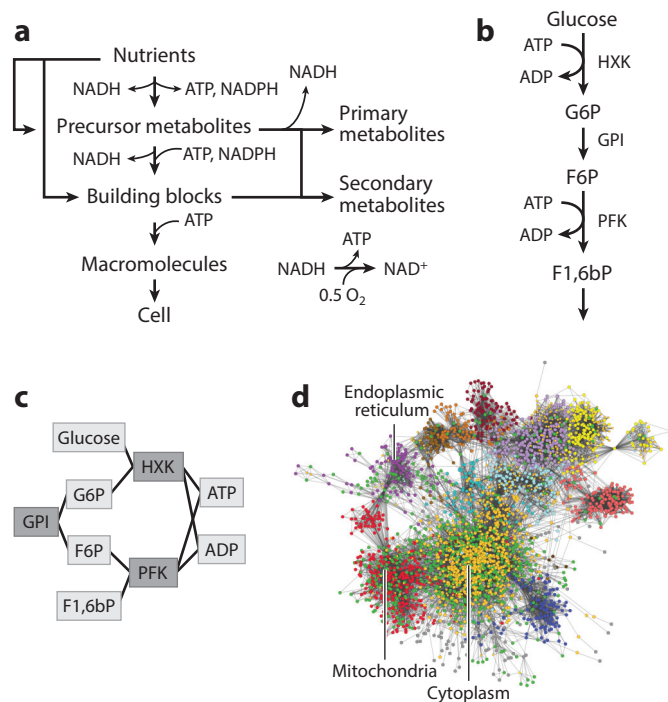


Figure 1

The structure and connectivity of metabolism. (*a*) Metabolism can be divided into catabolism, in which nutrients are converted into a set of 12 precursor metabolites, adenosine triphosphate (ATP), and nicotinamide adenine dinucleotidephosphate (NADPH), and anabolism, in which these precursor metabolites are converted into building blocks that are further converted into macromolecules with the consumption of ATP and NADPH. The precursor metabolites are glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), ribose-5-phosphate, erythrose-4-phosphate, glyceraldehyde-3-phosphate, 3-phospho-glycerate, phosphoenolpyruvate, pyruvate, acetyl-CoA, 2-oxoglutarate, oxaloacetate, and succinyl-CoA. There are approximately 50 building blocks, which include amino acids, fatty acids, and nucleotides. Catabolism and the biosynthesis of building blocks results in the net production of nicotinamide adenine dinucleotide (NADH), which can be consumed either through the conversion of precursor metabolites into primary metabolites or through oxidative phosphorylation, in which NADH is oxidized by molecular oxygen via the electron transport chain and ATP is formed. Secondary metabolites are formed from precursor metabolites and/or building blocks, for example, amino acids. (*b*) A canonical pathway in metabolism, glycolysis (*top*), is illustrated. (*c*) Illustration of how cofactors like ATP and ADP connect different enzymes of glycolysis (*top*) into a metabolic network. (*d*) Illustration of the genome-scale connectivity in metabolism. The network illustrated is a representation of yeast metabolism (Sanchez BJ, Zhang C, Nilsson A, Lahtvee P-J, Kerkhoven E, et al., unpublished manuscript). Green nodes are metabolites, and the other nodes specify enzymes present in different compartments, three of which are indicated. Panel *d* provided by Benjamín Sánchez. Abbreviations: F1,6bP, fructose-1,6-bisphosphate; HXK, hexokinase; GPI, glucose phosphate isomerase; PFK, phosphofructokinase.

The identification of these canonical pathways laid the foundation for how we study and understand metabolism today and mapped the stoichiometry of the individual reactions in these pathways (**Figure 1b**). Despite 100 years of studying metabolism, however, many parts of cellular metabolism remain unknown. For example, the transporter of pyruvate into the mitochondria, a very important step for the complete oxidation of glucose via glycolysis and the TCA cycle in eukaryotic cells, was only recently discovered (2, 3). In particular, secondary metabolism is less well defined. Here, precursor metabolites or building blocks are converted to complex natural products

that cells use for communication or defense (**Figure 1a**). Secondary metabolism is extremely diverse, and plants and microorganisms have the ability to produce a wide range of different natural products, several of which are useful as pharmaceuticals, food ingredients, and pesticides.

Even though metabolism is traditionally organized into canonical pathways, there is a very high degree of connectivity among different reactions. A very large number of metabolic reactions use ATP, NADH, and NADPH, and the common usage of these cofactors ties together reactions that are otherwise parts of different canonical pathways (**Figure 1c**). The 12 precursor metabolites are also used in a large number of reactions, and glutamate and glutamine are used as amino donors in a large number of reactions involved in the biosynthesis of amino acids and nucleotides. Thus, in yeast, >10% of all metabolites participate in more than 10 reactions each, and >4% are involved in more than 20 reactions. ATP participates in >180 reactions; NADPH participates in approximately 80; and glutamate participates in approximately 70 (4). Metabolism is therefore highly connected and consequently forms a very dense biological network (Sanchez BJ, Zhang C, Nilsson A, Lahtvee P-J, Kerkhoven E, et al., unpublished manuscript) (**Figure 1d**). This high degree of connectivity has a significant impact on metabolism and, concomitantly, makes the study of the individual parts of metabolism difficult because perturbing a single metabolic pathway may impact the function of a large part of the complete network.

The high degree of connectivity in metabolism has three major implications. First, to ensure the proper function of cells, it is important to balance the activity of the many different metabolic pathways to the needs of the cell, for example, the need for building blocks, Gibbs free energy, and redox power. This balancing is generally referred to as the homeostasis of metabolism. With the high degree of connectivity of the many different cellular pathways, it is necessary to have extensive regulation at different levels, that is, at the levels of transcription, translation, and enzymatic function. All cells have therefore evolved complex regulatory systems that attempt to ensure metabolic homeostasis even when exposed to widely varying environmental conditions, including stress. The dysfunction of these regulatory systems has major implications for the cell, and thus there is much interest in studying them. Second, with the high degree of connectivity and the extensive regulation imposed to maintain homeostasis, it is inherently difficult to engineer metabolism, for example, to engineer microorganisms for the production of chemicals to be used as fuels, materials, and pharmaceuticals. Third, because of the high degree of connectivity in metabolism, it is difficult to obtain mechanistic insight into the underlying causes for many human metabolic diseases. However, this connectivity also means that almost any perturbation in cellular function will lead to an altered metabolism and therefore result in a metabolic signature. Studies of how metabolic networks respond to the development of human disease may therefore lead to the identification of novel biomarkers and to new therapeutic opportunities.

Here, three aspects of metabolism will be discussed, namely, global regulation, engineering of metabolism, and human metabolism in response to disease. This will be prefaced with a description of the concepts of systems biology, which in short is the use of mathematical models to study biological systems, followed by a discussion of how different types of mathematical models can be used to obtain a holistic understanding of metabolism. Much focus will be on studies of the yeast *Saccharomyces cerevisiae* and the bacterium *Escherichia coli*, as they are probably the best studied organisms in terms of metabolism.

2. SYSTEMS BIOLOGY AS A DISCIPLINE

Systems biology has evolved as a scientific discipline in which computational and mathematical modeling is used to study biological systems (5). These systems typically involve complex metabolic networks, as illustrated in **Figure 1d**, or signaling networks with a similar complexity. Systems

biology uses a holistic approach that studies the complete system, as opposed to molecular biology, which focuses on subsystems often studied via *in vitro* experiments (6). Another characteristic of systems biology is that it involves quantitative analysis, unlike the largely qualitative nature of molecular biology that focuses on hypothesis testing, which is used to determine whether a given (verbal) model describing the system is true or false (6). Despite the different approaches, systems biology is highly dependent on the extensive biological information that has been acquired through molecular biology, and systems biology studies often result in the generation of hypotheses that require confirmation using a reductionist approach.

There are two different approaches to systems biology (7): in top-down systems biology, different types of high-throughput generated data, often referred to as omics data, are analyzed in an integrative fashion, and in bottom-up systems biology, detailed models for specific processes, for example, enzymatic reactions, are assembled into a model describing the system being studied. The two approaches are complementary; top-down systems biology is useful for mapping cellular functions at the genome scale, whereas bottom-up systems biology enables detailed timescale resolution of the impact of individual components on overall system properties. Both approaches allow the identification of what are often referred to as the emergent properties of the biological system, that is, how the interactions between the many different cellular components give rise to biological functions that cannot be identified through a reductionist approach.

Bottom-up systems biology is in essence an older approach that developed from theoretical biology. In the 1950s and 1960s, systems theory was already being used to study biological systems, with Denis Noble's (8) computer model describing the function of the heart pacemaker being a landmark study. In the late 1960s and early 1970s, two lines of research led to the development of rigorous mathematical frameworks that today form the fundamentals of bottom-up systems biology. One approach was driven by chemical engineers who translated reaction engineering principles to model biological systems, with the main objective being to improve industrial fermentation process designs. This research was driven primarily by Arnold Fredrickson and Henry Tsuchiya (9) at the University of Minnesota, who developed an extensive modeling framework for cellular growth models.

