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Global strategies to integrate the proteome and metabolome

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A fundamental goal of proteomics is to assign physiological functions to all proteins encoded by eukaryotic and prokaryotic genomes. Of the many activities performed by proteins, the chemical transformations catalyzed by enzymes form the basis for most, if not all, metabolic and signaling pathways. Elucidation of these pathways and their integration into larger cellular networks require new strategies to rapidly and systematically identify physiological substrates of enzymes. Here, we review emerging technologies that aim to assign endogenous biochemical functions to enzymes by profiling the metabolome.

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Introduction

The metabolome, which refers to the full complement of metabolites within a cell, tissue, or organism [1], encompasses a diverse array of molecular chemotypes, including peptides, carbohydrates, lipids, nucleosides, and catabolic products of exogenous compounds. As a rich source of both signaling and structural molecules, the metabolome and, in particular, its composition and regulation, are understandably subjects of intense research interest. Akin to its more mature counterparts, genomics and proteomics, the burgeoning field of metabolomics aims to develop and apply strategies for the global analysis of metabolites in cells, tissues and fluids [2,3]. By integrating these 'small molecule' profiles with transcript and protein expression/activity patterns, researchers hope to discern the contribution that specific metabolites and metabolic pathways make to health and disease.

An early example of the value of metabolite analysis can be attributed to Garrod, who discovered that the darkening of urine, upon standing, from patients with alkaptonuria was the result of a build-up of homogentisic acid, which he attributed to an 'inborn error of metabolism' [4]. Of course, this discovery was predicated on having a straightforward bioassay (the color change associated with oxidation of homogentisic acid), a methodological advantage often not afforded by other changes in metabolism. Indeed, in the pursuit of global strategies to profile the metabolome, contemporary researchers are confronted with a daunting set of challenges that, in many ways, exceed those confronted by genomics or proteomics. For example, unlike transcripts and proteins, metabolites share no direct link with the genetic code and are instead products of the concerted action of many networks of enzymatic reactions in cells and tissues. Similarly, metabolites are not linear polymers composed of a defined set of monomeric units, but rather constitute a structurally diverse collection of molecules with widely varied chemical and physical properties. As such, metabolites do not readily lend themselves to universal methods for analysis and characterization. These distinguishing features make the metabolome a unique portion of biomolecular space that requires advanced methods for its analysis. Here, we review contemporary approaches for global metabolite profiling and highlight recent applications. We focus in particular on efforts to use metabolomic data to elucidate the endogenous functions of enzymes. Collectively, these studies underscore that the metabolome, by being both sensitive to changes in protein activity and, at the same time, contributive to higher-order cellular phenotypes, offers an information-rich conduit between the proteome and the physiological processes it regulates.

The general analytical tools of metabolomics

To date, metabolite profiling has primarily been performed using either NMR or MS techniques. NMR offers a rapid and non-invasive method to comparatively characterize metabolite expression patterns *in vitro* [5,6] or *in vivo* [7,8]. However, NMR exhibits limited sensitivity and resolution and is therefore capable of detecting only the most abundant metabolites in complex samples. To increase the breadth and depth of metabolite profiling, investigators have turned to MS methods [9•,10]. MS analyses can be performed either directly or in combination with GC or LC separation steps. 'Direct-inject' MS experiments have the advantage of being fast (minutes per sample) [9•], but, like NMR, suffer from limited resolving power. By contrast, GC- and LC-MS experiments are considerably slower (1–2 h/sample), but permit more than 1000 metabolites to be analyzed per run, and thus sacrifice speed in exchange for substantial increases in sensitivity and 'metabolome coverage'.

Table 1

Analytical tools of metabolomics.

