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REVIEW

Metabolic networks in motion: ^{13}C-based flux analysis

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Many properties of complex networks cannot be understood from monitoring the components—not even when comprehensively monitoring all protein or metabolite concentrations—unless such information is connected and integrated through mathematical models. The reason is that static component concentrations, albeit extremely informative, do not contain functional information *per se*. The functional behavior of a network emerges only through the nonlinear gene, protein, and metabolite interactions across multiple metabolic and regulatory layers. I argue here that intracellular reaction rates are the functional end points of these interactions in metabolic networks, hence are highly relevant for systems biology. Methods for experimental determination of metabolic fluxes differ fundamentally from component concentration measurements; that is, intracellular reaction rates cannot be detected directly, but must be estimated through computer model-based interpretation of stable isotope patterns in products of metabolism.

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Fluxes quantify the integrated network response of gene–protein–metabolite interactions

Metabolic networks, in particular microbial ones, are arguably the best characterized complex biological networks. We know most of the reactions, the enzymes that catalyze them, the genes that encode the enzymes, and the involved chemicals and cofactors. From this comprehensive knowledge of the components, the network topology of enzyme and metabolite interactions has been worked out to a great extent in many organisms. In contrast to protein–protein interaction or regulation networks with many yet unknown components, metabolic interactions can be mathematically represented at the genome scale (Price *et al*, 2004). The modern 'omics' arsenal assesses particular system variables through global monitoring of component concentrations. Proteomics or, more indirectly, transcriptomics record changes in the concentration of the catalyzing enzymes, whereas metabolomics attempts to monitor concentration changes of the small chemical species within a cell (Nielsen and Oliver, 2005).

The concentration state of these components, however, is not the true functional bottom line of cellular operation, and hence insufficient to assess how component interaction is organized into networks with newly emerging functions and capabilities (Hellerstein and Murphy, 2004). Beyond methods to quantify component concentrations, systems biology thus requires experimental methods for (i) elucidating component interactions (e.g. physical protein–protein (Cusick *et al*, 2005) and protein–DNA (Workman *et al*, 2006) or indirect epigenetic gene–gene interactions (Tong *et al*, 2001)) and (ii) quantitative monitoring of integrated network responses that result from the highly nonlinear interaction of the various components across functional levels (Aderem, 2005). By tracking single molecule, virus, or organelle movement inside cells, modern imaging techniques are an example of integrated response analysis, in this case of physical behavior (Damm and Pelkmans, 2006). A particularly relevant application is the quantitative analysis of the dynamics within macromolecular assemblies, for example the protein flux of and along the cellular cytoskeleton where molecular and genetic interactions generate mechanical forces (Wittmann *et al*, 2001; Danuser and Waterman-Storer, 2006).

For metabolic networks, the integrated network response is given by small molecule fluxes (i.e. *in vivo* reaction rates) that result from all catalytic protein–metabolite interactions and the regulatory interactions at the genetic, protein modification, allosteric, and kinetic level (Figure 1). Without resolving the details, ^{13}C-based flux analysis quantifies the integrated output of these component interactions (Stephanopoulos, 1999; Hellerstein, 2003; Sauer, 2004; Fernie *et al*, 2005), hence has become attractive for applications in microbes, plants (Schwender *et al*, 2004a; Sriram *et al*, 2004), and higher organisms (Hellerstein and Murphy, 2004).

After a decade of intense research and development, ^{13}C-based flux methods can routinely track steady-state fluxes in microbes grown on single-carbon substrates. In contrast to higher organisms, where current methods are restricted to the local detection of one or few relative fluxes or molecular turnover (Kelleher, 2001; Sherry *et al*, 2004), absolute fluxes through larger networks can be determined in microbes, hence the term fluxome was coined (Sauer *et al*, 1999). Here I will highlight how such network flux analysis is used to infer metabolic system behavior and design principles in microbes to illustrate that flux analysis is a key methodology for systems biology of metabolism.

The principle of ^{13}C-based metabolic flux analysis

In contrast to static, snapshot-like concentrations of transcripts, proteins, or metabolites, fluxes are the time-dependent

motion of metabolites through a network, hence cannot be measured directly. Instead, they must be inferred from measurable quantities through computer model-based interpretation. One measurable quantity is uptake and production rate (i.e. fluxes in and out of cells), which can be balanced in assumed reaction networks to provide first estimates on some intracellular fluxes (Stephanopoulos, 1999). Such stoichiometric flux analysis is inherently limited in its capacity to derive new conclusions on intracellular network operation because the results are strongly based on assumptions and not on data. To experimentally quantify pathway activity, additional intracellular information must be obtained from stable isotope tracer experiments. Typically, ^{13}C-labeled substrates are fed to a growing cell population until the isotope label is distributed throughout the network. As a function of the particular distribution of fluxes in an organism, specific labeling patterns occur in the metabolic intermediates (Wiechert, 2001; Sauer, 2004). The task now is to measure those ^{13}C patterns and to reconstitute the network distribution of flux from the measured data (Figure 2A).