The other line of research was driven by biochemists/biophysicists who were seeking to understand complex biological processes, such as signaling pathways and metabolic pathways, through the use of mathematical modeling. A key driver was Reinhart Heinrich at Humboldt University. In 1974, he and Tom Rapoport (10) described a modeling framework that allowed the quantification of flux control in metabolic pathways. A few months earlier, Henrik Kacser at the University of Edinburgh had published a similar mathematical framework with the same objective (11), and these two frameworks have now been combined into what is referred to as metabolic control analysis (MCA). MCA has since been significantly developed, particularly by Hans Westerhoff and Douglas Kell (12), and today is widely used as a conceptual framework for studying cellular processes and for classroom illustration of how fluxes are controlled in metabolic pathways.

A major limitation to the application of these modeling frameworks has been access to high-quality experimental data. The genomics revolution not only resulted in the provision, for the first time, of complete lists of components of the living cells but also resulted in the development of a number of high-throughput experimental techniques that formed the basis for establishing top-down systems biology. This type of omics data provides a wealth of information about the cell, in particular, when it is obtained for cells grown in different conditions. However, the difficulty of gaining biological information from gene lists was soon realized, and this led to the annotation of genes into functional groups, with gene ontologies (GO) as the most important contribution (13). A breakthrough in the analysis of omics data occurred when these data were analyzed in the context of annotated biological networks, as illustrated in a study of the galactose regulon of

yeast by Trey Ideker and coworkers (14). This led to the coining of the term systems biology by Leroy Hood (15) and Hiroaki Kitano (16, 17), who independently established the first institutes for systems biology in Seattle and Tokyo, respectively, in 2000. Today, systems biology finds wide application in basic studies of biology, engineering cells for the production of valuable chemicals, and understanding the molecular mechanisms underlying complex human diseases.

3. MATHEMATICAL MODELING OF METABOLISM

Mathematical models used to describe natural phenomena are generally referred to as laws of nature. However, they are also widely used by engineers to design complex physical systems. One key advantage of using mathematical models is that they can capture information in a very concise way, as is very well illustrated by the Michaelis–Menten equation for enzyme kinetics that essentially describes the kinetics of an enzyme using two key parameters. Mathematical models as a consequence are widely used in biology. However, as there are many aspects of cellular function that are unknown—not just the known-unknowns but even the unknown-unknowns—it has not been possible to build all cellular processes into a comprehensive mathematical model for a living cell. There have, however, been several attempts to build whole-cell models. The most notable of these attempts are (a) the so-called Cornell model for *E. coli*, developed by Mike Shuler (18) in the 1970s, which described cellular functions by 14 (later expanded to 20) key variables; (b) the E-CELL model, also for *E. coli*, developed by Masaru Tomita (19) in the 1990s, which allows the user to simulate cellular functions based on specific inputs; and (c) a model for *Mycoplasma genitalium*, developed by Markus Covert (20) and published in 2012, which represents the most comprehensive mathematical model for a whole cell. This latter model captures many key biological processes and enables the simulation of the linkage between metabolism and other biological processes involved in cellular growth, but it is still based on a coarse description of the different biological processes. This coarseness is inherent to mathematical models as they are an abstraction of the real system, and their key value is in testing hypotheses in biological systems that are represented in a simplified manner. A “complete” mathematical description of a biological system may be of less use, as illustrated in the dialogue with Mein Herr about the value of maps in the book *Sylvie and Bruno Concluded* by Lewis Carroll (1893): “‘And then came the grandest idea of all! We actually made a map of the country, on the scale of *a mile to the mile!*’ ‘Have you used it much?’ I enquired. ‘It has never been spread out, yet,’ said Mein Herr: ‘the farmers objected: they said it would cover the whole country, and shut out the sunlight! So we now use the country itself, as its own map, and I assure you it does nearly as well.’” Therefore, in biology, mathematical models do not aim to reconstruct all elements of the system studied but are instead used as research tools for gaining new insight and assisting in the design of experiments.

There are many different types of mathematical models, but for describing metabolism, these can be divided into two groups: kinetic models and stoichiometric models (or constraint-based models).

3.1. Kinetic Models

Through evolution, enzymes have fine-tuned their kinetic parameters to coordinate the distribution of fluxes emerging from the networks of metabolic reactions. From an analysis of the distribution of k_{cat} values for all enzymes in metabolism, it was found that enzymes involved in central carbon metabolism have the highest k_{cat} values, followed by enzymes of biosynthetic pathways (e.g., amino acid biosynthesis), whereas enzymes involved in secondary metabolism have the lowest k_{cat} values (21; Sanchez BJ, Zhang C, Nilsson A, Lahtvee P-J, Kerkhoven E, et al.,

unpublished manuscript). This finding is consistent with the typical flux requirements for cell growth; that is, higher fluxes are needed in central carbon metabolism, which provides energy and precursor metabolites, than in the biosynthesis of vitamins and cofactors. Enzymes of central carbon metabolism most likely also evolved earlier than enzymes of secondary metabolism and thus have had more time to acquire higher efficiency. Despite generally high k_{cat} values for enzymes in central carbon metabolism, it is interesting to note that they cover more than four orders of magnitude, that is, from approximately 1 to 20,000 per second (22).

The proper function of a metabolic pathway requires that the metabolite levels be adjusted such that flux through each of the enzymes in the pathway is balanced. If not, there will be a rapid increase in the level of certain metabolites, which could have detrimental effects on the overall function of the pathway. Metabolite levels are to a large extent determined by the environmental conditions but also by the kinetic parameters of the enzymes. Typically, only a few enzymes in a pathway are regulated allosterically, but this type of regulation is very important for pathway function; for example, in amino acid biosynthesis, there is extensive feedback regulation to ensure a balanced supply of the different amino acids required for protein synthesis. There have been many studies on the kinetics of enzymes, and much of this information is collected in the BRENDA database (<http://www.brenda-enzymes.org>).

However, most of these studies are based on in vitro analyses using purified enzymes, and for some enzymes, the resulting kinetic expression is quite complex, for example, for phosphofructokinase, which has several allosteric regulators. In pioneering studies on the mathematical modeling of the glycolysis of yeast and *E. coli* headed by Matthias Reuss (23, 24), it was necessary to significantly adjust parameters for enzyme kinetics determined in vitro to allow for the functional simulation of glycolysis. The authors validated their model using dynamic chemostat experiments in which all glycolytic intermediates, ATP, ADP, and AMP, were measured at a timescale resolution of seconds, for 3 min. They observed that certain glycolytic intermediates rapidly acquired new pseudosteady state levels; for example, in yeast, fructose-1,6-bisphosphate and phosphoenolpyruvate changed rapidly in the first few seconds, whereas other metabolites acquired a new pseudosteady state after 2–3 min (23). The detailed metabolite measurements were then used to build a kinetic model for glycolysis, and a key finding was that much of the allosteric regulation identified from in vitro studies does not seem to play a role in the studied conditions (23). This implies that many enzymes have evolved allosteric regulation to cope with extreme conditions, whereas normal environmental conditions do not require all elements of this regulation.

This finding of course has important implications for the mathematical modeling of metabolism because it may be possible to significantly simplify kinetic expressions and still preserve the proper function of complete metabolic pathways. Another key finding from this study on yeast glycolysis was that flux control seems to reside in glucose transport, which is consistent with earlier findings that the overexpression of glycolytic enzymes does not result in increased glycolytic flux either enzyme by enzyme or in combination (25, 26). However, increasing the drain of ATP—for example, by adding a weak organic acid such as benzoic acid that causes decoupling of the plasma membrane pH gradient—is known to increase glycolytic flux in yeast, a phenomenon often referred to as metabolic pull. Similarly, in *Lactococcus lactis*, introduction of an additional ATP-consuming reaction via expression of F_1 -ATPase also increased glycolytic flux by increasing ATP demand (27).

The important role of ATP in controlling glycolytic flux has been elegantly illustrated using a simple kinetic model by Bas Teusink (28). Similar to other catabolic pathways, glycolysis is characterized by first requiring the activation of substrates through phosphorylation. Invested energy is then recouped downstream in the pathway to result in a net production of 2 moles of ATP per mole of glucose converted to pyruvate. If there is a rapid increase in glycolytic flux, for example,

if yeast is transferred from conditions with glucose limitation to conditions with excess glucose, complete drainage of ATP can occur, leading to substrate-accelerated death or stuck fermentation. Yeast is very capable of handling such changes, and this capability can be demonstrated using a simple mathematical model for key glycolytic reactions. Teusink (28) showed that this can be explained by the feedback inhibition of hexokinase by trehalose-6-phosphate (T6P), produced from G6P by T6P synthase (Tps1p). This role of Tps1p could explain why a yeast strain with *TPS1* deletion cannot grow when glucose is in excess; it simply accumulates hexose phosphates and rapidly consumes ATP and inorganic phosphate (29).

