

Chemical strategies for the global analysis of protein function

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As the human genome project nears completion, biological research is entering a new era in which experimental focus will shift from identifying novel genes to determining the function of gene products. Rising to this challenge, several technologies have emerged that aim to characterise genes and/or proteins collectively rather than individually. Of particular interest is a new breed of strategies that employs synthetic chemistry to enrich our understanding of protein function on a global scale.

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Abbreviations

2DE	two-dimensional gel electrophoresis
ABP	activity-based probe
ICAT	isotope-coded affinity tag
MS	mass spectrometry

Introduction

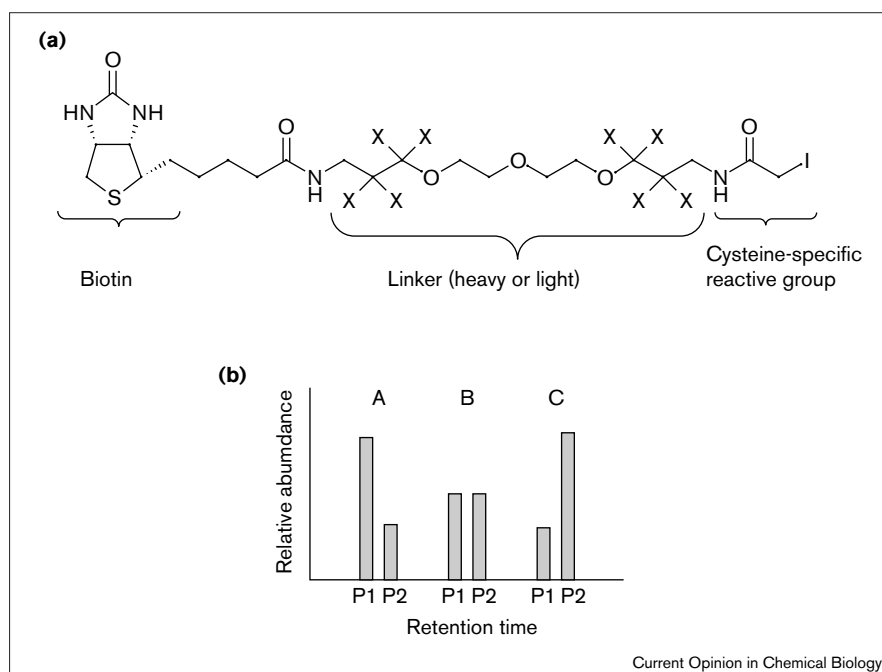
A fundamental goal of biological research is to understand complex physiological and pathological processes at the level of chemistry and molecules. Over the past two decades, deciphering the molecular correlates of health and disease has principally entailed an iterative cycle of isolating and characterising novel proteins and genes associated with a particular (patho)physiological event. Synthetic chemistry has often played a major role in this process, perhaps most visibly through facilitating the identification of protein targets of bioactive natural products [1,2]. Such chemical efforts have led to the characterisation of entirely new proteins and protein families, repeatedly underscoring the remarkable breadth and depth of molecular diversity that defines the living organism.

Presently, however, the biological community is experiencing a paradigm shift that promises to transform its most basic methods of experimentation. With the human genome sequence nearly completed, we are confronted with the realisation that soon there will be no 'new' human genes or proteins (at least at the primary structure level) left to discover. Instead, we will receive a monumental gift of molecular information composed of 30,000–150,000 human gene sequences, most of which encode proteins that have never been characterised. Thus, a daunting task awaits contemporary biochemists and cell biologists: the assignment of cellular and physiological functions to the protein products of all of these genes.