The most frequently employed isotope tracer method detects the ^{13}C patterns in 10–15 protein-bound amino acids (Figure 2A). As the carbon backbones of eight key intermediates are conserved in amino acids, protein is a stable and abundant source of labeling information, which enables

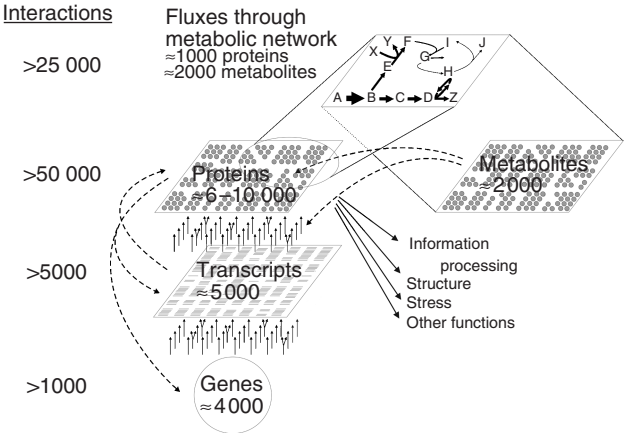

Figure 1 Schematic overview of the relationship between concentration-based, compositional, and functional units in metabolic networks. Regulatory interactions are indicated by dashed lines. Transcript–transcript interactions are based on average operon structures and ribosomal RNA interactions. Proteome interaction estimates include an average of 6–7 protein–protein interactions (Szallasi, 2006) as well as protein–DNA, protein–RNA, and protein–membrane interactions. Metabolic interactions include biochemical transformations and regulatory interactions between metabolites, RNA, and protein. The number of different proteins includes differences in folding, size, and covalent modifications.

