

Regulation and control of metabolic fluxes in microbes

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After about ten years of research renaissance in metabolism, the present challenge is to understand how metabolic fluxes are controlled by a complex interplay of overlapping regulatory mechanisms. Reconstruction of various regulatory network topologies is steaming, illustrating that we underestimated the broad importance of post-translational modifications such as enzyme phosphorylation or acetylation for microbial metabolism. With the growing topological knowledge, the functional relevance of these regulatory events becomes an even more pressing need. A major knowledge gap resides in the regulatory network of protein–metabolite interactions, simply because we lacked pertinent methods for systematic analyses – but a start has now been made. Perhaps most dramatic was the conceptual shift in our perception of metabolism from an engine of cellular operation to a generator of input and feedback signals for regulatory circuits that govern many important decisions on cell proliferation, differentiation, death, and naturally metabolism.

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Introduction

Compared to other biological networks, function and topology of metabolism are unusually well understood. Much of the contemporary focus is on the higher level cellular phenomenon of how small molecule fluxes emerge from the interaction between thousands of enzymes and metabolites in this network. Largely driven by advanced experimental [1] and computational methods [2] to estimate not only intracellular fluxes but also metabolite concentrations [3], novel biological insights are generated for microbes [4] and higher organisms [5]. In particular microbial metabolism became a paradigm network for systems biology [6]. In our view, the presently greatest challenge resides in unraveling, in a quantitative sense, the interplay between

the various regulatory mechanisms that control metabolic fluxes.

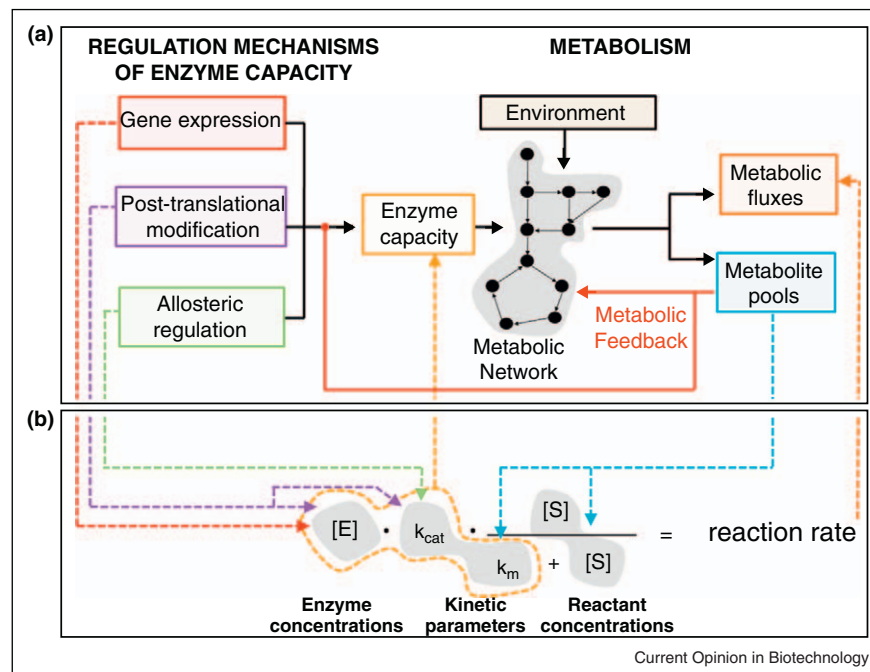
The key problem in understanding how metabolic fluxes are modulated is the complexity arising from multiple overlapping regulatory mechanisms and metabolic feedback into regulatory networks (Figure 1). The actual *in vivo* capacity of an enzyme to realize a catalytic flux is a function of its abundance and kinetic properties. Of the three layers of cellular regulatory mechanisms, *gene expression* acts only on enzyme abundance, *post-translational modification* may modulate abundance and kinetic parameters (e.g. by changing the degradation rate or protein phosphorylation, respectively), while *allosteric regulation* modulates exclusively the kinetic parameters through non-covalent binding of effector molecules to non-catalytic sites. The actual *in vivo* reaction rate (i.e. metabolic flux), however, depends also on the *in vivo reactant concentrations* (Figure 1b). Since these metabolite concentrations are a function of thermodynamics and reaction kinetics, the cell may modulate them only indirectly. Further complexity is caused by the reactant-mediated link to neighboring reactions (i.e. through altered reactant concentrations or competitive inhibition) and through feedback regulation into all three layers of regulatory mechanisms. While current large-scale efforts focus mostly on mapping the interactions between regulators and their targets within a given mechanistic class, for example, transcription [7] or phosphorylation networks [8], understanding metabolic regulation as a whole eventually requires the entire set of regulatory mechanisms to be considered.