In efforts to both accelerate the functional analysis of biomolecules and consolidate the resulting wealth of new data, researchers have developed several technologies that permit the study of genes and/or proteins collectively rather than individually. These global experimental approaches include genomics [3,4], the analysis of a cell's complete transcript repertoire (transcriptome), and proteomics [5,6], the analysis of a cell's complete protein repertoire (proteome). Genomics has evolved rapidly as a field, with gene chip technologies nearing the goal of quantifiably comparing, in a single experiment, the complete transcriptomes of two test samples [3,7]. Several recent studies have demonstrated how gene chips can be used to discover and predict new subclasses of cancer based on their distinctive patterns of gene expression [8,9]. Nonetheless, genomics methods possess some serious shortcomings, foremost of which is their reliance on mRNA levels as an indirect measure of protein quantity and function. In order to give cellular and physiological meaning to genomics data, one must accept that changes in transcript level correlate with changes in protein expression and activity, a potentially risky assumption in light of recent studies indicating that mRNA levels are generally poor predictors of protein abundance [10,11].

Largely because of concerns regarding the indirectness of genomics approaches, proteomics initiatives have emerged with the goal of characterising dynamics in the abundances of proteins themselves [5,6]. Although proteomics studies can rightly claim to generate data that more closely correlate with protein function, they also face formidable technical challenges that have limited their general utility when compared with genomics methods. To date, most proteomics experiments have relied on two-dimensional gel electrophoresis (2DE) using isoelectric focusing/SDS-PAGE and mass spectrometry (MS) as their separation and detection methods, respectively [12•]. Unfortunately, despite the considerable resolving power of 2DE, this technology has fallen far short of the ultimate goal of displaying in one experiment an entire cell or tissue proteome. Several classes of proteins have proven especially resistant to analysis by 2DE, including low and high molecular mass proteins, membrane proteins, proteins with extreme isoelectric points (pIs) and low abundance proteins [12•,13••]. Indeed, with the capacity and sensitivity of 2DE having been pushed to their limits, some proteomics researchers have concluded that alternative and/or complementary separation strategies must be developed in order to permit a global characterisation of the proteome [12•,13••]. Regardless, even if proteomics researchers can evolve separation strategies that allow access to the entire proteome, it is important to recognise that such global measurements of protein abundance would provide, like genomics, only an indirect assessment

Figure 1



An alternative method for quantitative proteomics by Gygi *et al.* [16••]. (a) The general structure of an ICAT reagent. Heavy reagent: d8-ICAT (X = deuterium); light reagent: d0-ICAT (X = hydrogen). (b) Mock proteomics data obtained with ICAT methodology. Relative abundances of proteins in two proteomes (P1 and P2) are determined from the ratios of differentially mass-tagged peptide pairs. Three peptide pairs are shown, with peptide A representing a protein more abundant in P1, peptide C representing a protein more abundant in P2 and peptide B representing a protein equally abundant in both proteomes.

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of protein function. Numerous post-translational events that regulate protein activity, including the large number of protein–protein and protein–small-molecule interactions perceived to modulate protein function *in vivo*, would still remain invisible to standard genomics and proteomics approaches.

In this review, we propose that the field of synthetic organic chemistry is uniquely suited to furnish proteomics initiatives with new concepts and tools that are both complementary and, in many cases, superior to those in use by today's researchers. Highlighted below are recent examples of chemical approaches that have yielded important new methods and reagents for the global analysis of protein function.

Chemical approaches for the global analysis of protein abundance

A robust method for the comparative quantification of differentially expressed proteins remains a major challenge in proteomics research. Surprisingly, the ability to compare protein abundances across proteomes is often taken for granted, despite the fact that most proteomics experiments to date have relied on general protein-staining methods for 'quantification'. At best, however, protein visualisation by Coomassie blue and/or silver staining should be considered semi-quantitative, with the former stain lacking sensitivity, the latter showing protein-dependent differences in staining intensity, and both stains exhibiting poor dynamic range [14]. More recently, fluorescent dyes for protein detection and quantification (e.g. Sypro dyes) have been generated that appear to circumvent several of the shortcomings of

traditional stains [15]. Nonetheless, the major long-term problem facing quantitative proteomics is not one of staining sensitivity and/or dynamic range, but rather of the separation capacity of 2DE. Efforts to maximise the number of proteins displayed on a single two-dimensional gel have demonstrated that loading more protein onto such gels does not necessarily augment the detection and quantification of lower abundance proteins [12•]. Instead, the overlapping of protein spots increases dramatically, creating a problem for quantitative proteomics that cannot be resolved by staining methods alone.