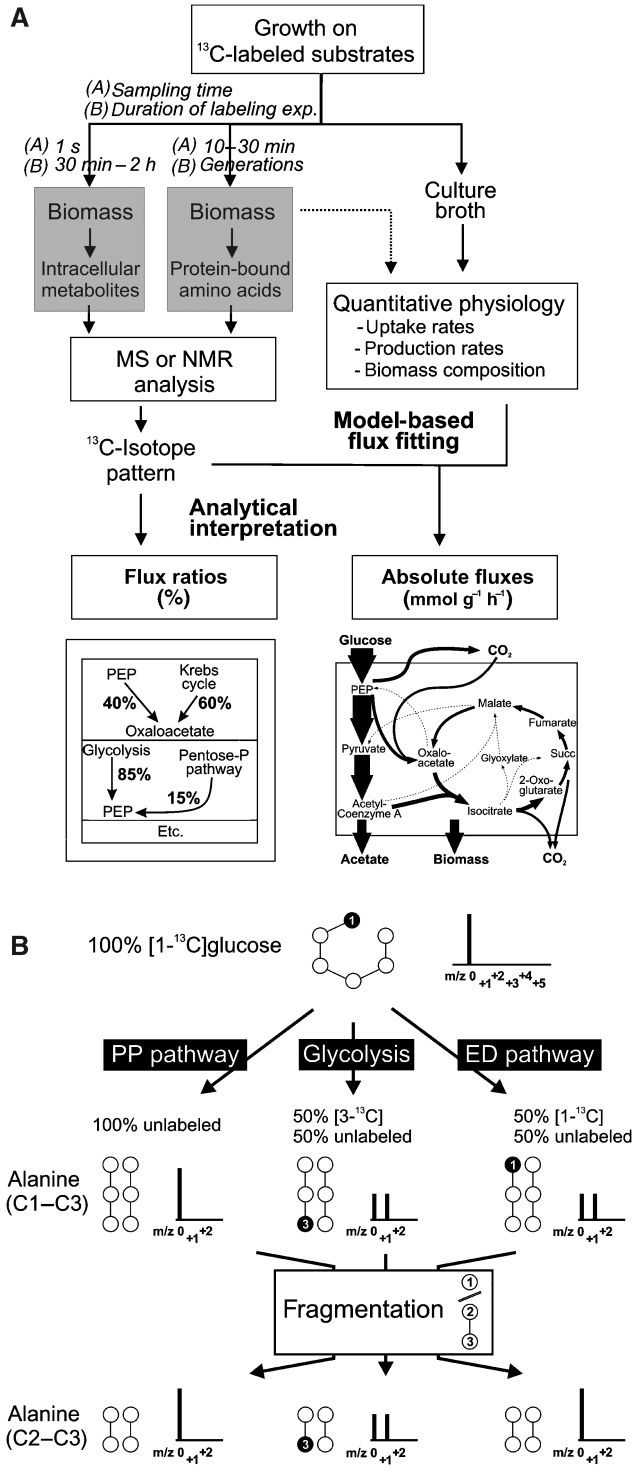

Figure 2 (**A**) Schematic flow chart of ^{13}C-based metabolic flux analysis. Exemplary results for flux ratios and absolute fluxes are given in the bottom boxes. (**B**) Example of inferring relative fluxes through the three initial pathways of glucose catabolism in *E. coli* from mass spectrometry data. A positional label is introduced by feeding [1-^{13}C]glucose, and ^{13}C-pattern are detected in alanine, which derives its carbon backbone directly from pyruvate. Although unique isotope pattern occurs in intact alanine molecules, the lack of positional information in the detected mass distribution cannot discriminate between glycolysis and the ED pathway. For discrimination of these two pathways, additionally the C2–C3 moiety of alanine must be analyzed, which occurs by fragmentation in some MS instruments. For flux ratios, the relative contribution of these pathways to the formation of alanine (pyruvate) is calculated directly from the detected abundance of the different mass isotope isomers by probabilistic equations. For absolute fluxes, a best-fit flux solution is obtained by extensive computations that seek to minimize the error between fitted intracellular fluxes not only to the six shown, but also to all other detected mass spectra and physiological uptake and production rates.

determination of central metabolic fluxes during steady-state growth from as little as 2 mg cells (Szyperski, 1995; Marx *et al*, 1996; Fischer and Sauer, 2003b; Grotkjaer *et al*, 2004). A potentially much richer source of information is the hundreds of free intracellular metabolites themselves that would allow resolving fluxes even beyond central metabolism and also to assess dynamic flux changes. Their comparatively low concentrations, diverse chemical nature, and high turnover rates in the range of seconds, however, severely hamper metabolite-based flux analysis, but several promising methods are currently under development (van Winden *et al*, 2005; Wiechert and Nöh, 2005; Nöh and Wiechert, 2006).

How does one obtain flux data from labeling patterns? At least two principally different approaches can infer fluxes from ^{13}C data that are obtained by either nuclear magnetic resonance (NMR) (Marx *et al*, 1996; Sauer *et al*, 1997; Portais and Delort, 2002) or mass spectrometry (MS) (Gombert *et al*, 2001; Fischer and Sauer, 2003b; Klapa *et al*, 2003). In the first approach, ^{13}C data, extracellular fluxes, and biosynthetic requirements are simultaneously integrated with computer models (Figure 2A). The flux distribution is typically identified by iterative fitting of fluxes to the measured data, whereby the difference between observed and simulated isotope spectra is minimized (Wiechert, 2001). Essentially, this is a parameter-fitting procedure, where the relation between unknown fluxes and measured data is described by mathematical models of varying complexity.

The second method relies on a direct and local interpretation of selected labeling patterns, for example, the mass distribution of pyruvate or its surrogate alanine (Figure 2B). For this purpose, a probabilistic equation is derived that quantifies the relative contribution of converging pathways to the formation of a particular metabolite (a flux ratio) from a particular combination of NMR or mass pattern (Szyperski, 1995; Fischer and Sauer, 2003b). Whereas the fitting method indirectly infers absolute flux values throughout the network, the flux ratio method provides direct evidence for the relative *in vivo* activity of a given reaction (Figure 2A). The drawback of this analytical method is its restriction to 10–15 preselected fluxes that are directly accessible from the data; hence these complementary approaches are ideally applied to the same ^{13}C data set (Emmerling *et al*, 2002; Hua *et al*, 2003; Fischer *et al*, 2004). Recently, the flux ratio approach was extended to the estimation of absolute fluxes that is applicable also at higher throughput (Fischer *et al*, 2004; Fischer and Sauer, 2005). Further details on flux analysis can be found in several recent reviews (Wiechert, 2001; Sauer, 2004; Schwender *et al*, 2004b; Wiechert and Nöh, 2005; Ratcliffe and Shachar-Hill, 2006).

In practice, the focus of flux analysis is typically not on the entire network of up to a thousand reactions, but rather on the 50–100 reactions of central metabolism. This core set of reactions establishes a ubiquitous and interconnected network that catalyzes the major material flows (Figure 3A). Superimposed on the metabolic network are multiple layers of control that ensure optimal usage of pathways and even the direction of flux to meet cellular requirements under different environmental conditions. To unravel and quantify such control mechanisms is a key goal of systems biology.