Author	Model system	Enzyme(s) disrupted	Analysis method (targeted/untargeted)	Metabolites identified (relationship to enzyme)
Raamsdonk *et al*. [6]	Yeast	Multiple	NMR (untargeted)	Unknown
Allen *et al*. [9•]	Yeast	Multiple	ESI-MS (untargeted)	Unknown
Rohde *et al*. [18••]	*Arabidopsis*	PAL1 and PAL2	LC-UV-MS (targeted)	Flavanols and amino acids (known substrates and novel 2° Metabolites)
Wu, *et al*. [19••]	Mouse	mBCAT	Tandem MS (targeted)	Amino acids (known substrates)
Saghatelian, *et al*. [21••]	Mouse	FAAH	LC-MS (untargeted)	Lipids (known and novel substrates)

Each of the profiling methods described above can be conducted in either a targeted or untargeted mode (Table 1). Targeted applications focus on the characterization of a specific class of metabolites, often exploiting their unique chemical properties — NMR-active atoms (e.g. ^{31}P), molecular masses and fragmentation patterns, etc. — to increase the sensitivity of detection and quantitation. By contrast, untargeted applications seek to broadly profile the metabolome by establishing conditions for the concurrent analysis of as many metabolites as possible. As we highlight in the following sections, targeted and untargeted methods offer complementary ways to interrogate the metabolome and assess the biochemical repercussions of specific genomic, proteomic and/or physiologic perturbations.

Biological applications of global metabolite profiling

As with genomics and proteomics, metabolomic studies typically prove most informative when performed in a comparative mode. Thus, while a static portrait of a cell or tissue metabolome may offer little in the way of hypothesis-testing or hypothesis-generating data, a comparison of the metabolomes of cells or tissues that differ in specific biological properties is likely to be much more enlightening. A significant portion of metabolomic research is focused on generating small-molecule portraits that distinguish health and disease (e.g. normal cell versus cancer cell). These efforts, which hold great promise for the discovery of new biomarkers for the diagnosis of human pathologies, have been the subject of several recent reviews [2,3,11•,12,13] and are not discussed further here. Instead, we focus on an equally, if not more enticing application of metabolomics — the assignment of endogenous functions to enzymes.

Assignment of enzyme function by global metabolite profiling

The elucidation of functions for the numerous enzymes encoded by prokaryotic and eukaryotic genomes represents a fundamental challenge for 21st century researchers. Genetic strategies such as RNAi [14] and targeted gene disruption (gene 'knockouts') [15], coupled with cell- and organism-based phenotypic screens, have provided powerful methods for the genome-wide analysis of the (patho)physiological functions of enzymes. By contrast, equivalent experimental strategies for the global characterization of *endogenous biochemical functions* of enzymes do not yet exist, and, as a consequence, the natural substrates for many enzymes remain unknown.

Substrate selectivities of enzymes are typically determined *in vitro* using purified preparations of protein. However, it is often difficult to ascertain the physiological significance of such 'test tube' biochemistry experiments for several reasons. First, many enzymes function as parts of large protein complexes and networks *in vivo* [16] that may be challenging to reconstruct or model *in vitro*. Second, enzymes are often regulated by post-translational events *in vivo* (e.g. phosphorylation, proteolytic processing) [17], which may alter substrate recognition and catalysis. Finally, the characterization of enzyme-substrate relationships *in vitro* is inherently limited by our current knowledge of cell metabolism and, therefore, ill-suited for the discovery of novel natural products regulated by enzymes *in vivo*. Metabolomics offers a potentially powerful strategy to address these limitations. Indeed, as highlighted below, several complementary analytical methods have recently been introduced for both targeted and untargeted metabolite profiling that, by providing access to a portion of biomolecular space (the metabolome) that is inaccessible to genomics and proteomics, enable the assignment of endogenous biochemical functions to a broad range of enzymes.