An alternative method for quantitative proteomics has recently been described by Gygi *et al.* [16••]. Instead of relying on protein-staining methods for quantification, these authors synthesised a chemical probe that they named an isotope-coded affinity tag (ICAT; Figure 1a), which permits the quantitative comparison of protein abundances between complex proteomes by MS analysis. Conceptually, the ICAT probe is a trifunctional molecule composed of a reactive group capable of covalently binding to a defined subset of amino acid side chains (a thiol-specific reagent is shown in Figure 1a); an isotopically coded linker (where X is deuterium or hydrogen in Figure 1a); and an affinity tag for the isolation of reactive peptides (biotin in Figure 1a). To quantitatively compare protein abundances between two proteomes, one proteome is treated with a 'light' (nondeuterated) version of the ICAT probe and a second proteome is treated with a 'heavy' (deuterated) version of this probe. The two proteomes are then mixed together and handled in an identical manner in all subsequent experimental steps; the combined proteomes

are digested with a protease (e.g. trypsin), the ICAT-labelled peptides isolated by avidin chromatography and the isolated peptide mixture analysed by liquid-chromatography–MS. The relative abundances of proteins in the two proteomes are quantified by determining the ratios of the MS signal intensities of differentially mass-tagged peptide pairs (Figure 1b).

An attractive feature of the ICAT method is that through the combined use of MS and tandem MS analysis, both the relative abundances and sequence identities of ICAT-labelled peptides can be determined in a single experimental operation. Additionally, by limiting the analysis of the proteome to cysteine-containing peptides, a significant reduction in complexity is achieved. In this manner, the ICAT method permits the comparative analysis of a relatively large fraction of complex proteomes without the need for a labour-intensive gel electrophoresis step.

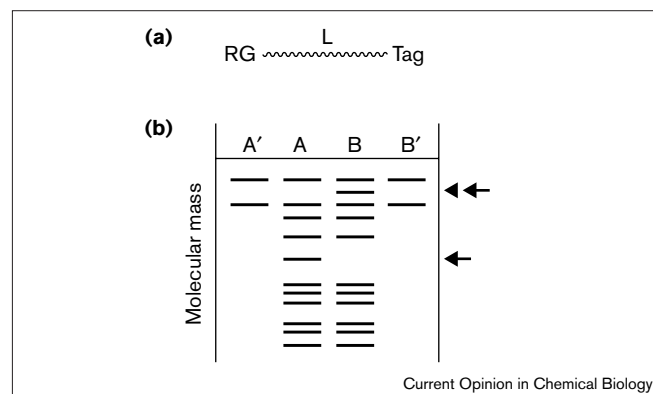
Chemical approaches for the global analysis of protein activity

‘Functional’ is a word used quite loosely by today’s genomics and proteomics researchers. Indeed, almost any type of transcript profiling experiment can be found to bear the name ‘functional genomics’. In most cases, however, changes in transcript and/or protein level only achieve a state of ‘functional’ significance if the corresponding protein’s activity also changes. In the simplest example, a twofold increase in transcript level should lead to twofold more protein, which in turn should lead to twofold more protein activity. Because of the complexity of the living cell and organism, however, such simple scenarios may prove to represent more the exception rather than the rule. Numerous forms of post-transcriptional and post-translational regulation take place both inside and outside the cell, and each of these regulatory processes to some degree dilutes the value of genomics/proteomics data that reports changes exclusively in transcript/protein levels. As such, one might conclude that the ideal proteomics method would monitor, in a single experiment, the activities of all of the proteins in the proteome.