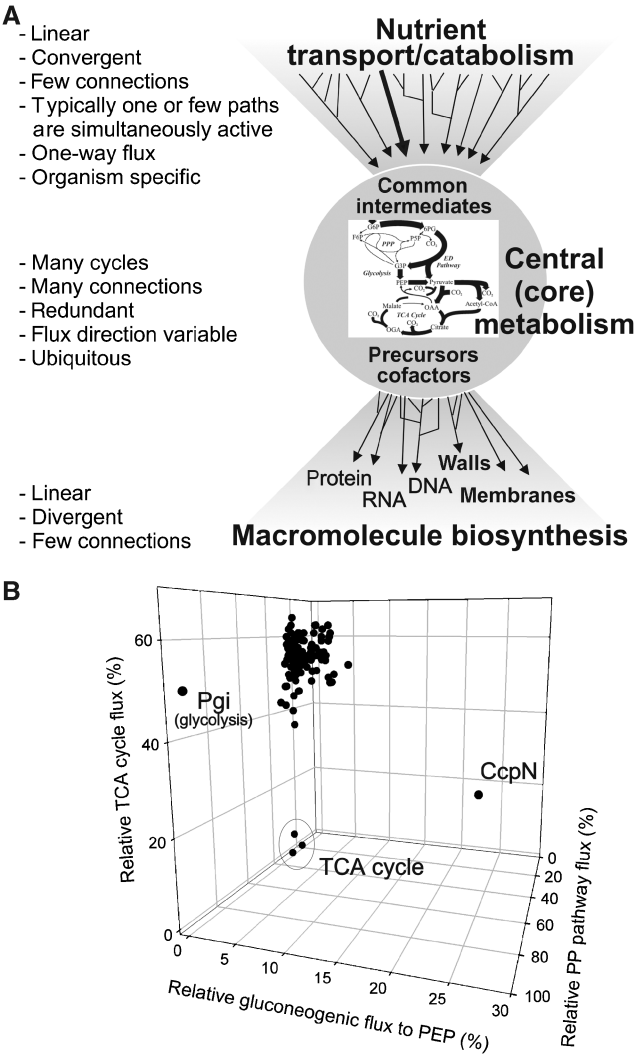

Figure 3 (**A**) Bow-tie abstraction of metabolic network organization (after (Csete and Doyle, 2004; Stelling *et al*, 2006). (**B**) Metabolic fluxes through three major pathways in the central metabolism of 137 *B. subtilis* knockout mutants. The phosphoglucose isomerase (Pgi) mutant and three mutants in genes encoding for enzymes of the TCA cycle are expected outliers because one of the plotted pathways was blocked. All other mutants cluster in a distinct region of this 3D flux space with the sole exception of the novel transcriptional regulator CcpN (Servant *et al*, 2005). Data are taken from Fischer and Sauer (2005).

Network operation versus pathway concept

The traditional use of isotopic tracer experiments was the structural identification of biochemical reactions that constitute the metabolic pathways that now populate our textbooks. In the heydays of metabolic biochemistry in the 1940–1960s, tracer experiments were the key method to prove *in vivo* operation of individual pathways within the bewildering complexity of the then largely unknown metabolic network. These experiments essentially followed the logic that first hypothesis on the chemical reactions within a pathway were generated. Subsequently, tracer experiments were designed such that tracing isotopically labeled atoms to particular positions of pathway products could discriminate between the

initial hypotheses. The initial dominance of radioactive tracers has been almost completely replaced by stable isotopes and their analysis through NMR or MS, both for pathway elucidation (Bacher *et al*, 1999) and for flux analysis (Szyperski, 1998).

To facilitate comprehension, textbooks structure metabolic networks into pathways and cycles. Within the reality of the network, however, our familiar pathways are biochemical concepts that often include assumptions on functionalities. Although incredibly helpful for teaching, these assumptions might be oversimplifications or simply incorrect under certain conditions. Here, I argue that experimental flux analysis is crucial to observe and eventually understand operation of networks. By placing pathway activity in a quantitative network context, this goes beyond pathway identification. A prominent example is the pentose phosphate pathway, whose generally considered function is supply of precursors and redox equivalents for biosynthesis. In many organisms, however, its function is more properly described as a second catabolic pathway (Fuhrer *et al*, 2005). In the next paragraph, methodological aspects of elucidating network topology are outline.

In principle, flux analysis takes the network perspective, but the models used for flux analysis often exclude certain pathways/reactions on the basis of genetic evidence because it simplifies ^{13}C data interpretation. Such qualitative expression level information, however, does not necessarily exclude the presence of active protein, and some discrepancies in reported flux data can be traced back to incorrect network structures (van Winden *et al*, 2001a). If carried out with proper care, ^{13}C-based flux analysis offers the experimental capacity to actually determine the topology of active pathways and reactions from data. Particularly valuable is flux ratio analysis, as it yields direct and independent evidence for the *in vivo* operation of pathways in central metabolism (Szyperski, 1995; Emmerling *et al*, 2002; Fischer *et al*, 2004). For network flux-fitting procedures, two methods can support the inference of network topology: (i) optimal experimental design for labeling experiments that provide maximum information for particular regions of the network (Möllney *et al*, 1999; Petersen *et al*, 2000; Fischer *et al*, 2004) and (ii) rigorous identifiability analysis of an existing data set (van Winden *et al*, 2001b; Isermann and Wiechert, 2003; Rantanen *et al*, 2006). When adding noise to the data, the latter is extended to statistical identifiability analysis that reveals how well a particular flux is actually determined from a data set, which can also be done for subnetworks (Antoniewicz *et al*, 2006). Model discrimination based on statistical evaluation of how well different network models fit a data set is then used to identify the correct topology of active reactions (Klapa *et al*, 1999; Dauner *et al*, 2001; Arauzo-Bravo and Shimizu, 2003; Yang *et al*, 2005). Thus, several methods support inference of the condition-dependent network topology and the following two paragraphs highlight how flux data provide new network insights.