With a so-called turbo design, in which energy is initially invested to obtain even more energy later, glycolytic flux must be balanced with ATP consumption, and if there is no initial “brake” on glycolysis, that is, the feedback regulation of hexokinase, then a sudden shift to excess glucose results in imbalance. Mammalian cells do not have trehalose biosynthesis and therefore do not have this feedback regulation. Instead, they inhibit hexokinase by G6P, which appropriately handles the relatively small perturbations in glucose concentrations observed by these cells. However, for the large perturbations that yeast may experience, it is necessary to have a more complex feedback loop such as the one involving T6P. Recently, Teusink and coworkers (30) performed a far more thorough analysis of the *TPS1* deletion mutant, including a more detailed mathematical model, and found that the mutant could operate with stable glycolysis under the right initial concentrations of glycolytic intermediates. The extensive analysis of the *TPS1* deletion mutant, involving mathematical modeling, metabolic flux analysis, and metabolome analysis, is an excellent example of how mathematical modeling can contribute to novel physiological insight (31).

Another seminal example of how kinetic modeling can be used to gain insight into metabolism is a joint study by the groups of Edda Klipp and Stefan Hohmann (32) on how the Hog1p pathway is involved in the regulation of glycerol production in response to osmotic stress in yeast. Hog1p is a mitogen-activated protein (MAP) kinase that regulates the expression of genes involved in stress tolerance, including genes associated with the production of glycerol as an osmoprotectant. As with other MAP kinases, Hog1p is activated by a kinase cascade involving a series of kinases (MAPK kinase and MAPKK kinase); Heinrich (33) had earlier shown, using a simple modeling scheme, that such a cascade of kinases and associated phosphatases allows for a delay in the onset of regulation in response to environmental changes. This creates stability if there are rapid changes in environmental conditions and therefore explains the evolution of kinase cascades in many signal transduction pathways. In their model of the Hog1p pathway, Klipp and coworkers (32) combined a model for the Hog1p kinase cascade with a gene expression module, a metabolism module describing a few key enzymatic reactions, a phosphorelay module responsible for activating the Hog1p pathway, and finally, a module describing biophysical changes in the cell, such as water flow across the cytoplasmic membrane. Upon combining these different modules into a coherent model, it was possible to simulate the dynamic response of yeast cells to osmotic stress in great detail and even to predict the metabolic effects of deleting key metabolic genes (32). Their model was later expanded to include more features and could then be used to follow how osmotic stress rapidly reroutes glycolytic flux towards glycerol (34).

3.2. Genome-Scale Metabolic Models

Genome-scale metabolic models (GEMs) are *in silico* reconstructions of the complete metabolism of a given organism (**Figure 2**). These models are built in a bottom-up approach in which genome information is combined with knowledge about the metabolic capabilities of the cell to reconstruct a complete metabolic map. In this map, the stoichiometry of each metabolic reaction is specified, including specification of the cofactor usage, and each metabolic reaction is linked to an enzyme.

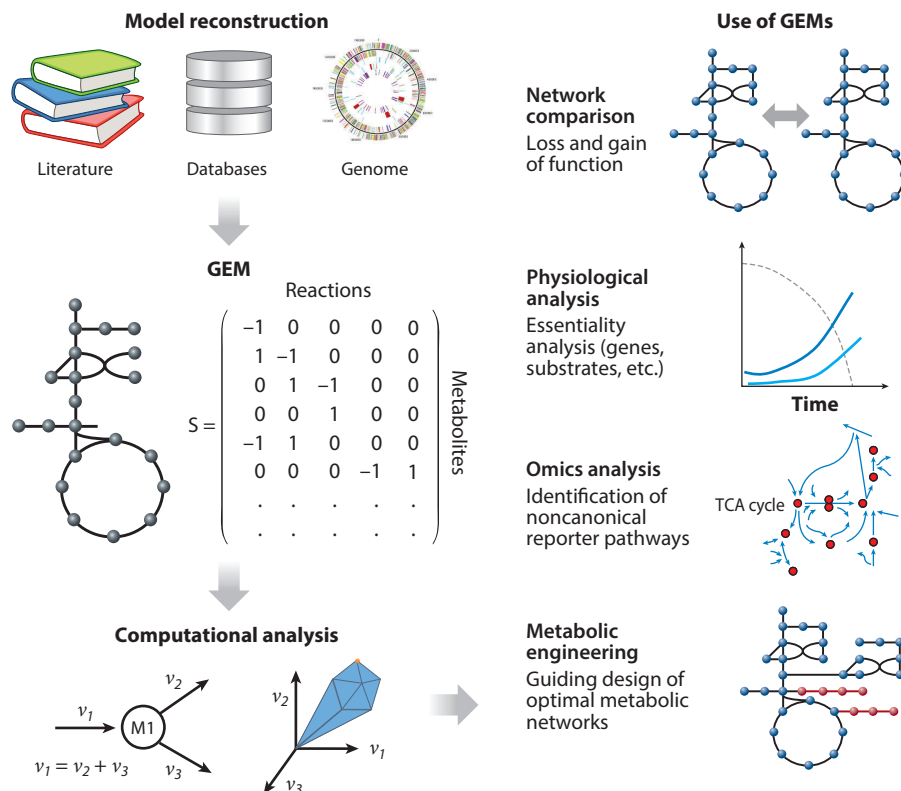


Figure 2

The concept of genome-scale metabolic models (GEMs). GEMs are reconstructed using information from the literature, databases, and an annotated genome sequence. The GEM is represented by a metabolic network and by a stoichiometric matrix, which can be used for computational analysis. Here, flux balance analysis, in which the fluxes around each metabolite are balanced, is used and allows for the identification of a feasible flux space (*blue cone*). A reconstructed GEM can be used for topology (or network) comparison (e.g., between healthy and diseased cells); physiological analysis (e.g., the simulation of growth and performing essentiality analysis); integrative analysis of omics data, in which the GEM is used as a scaffold for identifying reporter pathways and/or reporter metabolites; and identification of metabolic engineering targets (i.e., reactions that should be added to or removed from the network to improve the production of a specific chemical). Abbreviation: TCA, tricarboxylic acid.

Thus, the model provides gene-protein-reaction connectivity, which allows for a direct link between the genotype and metabolic capability. Bernhard Palsson and coworkers reconstructed the first GEMs for *Haemophilus influenzae* (35), *E. coli* (36), and *Helicobacter pylori* (37). Shortly thereafter, a GEM was reconstructed for *S. cerevisiae*, which was the first GEM for a eukaryotic organism (38, 39).

Today, GEMs have been reconstructed for a large number of microorganisms (40) covering all key phylogenetic groups (41), various plants (42), mammalian model organisms such as the mouse, and human cells, as discussed below. The process of GEM reconstruction often results in the identification of nonannotated parts of metabolism, and it is therefore a driver of biological discovery and/or the curation of conflicting experimental data. **Table 1** summarizes key characteristics of GEMs for a few microorganisms (42–47). A particular strength of these models is

Table 1 Properties of key microbial genome-scale models

Organism	No. of genes	No. of reactions	No. of metabolites	No. of compartments	Reference
<i>Haemophilus influenzae</i>	—	488	343	2	35
<i>Escherichia coli</i> ^a	1,366	2,251	1,136	3	43
<i>Saccharomyces cerevisiae</i> ^b	916	3,493	2,218	14	44
<i>Aspergillus niger</i>	871	2,240	782	3	45
<i>Penicillium chrysogenum</i>	1,006	1,471	1,235	4	46

^aMost recently updated model. Several models presented in the past (47).

^bYeast 7.0. The large number of metabolites is due to an expansion of lipid metabolism. Continuously updated at <http://yeast.sf.net>. Previous models discussed in Reference 48.

that they represent a knowledge base; that is, extensive information can be collected, and when more information is obtained, the models can be expanded to include additional reactions or additional cellular compartments. Thus, many GEMs have been updated/expanded, and for *E. coli* and *S. cerevisiae*, there is a continuous advancement in the coverage of these models. The most recent GEM for *E. coli*, updated based on new experimental data obtained from the phenotypic screening of 1,075 knockout strains, comprises 1,366 genes, 2,251 metabolic reactions, and 1,136 unique metabolites (43). Several different research groups have been involved in expanding the yeast GEM, resulting in the establishment of a consensus GEM (49). This consensus yeast GEM has been further expanded and is now continuously updated by a community effort (44, 50); thus, it is a continuously expanding knowledge base for yeast metabolism (48).