Although the aforementioned goal may seem excessively ambitious, several lines of recent research suggest that a significant fraction of the proteome can be monitored on the basis of activity rather than abundance. A common thread among these ‘activity-based’ proteomics efforts is the use of synthetic chemistry to create probes that directly monitor the functional state of large enzyme families. Such activity-based probes (ABPs) can generally be defined as reagents that meet the following criteria:

1. React with a broad range of enzymes from a particular class directly in complex proteomes.
2. React with these enzymes in a manner that correlates with their catalytic activities.

Figure 2



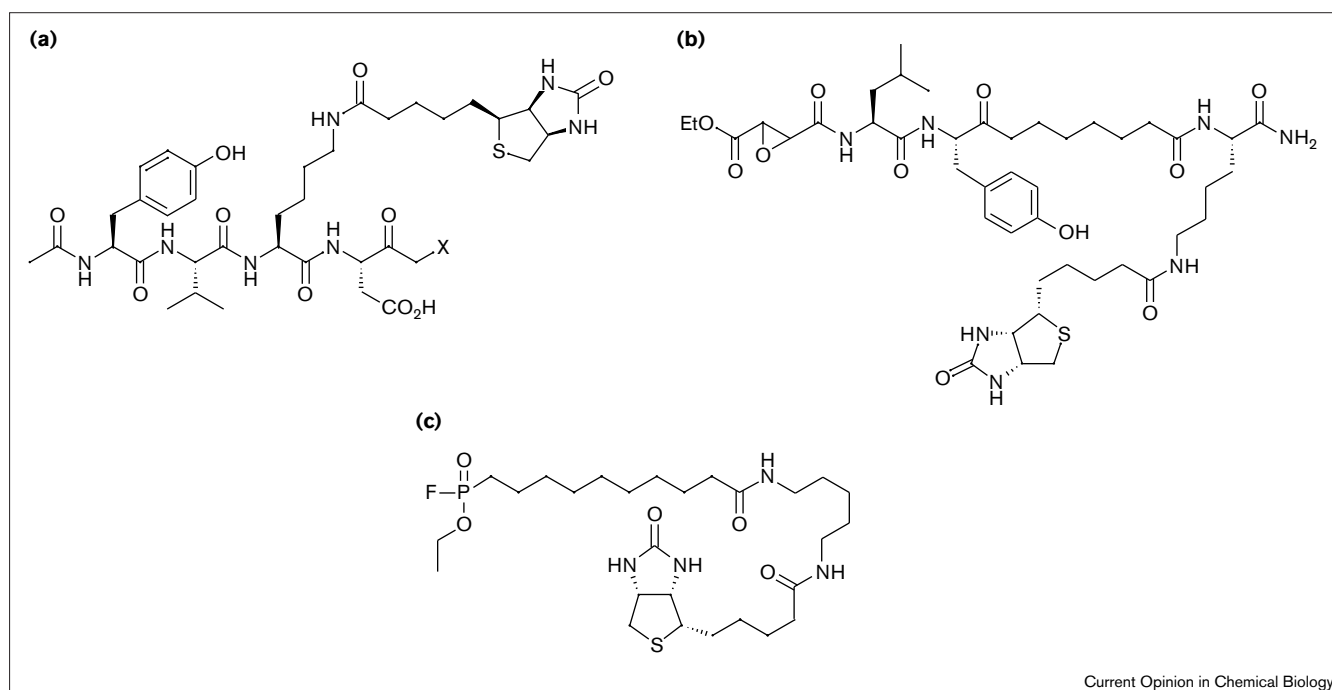
The use of synthetic chemistry to create probes that directly monitor the functional state of large enzyme families. (a) The general structure for an ABP. RG, reactive group; L, linker; Tag, biotin, fluorescein, etc. (b) Mock proteomics data generated with ABPs. Active enzymes labelled by a biotinylated ABP are visualised using an avidin-based chemiluminescence blot assay. The reactivity of an ABP with two representative proteomes (A,B) is depicted, with protein activities selectively expressed in either proteome A or proteome B highlighted by single or double arrowheads, respectively. Notably, samples of each proteome preheated prior to treatment with the ABP (A' and B') serve as simple and effective controls to distinguish specific (activity-based) from non-specific reactivity [23**].