Assignment of enzyme function by targeted metabolite profiling

Two recent studies highlight the power of targeted metabolite profiling for elucidating enzyme function *in vivo*. Rohde and colleagues set out to distinguish the endogenous activities of phenylalanine ammonia lyase (PAL) enzymes in *Arabidopsis thaliana* [18••], which participate in the biosynthesis of the phenylpropanoid class of natural products (e.g. lignins, flavonoids, coumarins). Four distinct *PAL* genes are present in the *Arabidopsis* genome, and the authors selected two, *PAL1* and *PAL2*, for mutation. Neither the *PAL1* nor *PAL2* mutant exhibited any obvious changes in morphology or plant physiology, prompting the authors to probe for potential molecular alterations in these mutant plants. Interest-

ingly, despite its benign external phenotype, the *PAL1* mutant was found by targeted metabolite profiling (HPLC-UV analysis) to possess significantly lower levels of several phenolic natural products, including hydroxycoumarin (scopolin) and a novel class of feruloyl malates coupled to coniferyl alcohol (Table 1 and Figure 1a). These phenolics were not altered in the *PAL2* mutant, but were further decreased, along with several flavonol glycosides, in the *PAL1/PAL2* double mutant, suggesting that PAL enzymes can compensate for one another *in vivo*. Consistent with this premise, transcript levels of the *PAL1* gene were increased in a *pal2* mutant, and *vice versa*. Finally, the authors performed a complementary series of gene expression studies using cDNA microarray and near genome-wide cDNA-amplified fragment length polymorphism (AFLP) transcript profiling, resulting in the identification of several enzymes with altered expression in *pal* mutants, including those involved in amino acid metabolism. These genomics studies led to the focused analysis of additional metabolites, including both aromatic and non-aromatic amino acids, which were significantly elevated in the *pal* mutants. These findings highlight that targeted metabolite profiling can uncover 'molecular phenotypes' resulting from the disruption of specific enzymes, even in cases where no obvious changes in morphology or physiology are observed. Notably, both primary (e.g. increases in phenylalanine) and secondary (e.g. decreases in scopolin) alterations in the metabolome were identified in the *pal* mutants, revealing the extent to which PAL enzymes are integrated into larger metabolic circuits of the plant cell.

Metabolite profiling also offers an exciting strategy for 'phenotyping' mutant organisms originating from forward genetics screens, as exemplified in a recent *N*-ethyl-*N*-nitrosourea (ENU) mouse mutagenesis study by Wu and colleagues [19••]. The authors screened ENU-treated mice for changes in plasma levels of amino acids and acylcarnitine using direct-inject tandem MS analysis, resulting in the discovery of a mutant line of mice that possessed dramatically elevated levels of branched chain amino acids (BCAAs; valine, leucine/isoleucine) (Table 1 and Figure 1b). These mice also exhibited reduced motility and body weight, suggesting a general failure to thrive. Similar metabolic and physiologic phenotypes are observed in human patients with maple syrup urine disease, which occurs as a result of defects in branched chain α-keto acid dehydrogenase (BCKD) activity. Interestingly, however, the mutant mice exhibited wild type levels of BCKD activity. Instead, these animals were found to possess a mutation in the gene that encodes the mitochondrial branched chain aminotransferase (mBCAT) enzyme, which converts BCAAs to branched chain α-keto acids (BCKAs), the natural substrates for BCKD. Curiously, the mBCAT mutant mice also possessed elevated plasma levels of BCKAs, the products of BCAT activity *in vivo* (Table 1, Figure 1b). This finding might reflect a second function of the mBCAT protein, which, in addition to converting BCAAs to BCKAs, also promotes the mitochondrial transport of BCKAs synthesized by the cytoplasmic form of BCAT (Figure 1b) [20]. These studies demonstrate the power of integrating targeted metabolomics with phenotypic screening to discover enzyme deficiencies that cause specific metabolic disorders.