Here we review progress since 2009 on metabolism-relevant regulatory networks in microbes, with a focus on publications that provide biological insights rather than new methods. For this purpose, we grouped papers into four categories: i) reconstructing regulatory network topology, ii) unraveling input–output regulatory logics and metabolic feedback, iii) assessing and quantifying the control exerted by regulation events, and iv) understanding the evolution of metabolic regulatory circuits.

Reconstructing the topology of metabolic regulatory networks

Topological knowledge of interactions between regulators and targets, and among regulators, is a prerequisite to understand regulation strategies. Driven by mature experimental methods to quantify gene expression and to map physical interactions between transcription factors and genes (i.e. chip-ChIP microarrays), reconstruction of transcriptional network topologies receives much attention [6].

Figure 1



A systems biology view of metabolism and its regulation. (a) Metabolic fluxes and metabolite pools emerge in the metabolic network from the complex interplay between cellular regulation of enzyme capacities, the environment, and metabolite pools themselves. Metabolite pools also provide a feedback mechanism from within metabolism to all modes of enzyme regulation. (b) Metabolic fluxes are net sums of underlying enzymatic reaction rates. Enzymatic reaction rates are themselves integral output of three biological quantities interacting at the level of enzyme kinetics: enzyme concentrations, kinetic parameters, and reactant concentrations, here exemplified by the classical Michaelis–Menten law. Enzyme concentrations ($[E]$) can be regulated by gene expression and post-translational modification; kinetic parameters such as turnover (k_{cat}) and substrate affinity (k_m) can be modulated by post-translational modification, allosteric regulation, and competitive inhibition; reactant concentrations ($[S]$) are metabolite pools that emerge from the overall metabolic system.

Curated databases are available for microbial model organisms, some with explicit links to metabolism [9]. Although transcriptional networks are presently the most advanced regulatory network reconstructions, recent investigations of the key *Escherichia coli* transcription factors of central and amino acid metabolism, *Cra* and *Lrp*, revealed 144 and 121 previously not annotated binding sites [10,11]. This 7-fold increase relative to previous knowledge suggests there are still wide gaps in the network topology. Computational analyses of the *E. coli* transcription topology highlight a hierarchical organization of general and specific transcription factors in which the diverse catabolic, anabolic, and central metabolic pathways are targeted by distinct combinations of regulators [12,13]. Notably, reconstruction of transcription networks increasingly embraces the complexity of transcription unit architectures by including regulatory events beyond transcription factors such as alternative sigma factors [7,14], anti-sense transcription [14], and transcriptional attenuation [15]. In contrast to transcription, translational regulation is mostly studied on a case-by-case basis. A recent example draws attention to the role of the small RNA *fnrS* in targeting mRNA of enzymes involved in anaerobic metabolism, acting in cooperation with the transcription factor Fnr [16].

On a different layer of regulation, much progress has been made in reconstructing networks of post-translational modification, primarily by advanced mass spectrometry-based proteomics. The most relevant protein modification is arguably reversible phosphorylation, where phosphoproteome complexity reflects evolutionary progress from few hundred phosphorylated proteins in bacteria (around 5% of all cellular proteins) [17] to thousands in eukaryotes [18]. Frequent phosphorylation of bacterial enzymes suggests a particular relevance for metabolic regulation [17,19,20]. The current focal point for comprehensive mapping of phosphorylation networks is the eukaryotic model organism *Saccharomyces cerevisiae*. An early study with a metabolic focus led to the identification of 80 phosphorylation sites in 48 different mitochondrial proteins, mostly enzymes [21]. Mass spectrometry phosphoproteomics from large-scale genetic perturbations brought a major advance by identifying 8814 regulated phosphorylation events in 124 kinase/phosphatase deletion mutants of yeast [8]. Functional interactions between these kinases and regulatory proteins were mapped by epistatic miniarray profiles from growth rates in pair-wise deletion combinations, revealing rather frequent occurrence in the same complex and/or function in