Well beyond classical tracer studies, modern ^{13}C-based flux analyses unraveled many surprises on the operation of the supposedly well-understood central metabolic network. A prominent example of unexpected activity of a principally known pathway is the Entner–Doudoroff pathway (Fuhrer *et al*, 2005), in particular in actinomycetes, where other data had suggested that different pathways would be operational instead (Gunnarsson *et al*, 2004; Borodina *et al*, 2005). A seemingly widespread phenomenon is gluconeogenic flux during otherwise glycolytic metabolism around the PEP–pyruvate–oxaloacetate node, although expression data suggest that the corresponding genes are not actively transcribed. Simultaneous operation of glycolytic and gluconeogenic reactions at this key node led to, in some cases, substantial loss of energy via ATP-dissipating futile cycles in *Escherichia coli* (Emmerling *et al*, 2002; Yang *et al*, 2003), *Bacillus subtilis* (Sauer *et al*, 1997), *Corynebacterium glutamicum* (Petersen *et al*, 2000), and others (Fuhrer *et al*, 2005). From the pathway concept that attempts to assign specific functions, such futile cycling is neither predicted nor understood, but may offer a flexible control strategy to rapidly reorganize network fluxes upon environmental changes.

The discrepancy between the network reality and the traditional pathway concept is highlighted by the recent ^{13}C-based discovery of the PEP-glyoxylate cycle in *E. coli* (Fischer and Sauer, 2003a), which was hypothesized earlier on theoretical grounds (Liao *et al*, 1996; Schuster *et al*, 1999). The key reactions of this bifunctional anabolic and catabolic cycle are PEP carboxykinase and the glyoxylate shunt, whose previously known functions are gluconeogenesis and anaplerosis, respectively, during growth on substrates that feed into the tricarboxylic acid (TCA) cycle. Their conjoint operation during glucose metabolism, however, effectively generates a novel cycle whose catabolic function is in sharp contrast to their known individual functions. The cycle's overall stoichiometry is almost identical to the classical TCA cycle, the previously exclusive textbook route for full oxidation of hexoses to CO_2. Thus, even the seemingly well-understood central metabolism in microbes bears surprises, and the network perspective is expected to be particularly critical for higher cell or organ metabolism.

Regulation and control of flux

Beyond qualitative, mostly genetic knowledge, our current understanding on how cells actually control their fluxes is limited. Lacking the beauty of genetic on/off regulation, overlapping layers of genetic and metabolic regulations, often with opposite directions, influence metabolic fluxes in a highly condition-dependent manner. Current key questions are as follows: which mechanisms control flux through a particular pathway? To what extent? When is a given mechanism relevant? How can we manipulate fluxes? Such questions are currently revisited by two approaches that link particular control mechanisms to experimental flux data.

The first approach follows the logic of classical metabolic control analysis (MCA), which defines a quantitative link between flux through a particular pathway and the activity of its constituent enzymes (Fell, 1997). Extended to regulation, the current focus is to quantitatively disentangle metabolic from hierarchical (all processes that determine active enzyme concentrations) regulation of flux (ter Kuile and Westerhoff, 2001). Downregulation of glucose influx in *E. coli* was thus shown to be either fully hierarchical or mixed metabolic/

hierarchical, depending on whether nitrogen or glucose starvation was imposed, respectively (Rossell *et al*, 2005). Extending such regulation analysis from individual reactions to the sequential reactions of glycolysis, flux regulation induced by nutrient starvation was demonstrated to be inhomogeneous in the various constituent enzymes of the pathway, varying from fully hierarchical to exclusively metabolic (Rossell *et al*, 2006). This MCA-based regulation analysis can quantitatively describe the different levels of flux regulation through single reactions or linear pathways, but not yet for the distribution of flux through the network.

The second line of research attempts to identify and quantify the mechanisms that actually control the distribution of flux between different pathways. In particular, transcriptional and protein level regulation of flux has become a focal point, primarily because it can be tackled through flux analysis in mutants. Although many regulator knockout mutants have a strong physiological phenotype, flux analysis can identify specific flux changes in the network of such mutants. Such specific flux changes imply that the deleted regulator modulates the relative distribution of flux in the network by inducing or repressing particular pathways.

