Chemical Strategies for Functional Proteomics

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Abbreviations: 1D, one-dimensional; 2DE, two-dimensional electrophoresis; ABPP, activity-based protein profiling; EDT, ethanedithiol; ESI, electrospray ionization; FAAH, fatty acid amide hydrolase; FP, fluorophosphonate; ICAT, isotope-coded affinity tag; IMAC, immobilized metal-affinity chromatography; LC, liquid chromatography; MS, mass spectrometry; PhIAT, phosphoprotein-specific isotope-coded affinity tag; PMA, 12-phorbol 13-myristate acid; PNA, peptide nucleic acid; PTP, protein tyrosine phosphatase
Summary

With complete genome sequences now available for several prokaryotic and eukaryotic organisms, biological researchers are charged with the task of assigning molecular and cellular functions to thousands of predicted gene products. To address this problem, the field of proteomics seeks to develop and apply methods for the global analysis of protein expression and protein function. Here, we review a promising new class of proteomics strategies that employs synthetic chemistry to create tools and assays for the characterization of protein samples of high complexity. These approaches include the development of chemical affinity tags to measure the relative expression level and post-translational modification state of proteins in cell and tissue proteomes. Additionally, we will discuss the emerging field of activity-based protein profiling, which aims to synthesize and apply small molecule probes that monitor dynamics in protein function in complex proteomes.
Introduction

In response to the availability of complete genome sequences for numerous organisms, the field of proteomics has emerged with the goals of developing and applying methodologies that accelerate the functional analysis of proteins (1, 2). Strategies in proteomics can generally be divided into two categories that have complementary objectives: 1) the global characterization of protein expression, and 2) the global characterization of protein function. Large-scale efforts to measure protein expression have typically relied on a combination of two-dimensional gel electrophoresis (2DE), protein staining, and mass spectrometry (MS) for protein separation, detection, and identification, respectively (3). 2DE-MS methods are capable of simultaneously evaluating the relative abundance and modification state of numerous proteins from endogenous sources, thus permitting the identification of new proteins associated with discrete physiological and/or pathological states [e.g., nucleoside diphosphate kinase A as a marker for reduced metastatic potential in human prostate cancer cell lines (4)]. However, several important protein classes, including low-abundance and membrane-associated proteins, remain difficult to analyze by current 2DE approaches (3, 5, 6). Additionally, by focusing on measurements of protein abundance, 2DE-MS methods provide only an indirect assessment of protein function and may fail to detect important post-translational forms of protein regulation such as those mediated by protein-protein and/or protein-small molecule interactions (7).

To expedite the functional analysis of proteins, methods have also been introduced to examine protein activity on a global scale. These technologies include
large-scale yeast-two hybrid screens (8, 9), which aim to construct a comprehensive map of protein-protein interactions that occur in the cell, and protein microarrays (10, 11), which offer a platform to rapidly assess the function of recombinantly expressed proteins. Although capable of attributing specific molecular activities to individual protein products, these methods require that proteins be studied in artificial environments and therefore do not directly assess the functional state of these biomolecules in their native settings.

Recently, a breed of chemical strategies has emerged that utilizes organic synthesis to create new tools and assays to advance the field of proteomics (12, 13). In this review, we will describe chemical approaches for both abundance-based and activity-based proteomics, with an emphasis on methods that permit the quantitative comparison of proteins, including low-abundance and membrane-associated proteins, in samples of high complexity.

**Chemical approaches for determining the abundance and post-translational modification of proteins in complex proteomes**

The most common method currently employed by proteomics researchers to monitor changes in protein abundance is 2DE-MS, in which proteins are typically visualized and quantified by staining. Traditional staining methods, including Coomassie Blue and silver staining are cost-effective, but offer limited dynamic range and sensitivity (14). To improve these features, fluorescent dyes like SYPRO-ruby have recently been developed (15). Nonetheless, independent of the staining method employed, 2DE
methods suffer from a lack of resolving power that hinders the detection of several important classes of proteins, including membrane-associated (5) and low abundance proteins (3).

The isotope-coded affinity tag (ICAT) method – a chemical approach to quantify protein abundance in complex proteomes. As an alternative to 2DE-MS, a gel-free method for quantitative proteomics has been introduced that relies on chemical labeling reagents referred to as isotope-coded affinity tags (ICAT) (16). These chemical probes consist of three general elements: a reactive group capable of labeling a defined amino acid side chain (e.g. iodoacetamide to modify cysteine residues), an isotopically coded linker, and a tag (e.g., biotin) for the affinity isolation of labeled proteins/peptides (Fig. 1A). For the quantitative comparison of two proteomes, one sample is labeled with the isotopically light (d0) probe and the other with the isotopically heavy (d8) version. To minimize error, both samples are then combined, digested with a protease (i.e. trypsin), and subjected to avidin affinity chromatography to isolate peptides labeled with isotope-coded tagging reagents. These peptides are then analyzed by liquid chromatography-mass spectrometry (LC-MS). The ratios of signal intensities of differentially mass-tagged peptide pairs are quantified to determine the relative levels of proteins in the two samples.