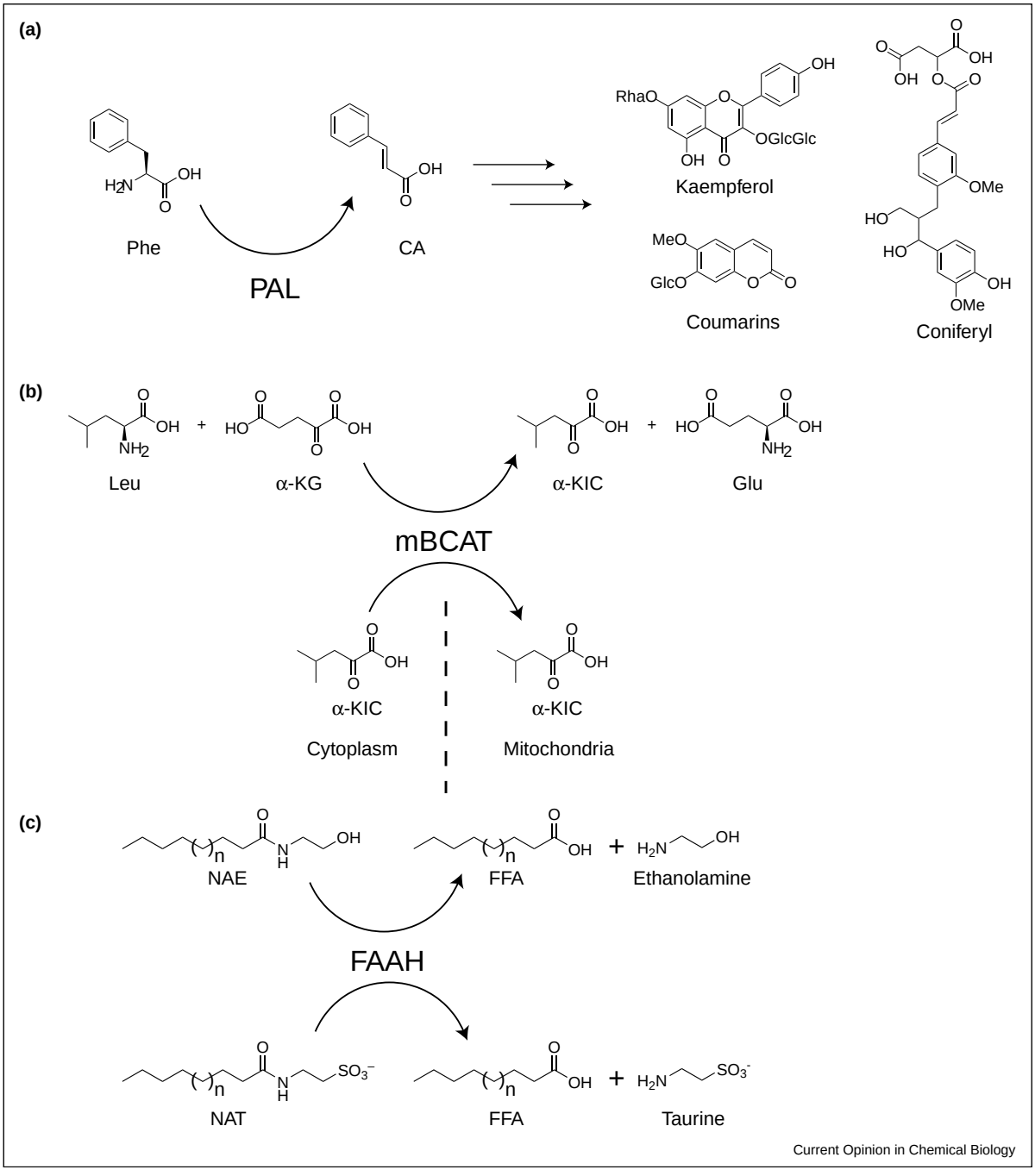
Assignment of enzyme function by untargeted metabolite profiling

As described above, targeted metabolite profiling has proven quite adept at elucidating the activities of enzymes *in vivo*. However, in these studies, prior knowledge of enzyme function was exploited to focus experimental efforts on specific portions of the metabolome (e.g. UV-active phenolics for the PAL enzymes; amino acids for the BCAT enzyme). To facilitate the functional analysis of uncharacterized enzymes, as well as the discovery of novel activities for known enzymes, untargeted metabolite profiling efforts have emerged with the goal of globally characterizing cell/tissue metabolomes. These untargeted methods fall into two general categories: metabolite fingerprinting and metabolite discovery.