the same pathway [22]. Typically, however, there is only a partial functional overlap between these interacting pairs of regulators, as was demonstrated by transcriptomics data from 150 single and 20 double kinase/phosphatase mutants [23]. An amazing 1844 of such physical interactions between protein and metabolite kinases with regulatory subunits and phosphatases were identified by mass spectrometry [24^{*}]. These data render the yeast phosphorylation network the currently most comprehensive – nearly 60% of the about 6000 genes encode phosphoproteins [25]. Many metabolism-related phosphorylation events were unraveled, but in particular the Breitkreutz *et al.* [24^{*}] interaction study identified several new effector kinases with relevance in nitrogen and carbon metabolism. In sharp contrast to this great progress in mapping network topology, function of specific phosphorylation events has rarely been demonstrated and quantified, leaving a major challenge for the near future.

Beyond phosphorylation, further post-translational modifications of lysine residues in metabolic enzymes were recently demonstrated in microbes; that is, succinylation [26], acetylation [27,28^{**}], and conjugation with the degradation-regulating, ubiquitin-like protein PUP [29,30]. While succinylation was only described for three *E. coli* enzymes, about 50% of the 191 acetylated *Salmonella* proteins were metabolic enzymes. Functionally, acetylation was shown to control the direction of glycolytic versus gluconeogenic flux and the branching between tricarboxylic acid (TCA) cycle and glyoxylate bypass [28^{**}]. Although functional evidence is lacking, succinylation of the glycolytic glyceraldehyde-3-P dehydrogenase and the TCA cycle isocitrate dehydrogenase in *E. coli* suggests a somewhat similar role [26]. Similarly, the pupylation network of *Mycobacteria* was systematically reconstructed by proteomics, revealing that about half of the 41 identified pupylated proteins were central metabolic or respiratory enzymes [30]. While pupylation has so far been described in only few species and only few succinylation events are known, acetylation represents a more general metabolic regulation mechanism conserved from bacteria [28^{**}] to mammals [27]. At present it remains unclear, however, how specificity in the response to different nutritional conditions can be achieved when only a single pair of lysine acetyltransferase and deacetylase exists like in *Salmonella* [28^{**}].

Finally, reconstruction efforts have begun also to investigate the topology of protein–metabolite interactions. In the absence of a dedicated methodology in the past, we had to rely on serendipitous discovery of allosteric regulation of enzymes or regulatory proteins, although such allosteric interactions are major determinants of dynamic feedback [6,31,32^{*}]. Two recent lines of work demonstrate approaches to systematically identify protein–metabolite interactions, one by starting from

lipid metabolites to screen for *in vitro* binding proteins [33,34] and the other starting from affinity-purified proteins to identify the *in vivo* bound metabolites by mass spectrometry [35^{*}]. In particular the *in vivo* analysis revealed an unexpected wealth of binding partners in ergosterol biosynthesis in yeast. Presently, these methods are limited to non-polar metabolites owing to the problem of losing polar ligands during washing. A more indirect alternative is statistical inference of gene–metabolite interactions through Bayesian integration (calculating probabilities that a hypothesis is true) from coordinated changes in concentration of transcript and metabolite data [36]. Since these interactions encompass direct and indirect relationships, they provide testable hypotheses on regulatory protein–metabolite interactions. Although protein–metabolite interactions are lagging far behind all areas of topology mapping, it is crucial in particular for a better understanding feedback regulation and a start has been made.

For metabolism, all the above network topologies relate to the level or activity of enzymes that modulate *in vivo* fluxes (Figure 1). Beyond the better-known protein localization into different compartments, it hence came somewhat as a surprise to find widespread physical reorganization of metabolic enzymes into large aggregates upon nutrient starvation as a regulatory mechanism, again in yeast [37]. While we have already discussed the importance of protein–protein interactions for phosphorylation-based regulation [24^{*}], here it is the very machines of metabolism themselves that aggregate reversibly into discrete physical structures. The high proportion of central metabolic and biosynthetic enzymes in these large complexes indicates a nutrient-specific function in substrate channeling, flux control, or enzyme storage [37]. Similar proteinaceous microcompartments have been described for ethanolamine utilization in *E. coli* [38], the TCA cycle of *B. subtilis* [39], and the carbon fixation machinery of cyanobacteria [40]. These dynamic enzyme assemblies maintain somewhat separate concentrations of their intermediate, which facilitates fast turnover of labile or toxic compounds and may also prevent undesired crosstalk between different pathways [41].