GEMs can be used for simulations based on the concept of flux balance analysis (FBA). FBA is an old modeling concept that was first used in 1979 by Shuichi Aiba, a pioneer of biochemical engineering, for the analysis of citric acid production using a simple metabolic network (51). Several different research groups took this concept further for the analysis of more complex metabolic networks. David Fell (52), while studying human adipocyte metabolism in the late 1980s, was the first to use FBA together with linear programming to quantify metabolic capabilities of a metabolic network. Through linear programming, it is possible to simulate metabolic network operation even for very large networks. Indeed, Palsson (53) showed that FBA can be used to simulate the entire metabolic capabilities of *E. coli* using a GEM. FBA relies on balancing metabolic fluxes around each of the metabolites in the network, resulting in a set of constraints on the metabolic fluxes (Figure 2). When this balancing is combined with an appropriate objective function, a solution can be identified using linear programming. The most frequently applied objective function is the maximization of growth, which works very well for microorganisms, but other objective functions have also been evaluated, such as maximizing ATP production (54).

GEMs have found a number of different applications (55) (Figure 2); for example, they can be used directly to compare the metabolic capabilities between different cells. This comparison is relevant in biotechnology, in which it is important to choose the right cell factory for the production of a given chemical, but it is also valuable in studies of human metabolism, for example, for comparison of the metabolic capabilities of a cancer cell with those of its predecessor cell type. GEMs can also be used for physiological analyses, which involve the evaluation of gene essentiality, growth on different substrates, the ability to produce different metabolites, and so on. In the first GEM reconstructions, it was important to validate the models against physiological data, and these models provided relevant new insight into metabolic redundancies and explained the nonessentiality of many genes. Furthermore, the models also provided insight into the cellular

ability to grow on different carbon and energy sources, and an analysis of *E. coli* showed that there is a remarkable higher model-predicted growth rate on glycerol than what is experimentally observed (56). This deviation could be explained by the fact that GEMs do not consider regulatory constraints on metabolism, but it also clearly shows that *E. coli* has the metabolic capacity to grow faster on glycerol. This was confirmed through adaptive laboratory evolution (ALE) in which *E. coli* could be evolved to increase their specific growth rate on glycerol, reaching a value consistent with the model prediction (56).

This important finding provides three very important messages: (a) GEMs provide the total metabolic capabilities of a cell, but regulatory constraints may reduce this; (b) through ALE, it is possible for microorganisms to acquire new metabolic phenotypes that are consistent with GEM predictions; and (c) applying growth optimization as an objective is generally applicable for substrates encountered in the environmental niche of the organism but not for other substrates, for example, glycerol for *E. coli*. Work by Uwe Sauer (57) on *Bacillus subtilis* further demonstrates that regulation in bacteria often results in the suppression of growth on certain substrates, as the authors found that the deletion of various genes involved in regulation results in faster growth.

Even though GEMs basically rely on stoichiometric constraints, it is possible to add kinetic information about individual enzymes and thereby further constrain the flux through the individual reactions. However, it is challenging to add detailed kinetic expressions for all the reactions in GEMs without violating stoichiometric, thermodynamic, or physiological constraints. By using an advanced computational framework, Vassily Hatzimanikatis and coworkers (58) demonstrated how an *E. coli* GEM can be reduced to 146 intracellular reactions that still capture the complete metabolism. With this reduced model, they incorporated enzyme kinetics and thereby determined metabolite concentration ranges, which allowed the direct integration of metabolomics data (58). This analysis suggests that enzymes in metabolic networks have evolved to operate at different levels of saturation, which may give more flexibility and robustness to metabolic networks (58). The integration of kinetics into GEMs has also been demonstrated for a reduced model of yeast (22). Using a model covering central carbon metabolism, the enzyme concentration allocated for each reaction was estimated using the k_{cat} value. By constraining the total protein mass within the cell, this reduced model could predict the Crabtree effect in yeast, namely, overflow metabolism to ethanol (22). The analysis explains the intriguing usage of the energetically less efficient fermentation route, which generates only 2 moles of ATP per mole of glucose, instead of the energetically more efficient respiratory route, which generates approximately 20 moles of ATP per mole of glucose: The amount of ATP that can be produced per gram of protein is lower when using the catalytically inefficient components of oxidative phosphorylation, particularly the F_1F_0 -ATP synthase (22).

Although GEMs focus primarily on metabolism, there have been recent attempts to expand this modeling concept to allow description of protein synthesis and other cellular processes. For example, Palsson (59) developed a modeling framework that allows description of protein synthesis for all *E. coli* enzymes and thereby links the metabolic network with transcription and translation. To integrate the usage of enzymes in the different metabolic reactions, a pseudostoichiometric coefficient was used that was determined by an average k_{cat} for all the metabolic enzymes. The expanded model contains a total of 76,589 reactions and has been shown to have greatly improved predictive power. In another study, protein structure information was incorporated into an *E. coli* GEM, allowing for the correct prediction of growth at different temperatures (60). It is also possible to include nonchemical transformation processes into GEMs, as illustrated with a model describing the protein secretory pathway of *S. cerevisiae* (61). This protein secretory model accounts for the translocation of proteins between different compartments, such as from endoplasmic reticulum to Golgi, and for posttranslational modification processes, such as folding and glycosylation. Using

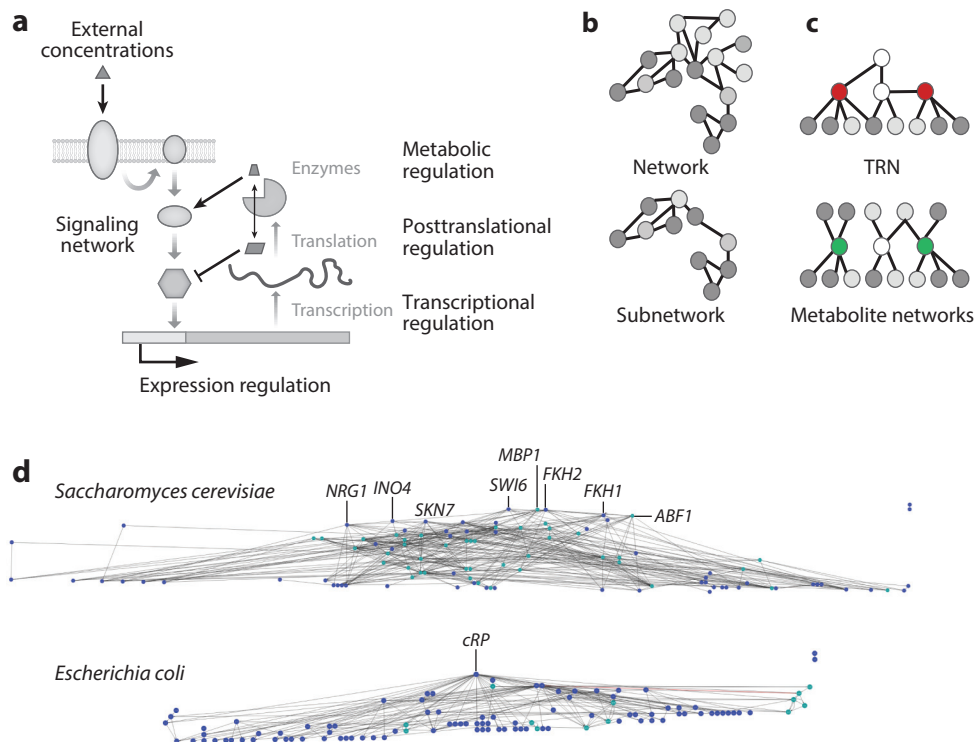


Figure 3

Illustration of different levels of metabolic control, integrative analysis, and transcription factor networks (TRNs). (a) The activity of an enzyme is determined by transcriptional regulation, posttranslational regulation, and metabolic regulation. (b) Overlaying transcription or proteome data on a biological network allows the identification of coregulated subnetworks. The significance of a change in gene expression/protein level is indicated by color; dark gray indicates high significance. (c) TRNs can be used to identify reporter transcription factors (red), and similarly, metabolic networks can be used to identify reporter metabolites (green). (d) Genome-scale TRNs for *Saccharomyces cerevisiae* and *Escherichia coli*. Panel b adapted with permission from Reference 73.

this model, it was possible to calculate the exact metabolic needs for the secretion of different proteins and thereby gain insight into how protein secretion is linked to metabolism (61).