Metabolite fingerprinting studies have been pioneered by Oliver, Kell and colleagues, who have showcased that both NMR and direct-inject MS methods can be used to rapidly generate global portraits of metabolic flux that occur following, for example, the deletion of specific enzymes in yeast [6,9•]. As was the case with the targeted metabolite analysis of PAL enzymes in *Arabidopsis*, here too 'phenotypically silent' mutants were distinguished based on changes in their metabolite profiles. The main difference, however, is that, in the studies by Oliver, Kell and colleagues, mutants were differentiated on the basis of their global patterns of metabolite expression without any knowledge of the structures of the metabolites under investigation. This strategy for classifying enzymes based on 'metabolic finger (or foot) prints' has both advantages and disadvantages. On the one hand, the fact that enzymes of related function (e.g. 6-phosphofructo-2-kinase 26 and 27 in yeast) were found to exhibit similar metabolite profiles suggests that enzymes that participate in the same metabolic pathway may be rapidly discovered by NMR or direct-inject MS profiling methods (Table 1). On the other hand, these analytical methods exhibit limited resolution and sensitivity and are therefore capable of detecting only the most abundant metabolites in cells or tissues. As a consequence, 'metabolic fingerprints' may prove too superficial to elucidate the endogenous biochemical functions of many enzymes, especially those that utilize medium to low abundance metabolites as substrates *in vivo*.

To enable a more in-depth and comprehensive survey of the biochemical changes that accompany enzyme inacti-

Figure 1



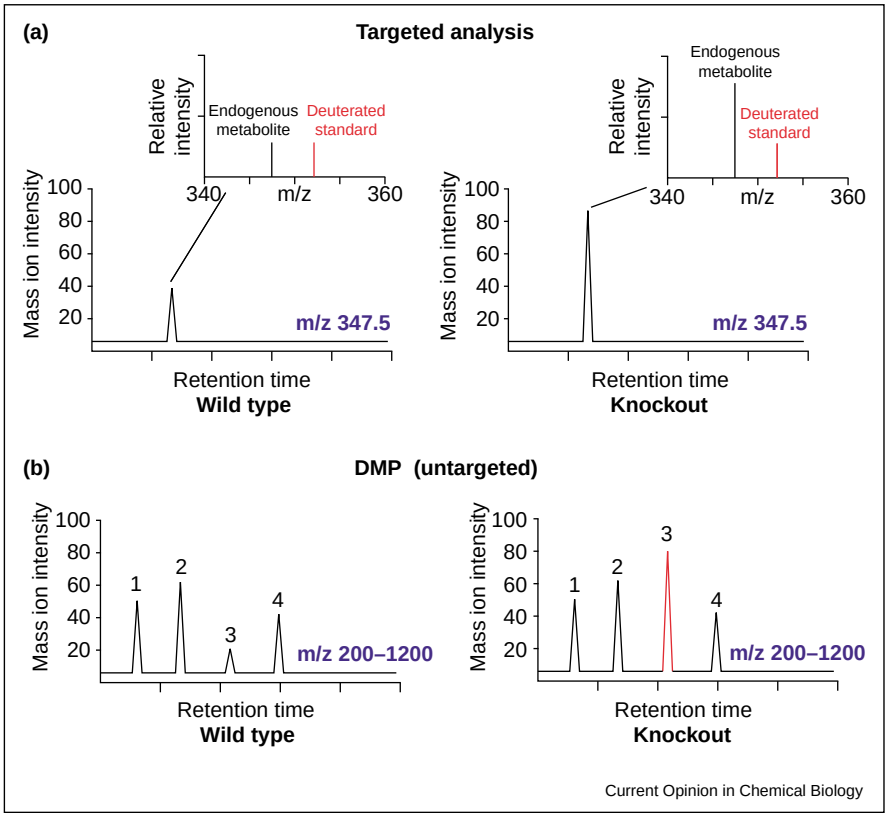
Examples of the utilization of metabolite profiling to elucidate the endogenous biochemical functions of enzymes. **(a)** Rohde and colleagues used targeted LC-UV/MS methods to characterize phenolic natural products that were altered in expression in *Arabidopsis* plants lacking specific phenylalanine ammonia lyase (PAL) enzymes [18••]. The deletion of PAL enzymes led to an accumulation of phenylalanine and a reduction in a variety of secondary phenolic metabolites, including kaempferol, hydroxycoumarin and coniferyl derivatives. **(b)** Wu and colleagues used targeted tandem MS methods to identify changes in plasma amino acid levels in ENU mutagenized mice, resulting in the discovery of a mitochondrial enzyme, branched chain aminotransferase (BCAT), that regulates both branched chain amino acids and α-keto acids by catalytic (upper arrow) and transport (lower arrow) functions, respectively [19••]. **(c)** Saghatelian and colleagues used untargeted LC-MS methods (DMP); see Figure 2 for more details) to identify several lipid substrates regulated by the mammalian enzyme fatty acid amide hydrolase (FAAH) *in vivo*, including known signaling molecules [*N*-acyl ethanolamines (NAEs)] and a novel structural class of brain natural products [N-acyl taurines (NATs)] [21••].