In general, surprisingly few regulators appear to have a specific impact on the distribution of flux. Out of 19 *B. subtilis* and seven *E. coli* transcriptional regulators tested, only two and one, respectively, exhibited a specific impact on the flux distribution in central metabolism (Fischer and Sauer, 2005; Perrenoud and Sauer, 2005). These and similar results support the above conclusions from regulation analysis that, in particular, central metabolic fluxes are rarely regulated at the expression level alone. An extreme case of transcriptional regulation is CcpN, a newly identified repressor of two gluconeogenic genes (Servant *et al*, 2005), whose knockout caused a severe flux redistribution in basically all major pathways in *B. subtilis* (Fischer and Sauer, 2005) (Figure 3B). The response regulator ArcA is a well-known repressor of the TCA cycle genes under oxygen limitation in *E. coli*. *In vivo* flux data demonstrated, however, that it also controls TCA cycle fluxes under fully aerobic and anaerobic conditions (Perrenoud and Sauer, 2005). Other transcriptional regulators, whose specific flux impacts were recently described, are the carbon repressor CreA in *Aspergillus nidulans* (David *et al*, 2005) and the virulence regulator PrfA in *Listeria monocytogenes* (Eisenreich *et al*, 2006). In particular, the parallel glucose sensing pathways in the model yeast *Saccharomyces cerevisiae* have attracted interest, and recent flux data identified several regulators whose knockout can be used to partly alleviate glucose repression of the TCA cycle (Blank and Sauer, 2004; Raghevendran *et al*, 2004).

With the availability of complete knockout mutant libraries for many model organisms, systematic flux analyses of mutants with defects in signal transduction and transcriptional regulation are underway. Beyond functional mapping of the hierarchical regulation network that controls metabolism, such quantitative data on the relevance of particular signaling events will be important for the construction of computer models. Obviously, quantitative understanding of flux control in the network would also enable precise and subtle re-engineering of cell factories (Vemuri and Aristidou, 2005), thus circumventing the current brute force knockout/overexpression strategies that perturb cellular operation in many, often unwanted ways.

Metabolic engineering of biotechnologically relevant flux states

The original driver for development of modern microbial flux methods was metabolic engineering that emerged about 15 years ago (Bailey, 1991). ^{13}C-based flux methods since became a key analytical technology in support of biotechnological applications (Stephanopoulos, 1999; Sanford *et al*, 2002). Beyond simple description of network responses to verify success of genetic manipulations or absence of non-obvious limitations, well-designed flux studies also enabled to devise new, non-obvious metabolic engineering strategies. A successful example was the discovery of substantial ATP-dissipating futile cycle fluxes through the PEP carboxykinase in lysine-producing *C. glutamicum* (Petersen *et al*, 2000). Subsequent deletion of this activity significantly improved lysine production (Petersen *et al*, 2001). An interesting recent example comes from the Wittmann Lab, where several comparative flux analyses on different substrates suggested insufficient NADPH supply through the pentose phosphate pathway for high lysine production on fructose-based substrates (Kiefer *et al*, 2004; Wittmann *et al*, 2004). From these results, the non-obvious strategy to overexpress the glucose-repressed fructose 1,6-bisphosphatase was devised, and shown to significantly improve product yields on the industrially relevant substrates fructose and sucrose (Becker *et al*, 2005).

Nevertheless, flux data rarely reveal a direct engineering target, primarily because fluxes result from multiple component interactions and genetic manipulations must be made at the component level through, for example, overexpression of a gene. Hence, it is necessary to integrate flux and potentially other 'omics' data by means of computational methods to identify the most promising engineering strategies and to quantitatively understand—and thus predict—complex network operation, which is discussed next.

Fluxes as input for or predictions of computer modeling

The most accurate representation of metabolic networks and their regulation are mechanism-based kinetic or stochastic models that reflect both the static network stoichiometry and the dynamic interaction of its components as described by kinetic parameters and reaction mechanisms. For small subnetworks, such detailed dynamic models can predict intracellular flux responses, and experimental flux data can be used to (i) interrogate those predictions or (ii) as input data for parameter estimation. However, the complexity of realistic networks and the lack of knowledge on the actual reaction mechanisms as well as the unavailable parameters limited so far the success of dynamic models (Stelling, 2004). By considering only the generally known reaction stoichiometry, static metabolic models can be generated at the genome scale with around a thousand reactions (Schilling and Palsson, 2000; Price *et al*, 2004; Borodina and Nielsen, 2005). Such stoichiometric models enable

qualitative predictions of an organism's metabolism in steady state with metabolic fluxes as the key variable. This constraint-based modeling approach (flux balancing analysis) does not attempt to predict precisely what the network does, but rather to distinguish feasible from unfeasible flux states, based on the constraints of connections and reaction reversibilities in the network.