4. INTEGRATIVE ANALYSIS OF OMICS DATA

The availability of high-throughput experimental techniques, often referred to as omics techniques, has allowed a more in-depth study of metabolism. In particular, it has become possible to begin to address the general question of how metabolic fluxes are controlled (Figure 3a), that is, at the transcriptional, translational, and posttranslational levels and/or at the level of metabolite–enzyme interactions. Analysis of omics data is traditionally performed using statistical and clustering methods, but these analytical methods are inherently naïve with respect to the underlying biology. Using biological networks for integrative analysis has made it possible to identify parts of large networks that are coregulated (62) (Figure 1b). In this context, metabolic networks are therefore well suited because they are reconstructed using detailed biochemical information (63). Other biological networks, including GO annotations that also reveal connections between genes,

can also be used to identify gene-enrichment groups or reporter features (63, 64) (**Figure 3c**), for example, reporter metabolites or reporter transcription factors. Many of these techniques can be easily implemented by employing the statistics programming language R, and many bioinformatics packages are available through Bioconductor (<http://www.bioconductor.org>), including the PIANO package for the identification of reporter features (65).

4.1. Transcriptomics

Today, transcription analysis is predominantly performed using RNA sequencing, a technique that has become possible with next-generation sequencing, but originally, DNA (or oligo) arrays were used. In the first study that described the development of this technology, the global reprogramming of yeast metabolism during the diauxic shift from growth on glucose to growth on ethanol was shown (66). The genome-wide transcription analysis showed for the first time how this change involves the upregulation of a large number of genes in the TCA cycle and respiration and the downregulation of genes in glycolysis (66). Following this study, DNA arrays and RNA sequencing have been used to study changes in metabolism in many different organisms, but due to the high degree of connectivity in metabolism, it is often difficult to learn new biology. It was therefore a breakthrough when the utility of GEMs, in performing integrative analysis of transcription data, was demonstrated. In most likely the first approach to integrate omics data with GEMs, my group used transcription data to constrain fluxes in the metabolic network and thereby improved the predictive strength of yeast GEM simulations (67). Shortly thereafter, we demonstrated how the network property of GEMs could be used for integrative analysis to find coregulated subnetworks and reporter metabolites that are hot spots in the metabolic network in terms of transcriptional changes in response to a certain perturbation (61). Since then, many other methods have been developed for the integration of transcription data into GEMs (68). With the expansion of GEMs to cover descriptions of protein synthesis in *E. coli*, it is possible to directly integrate gene expression data as input for simulations. This approach has proved to allow significantly improved model predictions, for example, for growth on different carbon sources and overflow metabolism, which results in acetate production during rapid growth on glucose (69).

Control of gene transcription can be explored by deletion of a given transcription factor (TF) and quantifying genome-wide expression, or determining TF binding to the promoter region of specific genes. The first approach gives a direct answer about the effect of a given TF on the cellular phenotype, but it is difficult to interpret the results as TFs interact with each other; that is, they may influence the expression of other TFs, and the binding of multiple TFs to the same promoter can occur. This complexity was illustrated in a large study by Sauer and colleagues (70), who quantified transcription and metabolic fluxes in 119 TF yeast deletion mutants under five different growth conditions. From their multiomics data, the authors mapped how different TFs interact in controlling TCA cycle activity and reconstructed a signaling cascade underlying this control (70). The second approach, determining TF binding to the promoter region of specific genes, provides direct information about TF binding, but this does not necessarily correlate with the control of transcription. TF binding can be determined by chromatin immunoprecipitation (ChIP) followed by the identification of the bound DNA, either by hybridization to DNA arrays (ChIP-chip) or by sequencing (ChIP-seq). ChIP-chip was first used for mapping TF binding in yeast (71), and to date, these data remain the most valuable resource for TF regulation of gene expression for this organism. One key finding from this analysis was that TF binding is extremely complex in eukaryotic cells. Some TFs have more than 2,500 targets (72), and many TFs target the expression of other TFs, resulting in a highly connected transcription factor network (TRN) (73). The ChIP-chip data are limited by the fact that only a single environmental condition was

studied. By mapping TF binding at different growth conditions, my group recently showed that it is possible to identify new functions for individual TFs (74). In this study, we used ChIP-exo, which relies on exonuclease cleavage of the DNA bound to the TF, and therefore gives a very high resolution of TF binding. This technique has also been used extensively by the Palsson group (75) to map genome-wide TF binding in *E. coli*, for example, to identify the TRN of amino acid metabolism.

A key question in metabolism is how flux through the individual reactions of the metabolic network is controlled (**Figure 3a**). Hans Westerhoff (76) expanded the concept of MCA by introducing two levels of flux control: (a) hierarchical flux control, meaning regulation at the levels of transcription, translation, and posttranslational enzyme modification and (b) metabolic flux control, meaning flux regulation determined at the level of metabolite-enzyme interactions. There have been several studies to identify flux control by transcription, one of the key components of hierarchical flux control, but these studies generally indicate that only certain key fluxes are controlled through transcriptional regulation. For example, in *B. subtilis*, only fluxes in the TCA cycle seem to be regulated by transcription, and there also seems to be little regulation at the metabolic level, indicating that allosteric regulation and posttranslational modification of proteins play an important role in flux control (77, 78). A similar finding was made in yeast, basically that only fluxes in the TCA cycle are transcriptionally regulated when metabolism changes from growth on glucose to growth on ethanol (79).

4.2. Proteomics

Proteome analysis was originally based on high-resolution gel electrophoresis, but with the advancement of mass spectrometry, it became possible to quantify thousands of proteins by gel-free analysis. This field has further advanced to enable measurement of protein modifications such as phosphorylation and acetylation. Using internal standards, it is possible to absolutely quantify protein concentration; when this quantification is combined with absolute measurements of mRNAs, it allows the generation of genome-wide quantitative data, namely, copies of mRNAs and proteins per cell (80). This has enabled the generation of proteome libraries, such as the quantification of 2,300 proteins in *E. coli* grown under 22 different growth conditions (81).

These types of quantitative data enable the detailed analysis of protein allocation to different metabolic pathways, and several recent studies have shown that protein allocation is extremely important for determining cellular phenotype. It was previously mentioned that the Crabtree effect in yeast can be explained by the reallocation of protein mass from the catalytically less but energetically more efficient respiration that includes F_1F_0 -ATP synthase to the catalytically more but energetically less efficient glycolysis (22). This type of trade-off between rate and yield is often found in biology and is consistent with the concept from nonequilibrium thermodynamics that flux is inversely proportional to thermodynamic efficiency. Thus, in a detailed systems biology study of two yeast species, a fast growing strain was found to have a lower biomass yield on glucose than a slower growing strain, and this could be explained by higher protein turnover in the faster growing strain (82, 83).

Increased protein turnover enables a faster response to changes in environmental conditions and results in increased levels of amino acids in the cells, thereby increasing the translation rate, but the trade-off is increased ATP costs and therefore a lower biomass yield (82). Protein costs form a key determinant of cellular phenotype, as has been illustrated by Naama Barkai and coworkers (84). By overexpression of an “unnecessary” fluorescent protein, at different levels in yeast, they quantified the effect of transcription and translation on cellular physiology under different growth conditions, with a key conclusion being that cells adapted to enforced protein production by

becoming larger. A limitation of their study, however, was a lack of quantitative analysis of the yeast proteome to evaluate whether the expression of the inert protein resulted in the reallocation of the yeast proteome.

Such a quantitative study has been performed for *E. coli* by Terry Hwa and coworkers (85). By expressing β -galactosidase with an inducible promoter, they could vary expression of the “unnecessary” protein and found that increasing β -galactosidase expression resulted in a reduced growth rate of the bacterium. The experimental results could be explained by a simple protein allocation model that was derived from the analysis of rRNA/protein ratios at different growth conditions, as well as from using data obtained from different levels of translational inhibition (85). Later, the same group used quantitative proteomics to study protein allocation to different metabolic pathways during growth under different conditions and for different deletion strains (86). From this analysis, they clearly showed that overflow metabolism in *E. coli*, which results in acetate production instead of respiration, is due to this metabolism being more energetically efficient per unit protein mass. This conclusion is consistent with the GEM-derived predictions concerning protein synthesis, as discussed above (69).

4.3. Metabolomics

One would intuitively think that the quantification of cellular metabolites would be the key method for studying cellular metabolism. However, there are two reasons why this is not the case: (a) It is inherently difficult to obtain quantitative data on the cellular metabolome, and (b) it is difficult to draw biological conclusions from metabolomics data alone. It is difficult to quantitatively measure intracellular metabolites because they are present in very low concentrations; their turnover rate is extremely high; metabolites are susceptible to chemical modifications; and many metabolites are not present in a free state within the cell. Thus, the turnover rate for most metabolites is on the order of seconds; for example, in yeast, it is approximately 3–10 s for ATP and most glycolytic intermediates. It is therefore necessary to quench metabolism extremely rapidly to obtain a representative sample (87). Another challenge is that due to the large chemical diversity of cellular metabolites, it is difficult to extract all the metabolites using the same solvent. The chemical diversity also makes it challenging to analyze all metabolites by one method. Although several different quenching and extraction methods have been developed, there is little consistency in terms of the recovery of metabolites between methods (82, 88). From a large interlaboratory experiment in which several different extraction and analytical methods were used, there was also poor consistency in terms of absolute measures for individual metabolites; however, metabolite ratios were similar across the different methods (82).