Unraveling the logic of regulatory networks and metabolic feedback

Beyond interaction topologies, knowledge of how regulatory networks compute input signals into output regulatory responses is necessary to fully understand regulation. This is of particular relevance for the intertwined and cross-talking regulatory networks. Because unraveling of input–output regulatory logics often requires pre-existent topological knowledge, current attempts mainly focus on transcriptional and signaling networks. The simplest and often used modeling abstraction for transcriptional networks is discretization of transcription factors (on or off) and target genes are expressed

according to equations composed of Boolean operators (i.e. AND, OR, NOT). Boolean networks can be easily combined with stoichiometric metabolic models for steady-state flux simulations. Boolean rules link environmental cues and the metabolic state to the transcription of a cell's genome [42] that can be automatically refined through cycles of matching experimental transcript and flux data [43]. Simulations suggest transcriptional regulation to control steady state fluxes in up to 20% of the reactions [44]. Different from the rather crude on/off abstraction of Boolean logic that is applicable to large networks, quantitative models of transcriptional regulation based on kinetic mechanisms or linear approximation can be used for small-sized or medium-sized networks. At the level of individual promoters that integrate different transcription factors, quantitative regulation of enzyme-encoding genes has been investigated by thermodynamic models *in vitro* [45] and *in vivo* [46]. Examples are quantitative input–output functions controlling uptake gene expression upon sugar availability in *E. coli* [47]. Crucial for inference of such quantitative input–output logics in more complex networks is the experimentally cumbersome quantification of transcription factor activities, which must be done by either fluorescent reporter promoters [46] or linear regression analysis of target gene expression on the basis of the known topology [48]. A further major challenge is unraveling the dynamic nature of regulatory responses. By considering temporal motifs, the timing of transcriptional regulation was demonstrated to often follow the topological order of metabolic pathways [49], although this particular case illustrates the danger of missing major mechanistic interrelations when focusing only on one layer of regulation [50]. In combination with modulated transcript half-lives, already the transcriptional network can perform complex tasks such as reliably responding to a slowly changing environment, while effectively ignoring fast fluctuations, as was nicely shown for yeast sugar catabolism [51]. Current efforts highlight transcriptional networks as the regulatory network of preference to study quantitative and temporal aspects of such input–output regulatory logics (see [52^{*}] for a recent review).

Faster than the minute-time scale of transcription network responses, signaling networks simultaneously integrate intracellular and extracellular signals and their input–output logic must additionally consider protein modification and metabolite interactions that occur on a seconds-time scale. Reconstructing such signaling networks at genome-scale comes within reach [53], but current applications are small-scaled. By explicitly including extracellular signaling in a kinetic model, it was demonstrated that *S. cerevisiae* evolved for economic stress protection: almost no response to fast pulse-like osmotic changes but well-adapted to periodic changes at a certain frequency [54]. Considering crosstalk between transcription and signaling is starting to reveal particular

input–output logics that control yeast metabolism, such as an incoherent feed forward loop that leads to a transient expression pulse of a glucose transporter [55] and a low-energy check point by the Snf1 kinase that controls transcription of enzymes in central and redox metabolism [56]. Overall, the fast time-scales of signaling networks render unraveling their input–output logics cumbersome, where quantitative and dynamic data are limiting.

While the previous sections dealt with regulatory mechanisms that feed into metabolism, we discuss in the following the opposite direction: feedback signals coming from metabolism into regulatory networks [57]. Frequently, metabolite-binding to transcription factors changes their activity [44,45,46], and metabolite-binding to catalytic RNAs (ribozymes) at so-called riboswitches can also modulate splicing, translation, or RNA stability [58]. The enrichment of kinases and phosphatases in metabolite–protein interaction screens [33,35^{*}] suggests that also post-translational modifications may be subject to metabolic feedback. In some cases, these metabolic feedbacks have been quantified through modeling, such as the intracellular dynamics of fructose-1-6-bisphosphate in *E. coli*, which is hypothesized to signal glucose limitation to central metabolism via the Cra-regulated transcription network [31]. According to control system theory, homeostasis and adaptation is best achieved when internal signals report the systems' state – metabolite levels or flux rates in the case of metabolism – to the regulatory circuits (Figure 1a, red arrow). But how does metabolic feedback through metabolite levels relate to the flux output of metabolism? Kinetic modeling demonstrated that *E. coli* senses concentration changes in few metabolites that report the central metabolic flux state to transcription factors [59^{**}]. Provided that a small amount of new nutrients can enter the cell, this distributed flux-sensing enables to mount appropriate responses to all carbon sources with only a handful of internal signals. Only few metabolites appear to qualify as potential flux sensors (i.e. their concentration is strictly correlated to the pathway flux) and at least some of them appear to be conserved across species boundaries [60]. Typically, such flux sensing is not restricted to transcriptional responses but involves also allosteric feedback at the level of enzymes [61]. First larger reconstructions of regulatory networks indeed highlight the strong involvement of internal metabolites in the general regulation of metabolism [62^{*},53].