These genome-scale stoichiometric models provide a biochemically and genetically consistent framework for systematic generation and testing of hypotheses on metabolic functions. Experimental data on central metabolic fluxes are then used either to further constrain the space of feasible solutions in the entire reaction network (Wiback *et al*, 2004; Kuepfer *et al*, 2005; Herrgard *et al*, 2006a) or to test model predictions (Segre *et al*, 2002; Almaas *et al*, 2004). Another important application is in the experimental design of large-scale flux experiments because model predictions allow one to focus efforts on the most meaningful experiments (Blank *et al*, 2005). Ultimately, the goal of metabolic modeling is integration of quantitative experimental data on fluxes, metabolites, and proteins to explain and predict metabolic regulation and cellular phenotypes. One attempt to this end is the second generation of genome-scale models that incorporate primarily literature-based transcriptional regulation using Boolean on/off rules (Covert *et al*, 2004; Barrett *et al*, 2005). Already with such relatively crude extensions of the network stoichiometry, large-scale transcript data can be integrated in the network context and potential regulation mechanisms may thus be identified (Herrgard *et al*, 2006b). Similar in scope is an approach to use probabilistic graphical models to either infer or explicitly include hypotheses on links between metabolic reactions and transcription (Yeang and Vingron, 2006).

More quantitative integration may be achieved through an important extension of the constraint-based framework that considers additionally thermodynamic principles (Kümmel *et al*, 2006). Such network-embedded thermodynamic analysis is a conceptual framework for quantitative integration of metabolite concentration and flux data. Beyond its capacity to verify consistency of flux and metabolite data and to predict concentration ranges of unmeasured metabolites, the results can also reveal putative sites of active (genetic or metabolic) regulation. Another development is hybrid models that combine stoichiometry and kinetics. By extending large stoichiometric models with a local kinetic model (for example of a particular branch point), the dynamics of a specific metabolic regulation process can be assessed in the context of the entire network (Petersen *et al*, 2003). For the lack of data, such hybrid models focus on one or few dynamic regulation sites. An alternative is to define a surrogate for the missing details of kinetic regulation on the premise that biological systems have evolved objective-based control programs that can be mathematically represented in large-scale models. Kinetic parameters and the underlying objectives of the metabolic control structure are then identified from flux data (Varner, 2000). Somewhat related is a first attempt to extend the static constraint-based concept of flux balance analysis to dynamic metabolite concentrations (Luo *et al*, 2006). All approaches mentioned in this paragraph can potentially integrate these two different data types (rates and concentrations), which opens up the road to analyze dynamics of network regulation.

Evolutionary 'design' of metabolism

In sharp contrast to the complexity of physical systems, such as sand dunes, where every particle can interact with each other, biological complexity is highly structured and functionally shaped through evolution. A key goal of systems biology is to identify the common evolution ('design') principles that underlie structure, regulation, and operation of networks. Several recent theoretical analyses provided new insights into structural design principles of metabolism. An almost obvious one is the 'bow-tie' structure of metabolism; that is, many parallel sequential pathways for nutrient degradation merge into a core set of reactions from which again a large number of biosynthetic pathways fan out (Csete and Doyle, 2004) (Figure 3A). This ubiquitous and highly interconnected core set of reactions is largely redundant with the classical central carbon metabolism (Ma and Zeng, 2003). Not based on flux but on promoter activity data, the just-in-time transcriptional program of metabolic pathways was recently identified as a regulation design principle of biosynthetic pathways in *E. coli* (Zaslaver *et al*, 2004). It describes the wave-like temporal expression, where enzymes at the beginning of a biosynthetic pathway are transcribed from promoters with shorter response times and higher maximal activity than enzymes that are further down the pathway.

With established methods for higher-throughput flux analysis under steady-state conditions (Fischer *et al*, 2004), we can now begin to ask key systems biology questions about the design principles of network operation. In contrast to the generally rather variable and/or noisy concentration data of transcripts and metabolites, large-scale flux studies from bacteria and yeast revealed a surprisingly rigid distribution of fluxes that appears to be a general design principle of metabolic network operation in microbes (Blank *et al*, 2005; Fischer and Sauer, 2005). Whereas the overall flux into cells and the rate of growth varied significantly between mutants, and thus most likely also transcript and/or metabolite concentrations, the relative distribution of flux into different pathways remained remarkably constant (Figure 3B). Apparently, metabolism is in a stable state that is robust towards random genetic perturbations, but responds flexibly to environmental stimuli.

Another unexpected observation was mutants with higher rates and higher efficiency of growth in *B. subtilis* when central regulators for developmental programs such as sporulation or flagella formation were deleted (Fischer and Sauer, 2005). This led to the hypothesis of the stand-by-mode design principle, where metabolism is kept in a suboptimal state in anticipation of changing environmental conditions. This stand-by-mode is probably more specific to microbes with developmental programs. Combining flux data with genome-scale stoichiometric models, there is currently significant interest to infer underlying 'rationales' of metabolic evolution by asking questions such as whether cells optimize their fluxes to maximize biomass formation (Fong and Palsson, 2004; Fong *et al*, 2006) or whether they minimize redirection or on/off regulation of flux

changes upon genetic perturbations (Segre *et al*, 2002; Shlomi *et al*, 2005).