This variability subsequently suggests that it is difficult to obtain trustworthy quantitative metabolome data and that one should therefore be careful in comparing kinetic parameters such as K_M values from Michaelis–Menten kinetics with metabolome measurements, for example. The approach of estimating kinetics, including the parameters, from dynamic in vivo metabolomics data, as performed by Reuss (23) in their analysis of yeast glycolysis (see discussion above), is therefore recommended for the kinetic analysis of metabolic pathways. To overcome the problem of measuring the large chemical diversity represented in the cellular metabolome, Uwe Sauer and Nicola Zamboni (89) have recently developed a method based on direct injection of cell suspensions into a mass spectrometer, which enabled high-resolution analysis of the *E. coli* metabolome.

Despite these advances in metabolome analysis it is still challenging to draw biological conclusions from metabolome data. For example, although GEMs can be used as a scaffold for the analysis of metabolome data (90), only a fraction of the metabolome is typically quantified. The limitation of metabolome analysis for gaining novel biological insight was discussed several years

ago (91), and this limitation exists because metabolite levels are determined by so many other factors, such as enzyme concentration, enzyme activation by posttranslational modification, and enzyme kinetics. The trend for analyzing metabolome data is therefore to combine these data with other omics measurements to confirm generated hypotheses, for example, from an analysis of transcriptome or proteome data.

4.4. Fluxomics

The quantification of metabolic fluxes can be performed by feeding a labeled substrate to the cell followed by measurement of the labeling distribution in intracellular metabolites and subsequent analysis of the data using a stoichiometric model of metabolism (92). This approach was pioneered by Wolfgang Wiechert (92), who was among the first to develop the computational framework required for analysis of the data and has further advanced the field significantly. Thus, it is currently used extensively for studies on both cell cultures and more complex biological systems such as tissues and organs (93). Traditionally, this method relied on a measurement of the labeling pattern for amino acids incorporated into proteins and therefore required a steady state of metabolism. It is now possible to quantify the fluxes during dynamic conditions through measurements of the labeling pattern in precursor metabolites, amino acids, and proteins and the use of a far more advanced modeling framework (94, 95). This concept of dynamic flux analysis has been used to study the dynamic response of cholesterol biosynthesis when primary rat hepatocytes are exposed to statins (96). The combined analysis of pathway intermediates and fluxes revealed that flux control resides primarily at 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the target enzyme for statins.

Fluxomics has been widely used for the analysis of microbial cells used for the industrial production of fuels and chemicals, but it has generally been difficult to use this type of data for the identification of bottlenecks or to guide further engineering of strains. However, the technique has been shown to be valuable for the analysis of human cellular metabolism, primarily because human cells rely on several carbon and energy sources. It is therefore possible to use fluxomics to obtain insight into the relative contributions of different pathways, for example, for the generation of energy and cofactors. For example, in a study of glutamine metabolism by cancer cell lines, it was found that glutamine is converted to acetyl-CoA through the reductive action of isocitrate dehydrogenase under hypoxic conditions (97). This finding explains the large uptake of glutamine by cancer cells at hypoxia conditions, which far exceeds their need for nitrogen: The cells use the carbon of glutamine for lipogenesis, whereas a major part of the glucose is converted to lactate due to the Warburg effect. Using the same type of analysis, it has also been found that blocking glucose oxidation, for example, by adding metformin, results in reductive glutamine metabolism in prostate cancer cells (98).

5. APPLICATIONS

The systems biology of metabolism has found application in both basic and applied sciences. Such applications have involved the engineering of cell factories for the production of fuels and chemicals and for facilitating the discovery of novel drug targets or biomarkers, an approach often referred to as systems medicine.

5.1. Basic Biology

All cells have evolved global regulatory systems to ensure that metabolic homeostasis is maintained. These regulatory systems sense the energy and redox status of the cell, which are manifested in

the relative levels of cofactor pairs such as ATP/ADP, NADH/NAD⁺, and NADPH/NADP⁺. As mentioned above, the extensive use of these cofactors in metabolic reactions is the primary reason for the high degree of connectivity in metabolism; thus, metabolism must be controlled globally to keep these cofactor pairs in proper ratios. Metabolism has evolved to use two different electron carriers, NADH and NADPH, because they are used in different parts of metabolism. Thus, NAD⁺ is used as a substrate and an electron acceptor in many catabolic reactions, and NADH donates electrons either to the respiratory system or to pyruvate when it is converted to fermentative products such as lactic acid. In both cases, there is a large thermodynamic driving force that can ensure the regeneration of NAD⁺; it is therefore possible to maintain a higher concentration of NAD⁺ compared to NADH in the cell. NADPH, however, is used in biosynthesis and is important for donating electrons to precursor metabolites that are being converted to building blocks, for example, in the conversion of acetyl-CoA to fatty acids and sterols.

NADPH is generated in very few oxidative metabolic reactions. The three main contributors are (a) the first two reactions of the oxidative pentose phosphate pathway (converting G6P to ribulose-5-phosphate), (b) the NADP⁺-dependent isocitrate dehydrogenase, and (c) the malic enzyme, which converts malate to pyruvate. All these reactions have a high thermodynamic driving force due to the splitting off of CO₂ and can ensure the generation of NADPH even though the concentration of NADPH in the cell is higher than that of NADP⁺. Because it is important for the cell to maintain different concentrations of these two cofactors, it is not possible to transfer electrons from NADH to NADPH without investing energy. In *E. coli*, this can be performed using a membrane-bound transhydrogenase that couples the conversion to the proton gradient, which is basically equivalent to the expenditure of ATP. Some organisms have a cytosolic transhydrogenase, but they always catalyze the transfer of electrons only from NADPH to NAD⁺.

Due to the central roles of NADPH and NADH in cellular metabolism, distortions in their metabolism or their interconversion can be detrimental to the cell; for example, the excessive use of NADPH during oxidative stress and in many human diseases has been shown to be linked to the altered metabolism of these cofactors (99). In biotechnology, it is also important to ensure a proper balance of these cofactors, and by engineering cofactor usage, my group previously showed it to be possible to improve yeast bioethanol production in this way (100). Specifically, we engineered ammonia assimilation to use NADH as a cofactor instead of NADPH; as this process accounts for approximately 50% of the NADPH usage in the cell, an alternative NADH sink was created in the cell. Under anaerobic conditions, the only way cells can dispose of excess NADH is through the conversion of glucose to glycerol, and by having the alternative NADH sink, glycerol production could be reduced by 50%, which allowed for an increased ethanol production (100).

To maintain the global regulation of metabolism, the cell has evolved many different regulatory pathways, and in eukaryotic cells, the two most important regulators of metabolism are AMP-activated kinase (AMPK) and target of rapamycin complex 1 (TORC1). **Figure 4** provides an overview of their interaction and their control of key metabolic processes. These two protein kinases regulate metabolism by modulating TFs, thereby controlling gene expression, but also through the direct phosphorylation of many enzymes. AMPK inactivates both acetyl-CoA carboxylase (ACC) and HMGCR by phosphorylation and is thereby a key regulator of lipid biosynthesis.

As illustrated in **Figure 4**, AMPK and TORC1 form a highly connected regulatory network impacting the regulation of many key cellular processes; it is therefore necessary with a systems biology approach to perform a detailed mapping of how these protein kinases interact with each other and with other key regulatory components of the cell. AMPK is activated by SIRT1 through the deacetylation of the upstream protein kinase LKB1, but AMPK also indirectly activates SIRT1 by activating mitochondrial oxidation and increasing the NAD⁺/NADH ratio in the cell (101).

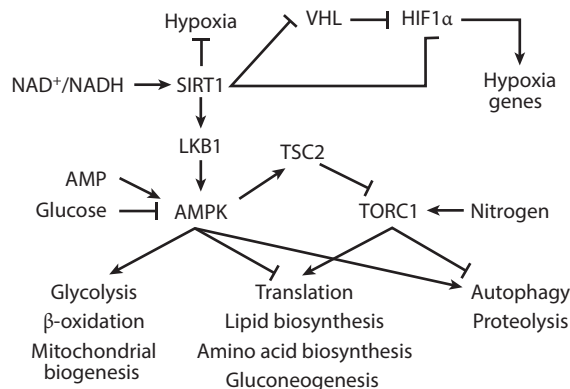


Figure 4

Illustration of the interactions between adenosine monophosphate-activated kinase (AMPK) and target of rapamycin complex 1 (TORC1) to control metabolism. AMPK is activated by AMP (which is indicative of low energy status in the cell) and inactivated by excess glucose. TORC1 is activated by the presence of nitrogen sources, and if both glucose and a nitrogen source are present, cell growth is activated (which requires protein translation and amino acid and lipid biosynthesis). If the energy status of the cell is low, energy-consuming processes (e.g., translation and biosynthesis) are repressed and energy generation (e.g., β -oxidation and mitochondrial biogenesis) is activated. The network includes two known tumor suppressors, TSC2 (tuberous sclerosis complex 2) and VHL (von Hippel-Lindau).