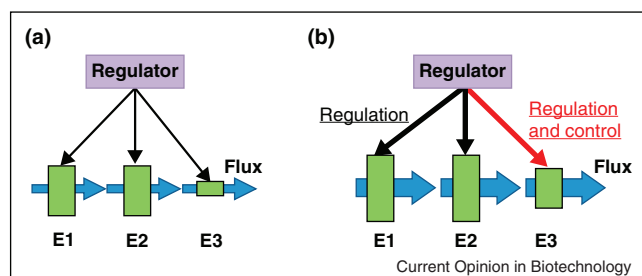
Lastly, even without dedicated regulatory mechanisms, substrate and product levels have a strong impact on the reaction rate and can have feedback-like consequences for the network (Figure 1). Quantitative metabolomics and ordinary-differential-equation-based modeling revealed substrate competition for the active site of saturated enzymes as a key determinant of fluxes in

nitrogen assimilation of *E. coli* [63^{••}]. Such competitive inhibition was shown for *E. coli*, whose metabolite concentrations exceeded the *in vitro*-determined K_m for most of the 100 tested substrate–enzyme pairs [64]. Consistent with the *E. coli* exception of near K_m substrate concentrations in lower glycolysis, not only metabolite concentrations in *S. cerevisiae* glycolysis but also TCA cycle were found to be near the K_m values [65[•]]. The consequence of near K_m concentrations was an inverse relationship between genetically induced fold-changes in yeast substrate metabolites and their enzymes upon local and global perturbations, suggesting that reaction rates are jointly limited by enzyme capacity and metabolite concentrations [65[•]]. Hence small variations in enzyme capacity are buffered by a converse change in substrate concentration that maintains a constant reaction flux (homeostasis) over a range of enzyme capacities (Figure 1). Whether indeed near K_m substrate concentrations prevail more generally in central metabolism and above K_m concentrations are the typical situation in the remainder of metabolism will be an issue for the near future.

Assessing and quantifying flux control of regulators

The previous sections summarized current progress in mapping out regulatory networks and the comparatively slower unraveling of their input–output responses to regulatory signals. From knowing that a particular regulation event is happening, however, we cannot infer its functional consequences because regulation is conceptually different from the control it exerts on a given biological functional output; that is, flux in metabolism. Here, the term regulation refers to a mechanism able to modulate enzyme activity by some molecular interaction (Figure 1). Instead, we speak of control when a regulator directly changes the overall pathway flux. Therefore,

Figure 2



Difference between regulation and control of metabolic fluxes. A linear metabolic pathway with three enzymes E1, E2, and E3 overarched by a regulator that modulates enzyme capacities (thickness of green rectangles). (a) Despite greater capacity of enzymes E1 and E2, capacity of enzyme E3 limits the overall steady state flux (thickness of blue arrows). (b) A regulatory event exerted by the regulator increases the three enzymatic capacities. Of the three observable regulatory events, only upregulation of E3 (red arrow) has an actual effect on the metabolic flux because it constrains the overall steady-state flux.

regulation is a local property influencing individual enzyme activities, while control is a global property effecting fluxes in the overall network (Figure 2) [66]. In metabolism, it is particularly important to distinguish both terms because control of metabolic fluxes is shared among many regulators that act simultaneously at different regulatory layers. Typically, only few of the many co-occurring regulatory events actually exert flux control at a given time and condition, hence it is crucial i) to assess whether a regulatory interaction has an impact on *in vivo* flux and ii) to quantify the extent of its flux control.