3. Display minimal cross-reactivity with other protein classes.
4. Possess a tag for the rapid detection and isolation of reactive enzymes (Figure 2a).

In this manner, a successfully designed ABP could permit the comparative measurement and identification of all of the active members of a given enzyme class present in two or more proteomes (Figure 2b).

To date, activity-based proteomics efforts have primarily focussed on two classes of enzymes: the cysteine and serine hydrolases. In retrospect, it is perhaps not surprising that the first generation of ABPs would target these hydrolytic enzyme families, as they display a well-documented sensitivity to class-selective irreversible inhibitors. The coupling of these inhibitory groups to chemical tags such as biotin has engendered a prototype ABP structure (Figure 2a, where RG is an inhibitory group and Tag is biotin). For cysteine hydrolases, ABPs bearing an α -halo or (acyloxy)methyl ketone substituent have been synthesised and used to profile the caspase proteases (Figure 3a; [17,18]), whereas ABPs possessing an activated epoxide have been generated that target the cathepsin proteases (Figure 3b; [19**]). In both cases, the ABPs possess peptide scaffolds that enhance selectivity for their respective subclasses of cysteine protease targets. Notably, these ABPs have been used to profile the kinetics of caspase activation during apoptosis [17] and to correlate changes in cathepsin activities with the progression of skin cancer [19**].

Figure 3



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Structure of ABPs. (a) Structure of a representative tetrapeptide ABP for targeting the caspase family of cysteine proteases. X, acyloxy or halide [29]. (b) Structure of an ABP for targeting the cathepsin family of cysteine proteases [19**]. (c) Structure of an ABP for targeting the serine hydrolase family of enzymes [23**].

The serine hydrolases represent an extremely large class of enzymes comprising proteases, lipases, amidases, esterases and transacylases. Indeed, analysis of the complete sequence of the *Drosophila* genome has revealed that serine hydrolases may constitute close to 5% of this organism's proteome, with over 100 members each of the trypsin, chymotrypsin and esterase/thioesterase classes being identified [20]. Interestingly, a clear expansion in the serine protease family is observed as eukaryotic organisms gain in multicellular complexity, with the yeast possessing one chymotrypsin/trypsin gene, the worm possessing 18 and the fly possessing several hundred. This trend implies that serine hydrolases will also constitute a major fraction of the mammalian proteome.

As a result of a shared catalytic mechanism, nearly all serine hydrolases are potently and irreversibly inhibited by fluorophosphonates [21]. Additionally, the reactivity of fluorophosphonates with serine hydrolases depends on these enzymes being in a catalytically active state, with both proenzymes ([22]; D Kidd, V Liu, BF Cravatt, EJ Sorensen, unpublished data) and inhibitor-bound enzymes [23**] showing greatly reduced reactivities with fluorophosphonates. Thus, the coupling of a fluorophosphonate moiety to biotin through an extended alkyl chain linker provided a first-generation ABP, fluorophosphonate-biotin (Figure 3c), which could, in principle, profile the activities of all of the members of the serine hydrolase family in a single experiment [23**]. In

general support of this notion, fluorophosphonate-biotin was shown to react with numerous serine hydrolases in an activity-dependent manner that could be kinetically monitored. A comparison of fluorophosphonate-biotin-reactive proteins across a panel of rat tissues highlighted the considerable breadth and diversity of serine hydrolase activities found in this mammalian organism. Importantly, fluorophosphonate-biotin readily labelled free (active) proteases but not inhibitor-bound (inactive) proteases. Considering that large families of endogenous protein inhibitors regulate serine protease activity *in vivo* [24,25], fluorophosphonate-biotin should prove especially useful for distinguishing such inhibitor-bound proteases from active proteases in complex proteomes. Finally, because fluorophosphonate-biotin-labelled proteins can be detected with a simple avidin-based, enzyme-linked chemiluminescence blot assay, experiments using this reagent (as well as other ABPs) have achieved an exceptional level of sensitivity and resolution, even in a one-dimensional analysis [23**].