SIRT1 is inactive during hypoxia, most likely due to a low NAD^+/NADH ratio, and is a repressor of HIF1 α (102), a TF that activates the expression of hypoxia-responsive genes. Because the expression of hypoxia genes is important for cancer cells, SIRT1 and AMPK play important roles during the onset of cancer metabolism. There have therefore been several attempts to develop drugs that activate SIRT1 for cancer treatment, and studies in several different model organisms have also shown that SIRT1 activation improves life span (103, 104). Supplementation with resveratrol, a polyphenol present in grapes and red wine, causes effects similar to those of SIRT1 activation and has a number of health benefits in both mice (105) and humans (106), but SIRT1 is not directly activated by resveratrol (107).

Another key regulator of metabolism is TORC1, which is activated by the presence of nitrogen sources, such as amino acids, and stimulates growth by activating a large number of biosynthetic processes and concomitantly inactivates autophagy and proteolysis. The protein kinase, a target of the anticancer drug rapamycin, is a key component in ensuring cellular proliferation when environmental conditions are favorable. Recently, it was shown that rapamycin may also increase life span (108), most likely through its activation of autophagy via TORC1 (109). This result is supported by the finding that the activation of autophagy through supplementation with spermidine improves life span (110).

The regulatory network shown in **Figure 4** is highly conserved in eukaryotic cells. Extensive knowledge of AMPK was first acquired from studies in yeast, and using a systems biology study involving transcriptome, proteome, and metabolome analysis, the regulatory functions of the yeast AMPK, Snf1, were reconstructed (111). In a similar study also involving phosphoproteomics, the interactions between AMPK and TORC1 in yeast were reconstructed and once again agreed well with findings in mammalian cells (112). AMPK/Snf1 is activated by phosphorylation and inactivated by dephosphorylation; recognizing the dynamics of its activation (by phosphorylation) is therefore necessary to understand how metabolism responds to perturbations in environmental conditions. Yeast is a well-suited model organism to study this as it is possible to generate several

different mutants that have, for example, an overexpressed upstream kinase and/or phosphatase and then measure the dynamic response to environmental changes in these mutants. This approach was used to build a kinetic model for the regulatory network around Snf1 and gain insight into how Snf1 controls some of its downstream targets (113). Yeast has also been used to study the role of TORC1 in the regulation of nucleotide and amino acid biosynthesis, and key targets of TORC1 have been identified by analyzing the phosphoproteome following changes in nitrogen sources and rapamycin addition (114).

5.2. Engineering Microbial Metabolism

Microorganisms have been used since ancient times for the production of fermented food and beverages, and after the identification of its antibiotic properties during World War II, the industrial-scale production of penicillin and many other antibiotics began. Today, a wide range of different chemicals is being produced using microbial fermentation (**Figure 5a**), for example, bioethanol used as a biofuel and lysine as a feed additive. With the introduction of genetic engineering, it has become possible to engineer microbial cell factories for the production of a vast range of other chemicals, an approach referred to as metabolic engineering (115–117). There are several success stories on developing novel bioprocesses through metabolic engineering of cell factories, for example, the production of lactic acid by a low pH-tolerant yeast strain to allow its direct use in the production of polylactate. From these developments and many other studies, it has become clear that due to the high degree of connectivity in metabolism, a holistic analysis is important for metabolic engineering. Such analysis involves a design-build-test cycle (DBT cycle) (118) in which strains are designed, built, and then tested repeatedly until a sufficiently efficient cell factory has been built (**Figure 5b**).

There are many examples of reconstructing heterologous pathways in different cell factories (118–131). Yeast and *E. coli* have, however, been the preferred platform cell factories to date (132), and **Table 2** provides a list of some prominent examples. Typically, a 3- to 5-year period of further development and an investment in excess of US\$50 million is required between the identification of a proof-of-principle strain, that is, a strain that can produce small amounts of the desired chemical, and the acquisition of a strain that meets the requirements for a commercially viable process (118). This development involves many rounds of the DBT cycle, and with recent

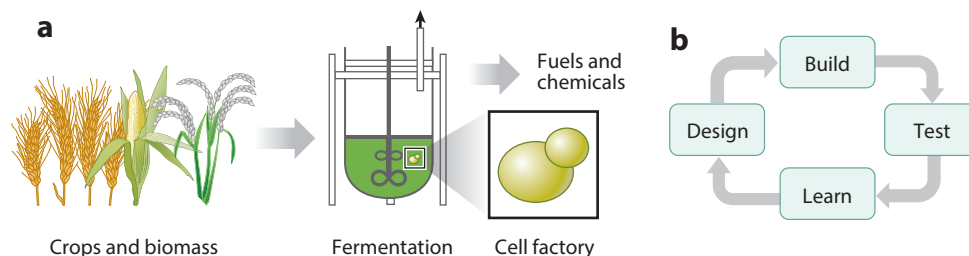


Figure 5

The concept of industrial biotechnology and metabolic engineering. (a) In industrial biotechnology, crops or biomass are used as feedstock for the production of fuels and chemicals. The biotransformation occurs in a bioreactor via microbial fermentation. In this process, a cell factory is used that is often genetically engineered to have its metabolism optimized for the conversion of the feedstock to the product of interest. (b) Optimization of the cell factory is performed through the design-build-test cycle of metabolic engineering. Generally, much is learned by engineering metabolism and testing different designs, and the knowledge can then be used for future design strategies.

Table 2 Examples of engineering metabolism for the production of valuable chemicals

Product	Cell factory	Product application	Reference(s)
Isobutanol	<i>S. cerevisiae</i>	Biofuel with better properties than ethanol	Primarily covered in the patent literature ^a
Alkanes	<i>E. coli</i>	Drop-in fuels for use in trucks and jets	119
	<i>S. cerevisiae</i>	Drop-in fuels for use in trucks and jets	120
Fatty alcohols	<i>E. coli</i>	Solvents or biofuels	121
	<i>S. cerevisiae</i>		120
Farnesene	<i>S. cerevisiae</i>	Biodiesel for use in trucks and jets	Primarily covered in the patent literature ^a
PDO ^b	<i>E. coli</i>	Polymer production, e.g., SORONA [®]	122
BDO ^c	<i>E. coli</i>	Polymer production	123
Lysine	<i>C. glutamicum</i>	Feed additive	124
Valine	<i>E. coli</i>	Feed additive	125
Santalene	<i>S. cerevisiae</i>	Perfume ingredient	126
Artemisinin acid	<i>S. cerevisiae</i>	Antimalarial drug	127
Cortisone	<i>S. cerevisiae</i>	Anti-inflammatory drug	128
Opioids	<i>S. cerevisiae</i>	Drugs to relieve pain	129
Resveratrol	<i>S. cerevisiae</i>	Antioxidant	130

^aPrimarily covered in the patent literature, which has been reviewed in Reference 131.

^b1,3-Propanediol.

^c1,4-Butanediol.

developments in genome engineering, such as CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 technology, the main challenge today is to rapidly phenotype new strain designs and use this information to improve design in the next round of the DBT cycle. The systems biology of metabolism therefore has a significant impact on metabolic engineering, because by modeling metabolism, it will be possible to design optimal metabolic networks for the production of a given chemical, as in other engineering disciplines.

With their representation of stoichiometric coupling in the whole metabolic network, GEMs are quite well suited for metabolic engineering design, and through the introduction of bilevel optimization, it is possible to identify metabolic engineering targets that couple product formation with growth (133, 134). This computational approach has been demonstrated to enable the improved design of *E. coli* for lactic acid production (135) and of yeast for ethanol (136) and succinic acid (137) production. More recently, Vassily Hatzimanikatis and coworkers (138) developed BNICE.ch, an advanced computational framework that allows both pathway identification and a detailed analysis of kinetic and thermodynamic constraints in metabolic networks. In addition to the use of GEMs for designing cell factories, these models play important roles in the integrative analysis of engineered strains using the techniques discussed above (139). The DBT cycle therefore also includes an element of learning, in which new information about the biological system being engineered is acquired and fed into the models.

Even though there are many examples of successful forward engineering, this approach depends on knowledge of the biological system to be engineered. For some complex traits, such as tolerance to high temperatures or low pH, it is difficult to identify suitable engineering targets. In these cases, ALE is very efficient because it allows the isolation of mutant strains with new acquired phenotypes, and causal mutations can be identified through genome sequencing. However, it is generally important to perform systems biology analysis of the evolved strains to identify causal mutations.