vation, we have recently introduced an LC-MS platform for untargeted metabolomics [21••]. In contrast to conventional LC-MS-based metabolite analysis, in which the levels of specific compounds are determined by selected ion monitoring using isotopic variants as internal standards, discovery metabolite profiling (DMP) operates as a global, 'standard-free' method in which the relative levels of many metabolites are quantified in parallel by directly measuring their mass ion intensities in a broad mass scanning mode (Figure 2).

We applied DMP to compare the central nervous system (CNS) metabolomes of wild type mice and mice lacking the enzyme fatty acid amide hydrolase (FAAH) [22], which degrades several neural signaling lipids *in vivo*, including the endogenous cannabinoid *N*-arachidonoyl ethanolamine (anandamide) [23]. Previous targeted LC-MS studies had shown that FAAH(−/−) mice possess highly elevated CNS levels of anandamide and related *N*-acyl ethanolamines (NAEs) [22,24]; however, whether other constituents of the brain metabolome were also altered in these animals remained unknown. DMP accurately estimated a ~10–15-fold increase in the concentration of NAEs in FAAH(−/−) brains and spinal cords, while revealing that most of the other lipid species, including free fatty acids, phospholipids and ceramides, were unchanged in these tissues. Significantly, DMP also detected a class of unknown metabolites that were dramatically (>10-fold) elevated in CNS tissues of FAAH(−/−) mice. Using a combination of analytical and synthetic chemistry techniques, we determined the structures of these novel, FAAH-regulated metabolites as conjugates of very long chain fatty acids and the amino acid derivative, taurine [*N*-acyl taurines (NATs)]. *In vitro* assays confirmed that NATs are direct substrates for FAAH, which hydrolyzed these lipids to their parent fatty acid and taurine constituents (Figure 2c). Although the function of NATs in the nervous system is currently unknown, taurine has been shown to act as a chemical transmitter [25,26], suggesting that FAAH may contribute to the production of this signaling molecule *in vivo*.