To this point, we have discussed ABPs only in terms of their ability to distinguish two qualitative states of enzyme activity: on (active) and off (inactive). Enzymes, however, clearly exist in more than these two extreme forms *in vivo*, often showing manifold degrees of activity depending on their surrounding molecular and cellular environment. Can the utility of ABPs be extended to the measurement of quantitative differences in the activities

of enzymes in complex proteomes? When attempting to answer this question, it is perhaps first worth considering the types of regulation that enzymes tend to experience *in vivo*. Three of the most common mechanisms for the post-translational control of enzyme activity are the complexation of enzymes with endogenous inhibitors (e.g. serpin–serine protease interactions), the complexation with endogenous activators (e.g. GTPase–GAP [GTP activating protein interactions [26]) and covalent modification (e.g. cleavage of a proenzyme, phosphorylation, glycosylation). In the first case, assuming that the form of inhibition is competitive, the rate of reactivity of ABPs should clearly be affected, as natural enzyme inhibitors will compete with ABPs for active-site occupancy. For the latter two cases, the degree to which changes in catalytic activity will be reflected in altered rates of ABP reactivity will depend on how given ABPs interact with their target enzymes. For example, the reactivity of fluorophosphonates with serine hydrolases depends primarily on two catalytic parameters: nucleophile strength and oxyanion hole stabilisation [27]. Thus, changes in serine hydrolase activity that result from alterations in one or both of these catalytic properties should correspondingly impact the rate of fluorophosphonate reactivity. In general support of this notion, studies with fatty acid amide hydrolase (a mammalian serine hydrolase) have shown that several mutants of this enzyme that display decreased catalytic activities also show similar degrees of reduction in their fluorophosphonate–biotin reactivities [28]. Nonetheless, if the post-translational regulation of an enzyme's activity influences catalytic parameters not accurately measured by a given ABP (e.g. for fluorophosphonate–biotin — the leaving group protonation step of the serine hydrolase reaction pathway) then, in such cases, the ABP may fail to distinguish the altered catalytic state of the enzyme.

Conclusions and future directions

The post-genome era promises to be a time of unprecedented discovery and invention for biological and medical research. The accumulation of raw molecular information should no longer represent a rate-limiting step towards advances in understanding, as past efforts encumbered with determining gene/protein identity will give way to research programs focussed on determining gene/protein function. This review has attempted to emphasise the need for new experimental strategies that can exploit genomic level data, highlighting several chemical approaches that aim to increase the quality of information obtained from proteomics research. These synthetic chemistry efforts have already advanced the field of proteomics in several ways, by providing tools for the following:

1. Simplifying the proteome to a manageable number of proteins.
2. Enhancing the sensitivity of proteome analyses.
3. Comparing protein abundances between proteomes.

4. Comparing protein activities between proteomes.

In the near future, one could envisage evolving a method that merges the ICAT strategy for quantitative proteomics with ABPs to allow the comparison, in a single experiment, of the relative activities and identities of all of the active members of an enzyme family or families present in two proteomes.

To date, ABPs have been successfully created only for hydrolytic enzyme families that possess catalytically essential active-site nucleophiles. Thus, the expansion of activity-based proteomics methods to include other enzyme families, especially those for which clearly defined irreversible inhibitors do not yet exist, stands as a major future challenge. Nonetheless, through a combination of directed and non-directed chemical approaches, a large fraction of the enzyme proteome may soon become accessible to profiling with ABPs, offering biochemical researchers the special ability to measure protein function, as well as protein abundance, on a global scale.

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