To address the first point, the most reliable method to determine intracellular fluxes is based on ^{13}C -experiments [1]. Since ^{13}C -flux analysis is now feasible at a throughput of several hundred parallel experiments, it can be used to assess the impact of genetically manipulated regulation events. Precisely this approach has been used to systematically identify transcription factors that control the distribution of flux through different metabolic pathways. Intracellular fluxes were quantified in more than 100 transcription factor deletion mutants of yeast [67[•]] and *E. coli* [68] under different conditions. Although up to 2/3 of the factors were active on some cellular process based on a detectable growth phenotype, only about a handful of them actually controlled the distribution of flux under a given condition in either organism. Although many transcription factors influence enzyme abundance and fluxes in particular pathways, only up to a handful controlled the distribution of fluxes per condition. The transcriptional control of flux distributions during sugar catabolism focused on the switch between respiratory and fermentative metabolism, which in the case of yeast involved a network of 23 of 119 tested transcription factors that fine-tuned the response under five different conditions [67[•]]. Since flux data can only reveal the affected pathways, it required ‘omics’ data from the relevant transcription factor mutants to derive testable hypotheses on the actual target genes that caused the observed flux increase. Rather than perturbing (potential) individual regulation events, the typical approach follows the reverse logic, inferring regulatory relevance from ‘omics’ data. Two good examples are i) the control of flux direction and branching in central metabolism of *Salmonella* through enzyme acetylation [28^{••}] and ii) unexpectedly strong carbon catabolite repression through the TCA cycle intermediate malate in *B. subtilis* [69]. To extend beyond central metabolism, genome-scale fluxes can be estimated through stoichiometric modeling, albeit with less confidence, and such data have been used to identify the relevant transcript [70] or enzyme [71] changes from flux-transcript (or protein) correlations under different conditions in yeast. While these analyses did not directly reveal the underlying mechanism, they allowed to hypothesize which fluxes are likely to be transcriptionally controlled [70]. The obvious next question is which regulators are responsible for this transcriptional control? Starting

from the regulatory network topology or inferring the most likely transcription factor for observed transcriptional changes [11], it should be possible to design conclusive experiments on flux control.

The second point is to quantify the extent to which a regulation process actually controls a flux under a certain condition. For this we need to know i) how much pathway flux is affected by enzyme activity and ii) how much enzyme regulators affect this activity. Historically, the first question has been addressed in the context of metabolic control analysis and, with a different focus, by biochemical systems theory [66]. The task boils down to estimating numerical coefficients with predictive power from data; that is, numbers quantifying the flux change that will happen upon modulation of enzyme activities at a given steady state. Based on an approximated kinetic model of central metabolism in hepatoma cells, glucose-6-phosphate dehydrogenase and oxidative phosphorylation were shown to exhibit negative and positive flux control, respectively, on the glycolytic flux [72]. Similarly, approximated kinetics highlighted the predominance of enzyme concentration rather than allosteric regulation in controlling glycolytic flux in *Lactococcus lactis* [73]. The second question on which regulator is relevant can only partially be addressed by such approaches because the mechanisms that regulate those putatively controlling reactions typically remain unknown. In this case, the approach of regulation analysis helps delineating which regulatory layer is responsible for establishing fluxes through a given enzyme [74]. In its time-dependent extension, it allows to derive quantitative coefficients for hierarchical (i.e. expression and post-translational modification) and metabolic regulation [75]. For the glycolytic enzymes of yeast, it was thereby shown that there is no particular temporal or enzyme preference for protein degradation or allosteric regulation during the first 4 h of nitrogen starvation. More resolution on the specific regulatory mechanism controlling reactions that limit flux is possible by using detailed kinetic models, when the regulation topology is known and rich data are available. This was done with a differential equation model populated with *in vitro* kinetic parameters of aspartate metabolism in *Arabidopsis*, which revealed allosteric interaction as being the key controlling event to decouple flux rerouting in competing pathways [76]. Using *in vitro* as well as *in vivo* estimated parameters, a kinetic model of ammonia assimilation in *E. coli* surprisingly revealed active-site competitive inhibition of glutamine synthetase by non-substrate metabolites as a key controlling mechanism during dynamic nitrogen perturbations [63**].