In summary, by globally profiling the metabolic consequences of FAAH disruption *in vivo*, DMP elucidated

Figure 2



Comparison of targeted and untargeted LC-MS methods for metabolite profiling. **(a)** General scheme for targeted LC-MS analysis, in which metabolites are detected by selected ion monitoring (shown for a metabolite of the mass of 347.5) and their levels quantified by comparing their mass signals to those of isotopically distinct internal standards. **(b)** General scheme for discovery metabolite profiling (DMP), an untargeted LC-MS approach, in which metabolites are detected in the broad mass scanning mode (e.g. 200–1200 mass units) and their levels quantified by measuring direct mass ion intensities (i.e. without the inclusion of internal standards). Enzyme-regulated metabolites are identified by comparison of mass ion intensities between wild type and knockout samples (e.g. metabolite 3).

both known and novel roles played by this enzyme in CNS metabolism (Table 1). Importantly, these endogenous activities could not be directly gleaned from an *in vitro* analysis of FAAH's substrate selectivity, which was unpredictive of the lipids regulated by this enzyme *in vivo* [21••]. These findings indicate that, to determine the endogenous functions played by an enzyme, one must not only evaluate its catalytic properties *in vitro*, but also distinguish which subset of these activities is uniquely attributable to this enzyme versus those that can be performed by other metabolic pathways *in vivo*. DMP should constitute a general strategy to achieve this objective, as this method can, in principle, be applied to identify the physiological substrates of any metabolic enzyme, even those that function in highly complex tissues such as the mammalian brain.

Conclusions and future directions

Enzymes regulate biological processes through the conversion of specific substrates to products. Therefore, of fundamental interest for every enzyme is the identification of its endogenous substrates. Here, we have briefly reviewed an exciting new branch of post-genomic research that aims to address this problem by forging direct connections between the proteome and metabolome. Strategies for global metabolite profiling, or metabolomics, have provided key insights into the endogenous functions of enzymes, including, for example, the discovery of novel structural classes of metabolites regulated either directly [21••] or indirectly [18••] by specific enzymes *in vivo*. These findings illustrate the unique value of metabolomics, which provides access to an information-rich portion of biomolecular space that is inaccessible to genomic and proteomic methods. Nonetheless, for metabolomics to realize its full potential as a global profiling strategy, several experimental challenges must be addressed. First, efforts must be made to improve the sensitivity of metabolomic experiments, while maintaining reasonable scope and throughput. As is the case with proteomics [27], a clear trade-off exists between the breadth and depth of analysis achievable in metabolite profiling studies. Targeted approaches, by focusing on specific classes of small molecules, can achieve remarkable sensitivity, but offer a restricted view of flux across the entire metabolome. By contrast, untargeted profiling methods provide a more global portrait of the metabolome, but often at the expense of visualizing low abundance metabolites. We have found that LC-MS methods coupled with a general fractionation step (e.g. organic extraction) offer a practical compromise, providing, on the one hand, significant breadth of metabolome coverage, while offering a level of sensitivity compatible with detecting low abundance metabolites. Still, it is important to note that our DMP studies have, to date, focused on the analysis of lipophilic metabolites and therefore should not be considered truly comprehensive in scope.

Tackling other portions of the metabolome constitutes a second major challenge, where efforts to develop fractionation and enrichment methods for specific classes of aqueous metabolites should prove particularly valuable. Finally, a third problem that uniquely faces the field of metabolomics (compared with genomics and proteomics) is the determination of metabolite structures. Unlike genes and proteins, metabolites are not linear polymers composed of a defined set of monomeric units, but rather constitute a family of biomolecules of near limitless structural diversity. The continued advancement of increasingly sensitive and high-resolution analytical tools, in combination with the advent of searchable databases that contain the chemical and physical features of all known metabolites, should facilitate the future characterization of metabolites. Additionally, as exemplified throughout this review, metabolite structure determination can be further expedited by integrating our knowledge of the proteome and metabolome (e.g. the fact that FAAH is an amidase assisted in the structure prediction of NATs). Thus, by developing methods that establish functional links between the proteome and metabolome, we may arrive at a day in the not too distant future where the characterization of novel metabolites and the enzymes that regulate them can be routinely performed in synchrony.

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