Future directions

To identify the actual control mechanisms, the need for integration of flux data with other genome-wide data is clearly recognized. As this can only be achieved through mathematical modeling, we can expect significant progress on computational methods for data integration in the near future, and several promising approaches were discussed in the modeling section. Based on the current methods, flux studies will likely identify further microbial design principles and modes of network operation. The available experimental flux methods themselves, however, suffer from a number of limitations that call for improvements.

A precondition of current flux analysis methods is metabolic steady state; that is, all fluxes must be constant over the course of the tracer experiment (Wiechert and Nöh, 2005). Although experiments can be set up to meet this requirement, continuously changing environments are a biological reality. Hence, development of dynamic flux methods is an obvious necessity that is currently followed by two approaches. In the first, the required steady-state condition is reduced by detecting the isotope patterns in free intracellular intermediates (van Winden *et al*, 2005). As the pool size of primary metabolites is orders of magnitude smaller than the normally analyzed proteinogenic pool of amino acids, isotopic steady state is reached much faster. Labeling experiments may thus be shortened from several hours (multiple cell divisions) to minutes, although exchange of label with large pools of unlabeled intracellular macromolecules may extend the labeling period to 1–2 h. This method will also be applicable to complex media, because central intermediates contain information-bearing label but not the normally used amino acids that are imported from the complex components. These benefits come at the cost of laborious protocols for rapid sampling of large cell quantities, instantaneous quenching of metabolic activity, and sensitive high-end MS analyses of chemically diverse species.

In contrast to the above detection of relative changes in the ^{13}C patterns as the information-bearing unit, the second approach exploits the kinetic information of label distribution during the first minute of isotopic instationarity (but metabolic stationarity) (Nöh and Wiechert, 2006). In addition to monitoring time-dependent accumulation of tracer molecules in intracellular metabolites, also (many) pool sizes must be known. The fluxes are then calculated through systems of differential equations from pool sizes and the rate of label accumulation. As the computational effort to solve the large differential equation systems is currently extremely high, further developments are necessary (Nöh *et al*, 2006). Both dynamic flux methods will allow tackling a number of key problems, for example, flux changes during culture transients or the cell cycle in synchronized cells.

Lastly, global tracking of small molecule fluxes in mammalian cells, perfused organs, or humans is highly relevant for monitoring and understanding of disease phenotypes, nutrition, or drug metabolism. Unfortunately, the current method repertoire is limited to the detection of one or few relative fluxes or macromolecular turnover (Hellerstein and Murphy, 2004; McCabe and Previs, 2004; Sherry *et al*, 2004). One problem is the continuously changing physiology where fluxes typically vary long before metabolite pools attain isotopic steady state (Kelleher, 2001). Another fundamental problem is network complexity with temporal and spatial separation of metabolic tasks between different compartments, cell types, and organs. As flux analysis depends on a mathematical framework for the interpretation of isotope tracer patterns, two key issues currently preclude network flux analysis in higher organisms. First, as a prerequisite for model construction, our structural knowledge on all possible distributions of tracer atoms in large, possibly multicellular networks is incomplete, in particular for tracers such as ^{2}H that exchange with water. Second and more importantly, it is extremely difficult to acquire sufficient data to resolve fluxes in such complicated models. A conceptually novel solution is model-independent fluxome profiling that was demonstrated for genetic variant discrimination in microbes from ^{2}H and ^{13}C experiments (Zamboni and Sauer, 2004), but is readily applicable to higher organisms when labeling patterns are detected in a sufficient number of intracellular metabolites. Akin to concentration-based 'omics' analyses, fluxome profiling relies exclusively on experimental data—isotope distributions in this case—and multivariate statistical analysis. The approach lacks the biochemical resolution of model-based flux analyses, but is quantitative in terms of relative flux differences. Hence, it can be used to discriminate genetic variants, drug toxicity, nutrition, or disease states of higher cells and organisms based on their functional flux phenotype.

Conclusions

Metabolic flux analysis based on stable isotope experiments is a quantitative method to assess gene, protein, and metabolite interactions within metabolic networks. It thus is an important complement to the detection of global transcript, protein, and metabolite concentrations—the network components. Although neither of the component 'omes' is a functional end point of cellular processes but rather contains potentially valuable indicators of such processes, fluxes are the integrated functional output of a metabolic network. Owing to their inherent network perspective, flux data are highly relevant for fundamental systems biology of metabolic networks and for all applications that focus on manipulating or monitoring metabolic behavior in areas like metabolic engineering, nutrition, and medicine.

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