Understanding evolution of metabolic regulatory circuits

Are the diverse and overlapping regulatory mechanisms that act on different metabolic pathways in different

organisms ‘frozen’ accidents of evolution or potentially understandable through design principles that would drive evolution towards a particular regulatory configuration? For the simple case of linear pathways, metabolic control theory predicts adaptive evolution to occur first at enzymes with highest flux control, typically in the initial reaction steps [77]. Since flux control is a property specific to a steady state and thus highly condition dependent, evolution theories based on fitness maximization appear to have higher predictive power [78,79*]. For the example of maximizing benefit and cost of protein expression in lactose catabolism of *E. coli*, predictive power was validated experimentally by evolving multiple cost–benefit solutions in different environments [80]. These and other studies suggest that initial regulatory changes occur at the level of gene expression, a view fully consistent with the computationally inferred rate of evolutionary rewiring in various networks from comparative genomics and proteomics data [81]: evolution proceeds continuously slower from transcriptional regulation, phosphorylation, genetic interaction, protein–protein interaction to the slowest changes in metabolism itself. Rapid optimization of metabolic gene expression was empirically shown by transcriptomic and proteomic data from laboratory evolution experiments within 1000 generations [79*]. Overall, the transcriptional network is remarkably robust to rewiring, as was demonstrated by systematic transcription factor knockout studies [67*,68] and addition of 600 rewired links between promoters and transcription factors of *E. coli* [82]. In addition to simply modulating the strength of a given regulatory link, complete absence or addition of entirely novel links can improve condition-specific fitness [68,82] and thus broaden the possibilities for adaptive evolution.

There are clearly common principles underlying the evolution of regulatory circuits that govern metabolism. While our current understanding of these principles is coincidental, many identified architectures optimize properties such as resource allocation [78,79*], robustness-to-noise [83], and response time [55,84] under the framework of evolutionary cost-and-benefit to increase an organisms’ fitness. A major obstacle in understanding the evolutionary fitness of regulatory circuits is our limited knowledge of detailed molecular mechanisms and kinetic parameters. Sidestepping the requirement for such detailed knowledge, a recently developed theoretical framework introduces an approach to formally define the design space of biological circuits [85]. By enumerating phenotypes with quantitatively distinct behaviors [32*], it represents a conceptual step towards linking a genotype to the phenotypic advantages it confers to the organisms. Verified through experimental data, this design space framework demonstrated that the non-obvious dimerization rate of the oxygen-sensing transcription factor Fnr of *E. coli* is crucial for optimal regulation of anaerobic metabolism [86].

Conclusions

After about ten years of renaissance in metabolic research, the currently most important contributions are providing novel understanding of how metabolic fluxes are established and regulated. Reconstruction of various regulatory network topologies is steaming. In particular reconstructions of post-translational modification networks such as protein phosphorylation [8,24*] or acetylation [28**], are now in full focus. The past two years clearly showed that we have underestimated the broad importance of such modifications for microbes and their intermediary and energy metabolism. With the growing knowledge of regulatory network topology, the functional relevance of these regulatory events becomes an even more pressing need, and is presently most advanced for transcriptional networks. A major knowledge gap resides in the regulatory network of protein–metabolite interactions, simply because we lacked pertinent methods for systematic analyses – but a start has now been made.

Perhaps most dramatic was the conceptual shift in our perception of metabolism from an engine of cellular operation to an engine that is also steering many processes in microbial [6] and higher cells [87]. Metabolism provides the input and feedback signals for regulatory circuits that govern many important decisions on cell proliferation, differentiation, death, and naturally metabolism itself. Pivotal for a deeper understanding of this active regulatory role are i) methods to detect and quantify protein–metabolite interactions and ii) computational methods to integrate regulatory events dynamically across all relevant levels of molecular interactions.

As we move towards a more quantitative representation of metabolic regulation, we must assess the actual control that an occurring regulatory event exerts on establishing metabolic fluxes. Beyond dynamic component concentrations and flux data – which need also further experimental method development – this requires validated and ready-to-use computational methods to extract control information from dynamic data. Since control is a highly condition-specific property, empirical assessment of control under many different conditions would be an endless task. More effective would be to fit kinetic models to data, perhaps using approximate kinetic laws, from which control can be estimated for all conditions that can be simulated. Lastly, such models may be key in fostering understanding of principles behind the evolution of different regulatory circuits, properly accounting for flux control across environmental scenarios and the fitness advantage it confers.

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