Thus, ALE was used for the isolation of yeast strains with improved growth on galactose (140), and the ALE-created strains had improved galactose metabolism compared with yeast strains that were engineered for improved galactose uptake, either by targeting regulatory components (141) or targeting a key enzyme of the Leloir pathway (142). From systems biology analysis, it was found that the proper functioning of the Leloir pathway requires homeostasis of the pathway intermediates, and paradoxically, overexpression of pathway enzymes individually or in combination results in reduced galactose uptake and cell growth (143). ALE has also been used to isolate yeast strains with the ability to grow at elevated temperatures (144). From a systems biology analysis of these isolated strains, causal mutations in *ERG3* were found. These mutations created stop codons that disabled Erg3p, an enzyme involved in ergosterol biosynthesis, and resulted in the accumulation of fecosterol, instead of ergosterol, in the cellular membranes. It was speculated that the bent structure of fecosterol created a more rigid membrane than the straight structure of ergosterol and thereby conferred improved tolerance to higher temperatures (144).

5.3. Human Metabolism

All human diseases impact energy and redox metabolism in one or more cell types, and due to the high degree of connectivity in metabolism, any human disease will carry a metabolic signature that can potentially be identified through analysis of the metabolome in tissues and/or plasma. However, because the number of chemicals present in plasma is very large, it is difficult to identify these metabolic signatures from metabolome analysis alone. It is therefore generally necessary to first analyze the metabolism of the diseased tissue and generate a hypothesis that can form the basis for targeted metabolomics. It is believed that this approach could lead to improved diagnostics and allow the stratification of patients into subgroups that can then be treated using precision or personalized medicine. Furthermore, by studying metabolism associated with disease development, it may be possible to identify novel drug targets. This approach is particularly well illustrated in the field of cancer treatment, in which studying metabolism has become a mainstream topic (145), and several metabolic enzymes have been identified as novel drug targets (146, 147).

With their holistic approach, GEMs are very well suited for the analysis of alterations in human metabolism in response to disease development (148). The first human GEMs were constructed in 2007 (149, 150) and formed the basis for the creation of a greatly expanded human GEM in 2013 (151). This model was further expanded to cover lipid metabolism in more detail, resulting in Human Metabolic Reaction 2.0 (HMR2.0) (152), which is currently the most comprehensive generic human GEM, covering 3,765 genes, 8,181 reactions, and 6,007 metabolites. HMR2.0 has been used as a scaffold for generating detailed models for adipocytes, hepatocytes, and myocytes (153–155). The adipocyte model was used to study metabolic changes in fat tissues in response to obesity, and model simulations revealed that lean subjects have larger dynamic changes in lipid bodies in adipocytes compared with obese subjects (153). The hepatocyte model was used to study metabolic alterations in subjects with nonalcoholic fatty liver disease (NAFLD), and these subjects appeared to have serine deficiency (154). This finding was later confirmed in a clinical study that showed improved health status for NAFLD subjects who were given serine supplements (156). The myocyte model was reconstructed using RNA-seq data from isolated myocytes, and the model was used to perform a meta-analysis of several transcriptome data sets and thereby identify new potential biomarkers for type 2 diabetes (T2D) (155).

Other tissue-specific GEMs have also been reconstructed using tissue-specific transcriptome analysis (157) or data from the Human Protein Atlas (HPA) (<http://www.proteinatlas.org>) (152, 158). HPA uses immunohistochemistry to identify the presence of proteins in more than 80 different human cell types and more than 20 cancer cell lines and is therefore an extremely valuable

resource that provides information about the proteome at the cellular level. A recent study using RNA-seq on 32 human tissues, however, showed that there is a good correlation between mRNA presence in a tissue and associated cell types (159), which is an important finding because most clinical studies are based on the analysis of tissue biopsies. There is generally a poor overall correlation between mRNA abundance and protein abundance (160), but for specific genes/proteins, good correlation has been found across different cell lines (161), indicating that the poor overall correlation is due to large variability in protein translation and degradation rates. Thus, mRNA data can be used as a good proxy for protein abundance (162), and the mRNA data from the 32 human tissues could therefore be used to create 32 tissue-specific models (159).

GEMs are particularly well suited for the analysis of cancer metabolism because these models can be used not only for integrative data analysis but for model simulations (163) and the detection of synthetic lethality in cancer cells (164). As discussed above, GEMs can be used to simulate cell growth if an appropriate objective function is specified, for example, maximizing cell growth. For normal human cells, it is difficult to specify an objective function, but it is reasonable to assume that cancer cells strive to maximize cell growth. This imperative has been well illustrated by Eytan Ruppin, who showed through GEM simulations that cell proliferation causes the Warburg effect in cancer cells due to a proteome constraint (165), just like the Crabtree effect in yeast (22). He also demonstrated the use of GEMs to identify novel drug targets for cancer treatment (166), and in a follow-up study, he specifically identified drug targets in argininosuccinate synthase (ASS1)-deficient tumors (167). ASS1-deficient cells have increased aspartate levels, which results in increased *de novo* pyrimidine biosynthesis, and it is possible to prevent cell proliferation by blocking this activity (167).

Cancer cells are very heterogeneous, which can impact treatment; to address this issue, my group used proteomics data from hepatocellular carcinoma (HCC) tumors from six individuals to generate personalized GEMs (152). Using these six GEMs, we identified antimetabolites—metabolite analogs that inhibit specific enzymes and are widely used in cancer therapy—for preventing cell proliferation in all six tumors; these could be potential drugs for HCC treatment. In this study, several known targets for cancer treatment were identified, but we also identified the carnitine carrier system as a new target, responsible for fatty acid transport to the mitochondria for β -oxidation. This target was verified experimentally using a cancer cell line (152). GEMs can also be used to identify cancer-specific metabolism; using data from The Cancer Genome Atlas, we found that clear cell renal cell carcinoma (ccRCC), the most dominant form of kidney cancer, has a distinct metabolism that is associated with chromosome 3p loss of heterozygosity (168). Furthermore, we found that ccRCC is characterized by a significant deregulation of heparan and chondroitin sulfate metabolism, leading us to analyze associated metabolites in plasma and urine, which led to the identification of a very strong diagnostic biomarker for metastatic ccRCC (169).

The gut microbiome is a complex biological system that has been connected to human disease development. The human gut harbors a vast number of different microbial species that interact with the host immune system and metabolism (170). This microbiome can be analyzed through metagenome analysis of extracted DNA (171). Several studies have shown that the gut microbiome composition is associated with disease development, for example, T2D (172, 173) and other metabolic diseases (174). Gut bacteria are involved in the biosynthesis of different vitamins that are important for overall metabolism of the host, but they also produce bioactive molecules such as β -carotene that can protect against atherosclerosis (175). The gut microbiome is to a large extent determined by diet, and dietary interventions can therefore alter its composition (176). Computational models have been used to identify species co-occurrence in complex microbiome ecosystems (177), and recently, GEMs have also been used to model the overall metabolism of the gut ecosystem. Using GEMs for three dominant gut species, we simulated the interaction

between these microbes when they colonized germ-free mice (178). This concept has also been used to model the human gut microbiome so that metabolic changes in response to dietary interventions can be described (179). The analysis involved two groups of subjects, one with a high gene count and a diverse gut microbiome (i.e., a high microbial gene richness) and one with a low gene count and a compressed gut microbiome. From the analysis, we found that the low-gene-count group was at elevated risk of developing T2D and other metabolic diseases when overweight, most likely due to inefficient digestion of food in the gut resulting in elevated amino acid levels in the plasma, a well-known risk factor for the development of the abovementioned diseases (179). Our study therefore directly points to a connection between gut microbiome metabolism and plasma chemistry. Recently, gut microbiota composition has also been shown to impact the response to anticancer treatment using immunotherapy (180) and can in some cases even be associated with the presence of a specific species (181).

SUMMARY POINTS

1. Due to the high connectivity in metabolism, it is necessary to use mathematical models for its analysis, that is, systems biology.
2. Kinetic models can be used to obtain insight into the dynamic operation of pathways in response to environmental perturbations.
3. GEMs are knowledge bases of metabolism that can be used for both simulation and data integration.
4. Omics technologies are well suited to study metabolism because they make it possible to obtain a holistic view. However, these data often require integration with biological network models to obtain new biological insight.
5. Cells have evolved complex regulatory systems to ensure metabolic homeostasis. These regulatory systems are intertwined with metabolic readouts, which makes it difficult to dissect their topology and dynamic operation.
6. Due to the high connectivity in metabolism and the extensive regulation, it is difficult to engineer cells to overproduce specific chemicals, but systems biology allows for identification of nonintuitive targets for engineering.
7. Most human diseases are associated with changes in metabolism that can be captured as a metabolic signature in plasma or urine, and through systems biology analysis, it is possible to identify such metabolic signatures.

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Errata

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