ICAT circumvents several of the previously described limitations of gel-based methods, providing improved access to important portions of the proteome, like membrane-associated and low abundance proteins. For example, ICAT has been used to
compare the microsomal fractions of normal and 12-phorbol 13-myristate acid (PMA)-treated samples of the human HL-60 leukemia cell line (17). In this *in vitro* model of cellular differentiation, the ICAT method was capable of measuring the relative levels of 491 proteins, many of which were membrane-associated proteins and/or proteins of moderate to low abundance. Notably, this study identified previously unknown isoform-specific changes in protein kinase C that occurred during PMA-induced differentiation.

Recently, the ICAT technology was converted to a format for the solid phase capture and release of chemically tagged peptides (18). In this study, the solid phase isotope-tagging reagent consisted of a thiol-specific reactive group attached *via* an isotopically modified amino acid (either d0 or d7 leucine) to an o-nitrobenzyl-based photocleavable linker bound to an aminopropyl-coated glass bead (Fig. 1B). Each of the two proteomes under comparison was digested with trypsin and its cysteine-containing peptides captured with either the light or heavy form of the solid phase reagent. The light and heavy beads were then combined, washed, and exposed to UV light to induce photocleavage of the linker. The isotopically labeled peptides, now present in solution, were then analyzed by LC-ESI-MS-MS. Compared to the original solution phase ICAT approach, the solid phase strategy required less sample handling and provided greater sensitivity for quantitative protein analysis. On the other hand, because solid phase ICAT involves proteolysis prior to probe labeling, the solution phase ICAT method may still be preferred in cases where the separation of labeled proteins is desired. For example, solution phase ICAT methods have been used in combination with 2DE to concurrently
quantify changes in protein expression and modification state that occur in the yeast proteome in response to a metabolic shift (19).

Chemical methods to measure protein phosphorylation in complex proteomes. Building on the success of ICAT, related chemical proteomics strategies have been introduced to evaluate the posttranslational modification state of proteins. In particular, several chemical reagents have been developed to measure the phosphorylation state of proteins in complex proteomes (20). Traditional methods for detecting protein phosphorylation include metabolic radiolabeling with $^{32}$P inorganic phosphate (21,22) and affinity chromatography with either immobilized metal-affinity chromatography (IMAC) (23) or phospho-specific antibodies (24). However, each of these techniques exhibits shortcomings for quantitative proteome analysis (Table 1). For example, metabolic labeling with $^{32}$P requires a viable cell source and therefore is not applicable for the proteomic analysis of human tissue specimens. Additionally, transitioning from the detection of $^{32}$P-labeled proteins on 2DE gels to the molecular identification of these proteins can be challenging without the availability of target-specific enrichment reagents (e.g., antibodies). Affinity chromatography procedures like IMAC and phospho-specific antibodies have typically suffered from high levels of background binding by nonphosphorylated peptides and poor quantitation [notably, however, recent advances in IMAC methodologies may help to overcome these deficiencies (25)].

Two chemical tagging strategies for quantitative phosphoproteome analysis have recently been described. The first approach, concurrently put forth by two independent
research groups (26, 27), involves the sequential base-catalyzed β-elimination of the
phosphate group and nucleophilic addition of an affinity tag to the resulting
dehydroalanine residue (Fig. 2). In both methods, cysteine residues on proteins were first
oxidized with performic acid to prevent crossreactivity in subsequent steps. Then,
treatment with base transformed phosphoserine and phosphothreonine residues into
Michael acceptors susceptible to nucleophilic attack with ethanedithiol (EDT). A reactive
biotin reagent was then coupled to the free thiol end of EDT-modified sites, permitting
the purification of the originally phosphorylated proteins by avidin-affinity
chromatography (enriched as either whole proteins or as peptides if preceded by digestion
with trypsin). Affinity-isolated biotinylated peptides were then analyzed by ESI-LC-
MS/MS, permitting the identification of the corresponding proteins, as well as the
specific sites of phosphorylation on these proteins. In the study by Goshe and
colleagues, this method was adapted for quantitative analysis of phosphoproteomes by
incorporating phosphoprotein-specific isotope-coded affinity tags (PhIAT) (27). These
PhIAT reagents consisted of two isotopic derivatives of EDT [a light (d0) and heavy (d4)
version], each of which served as the nucleophile for one of the two proteomes under
comparison (Fig. 2). The light and heavy PhIAT-modified proteomes were then
combined, processed, and analyzed by ESI-LC-MS/MS as previously described for
ICAT.

Because the strategy detailed above requires the β-elimination of a phosphate
group in order to expose a site for affinity tagging, it is not capable of monitoring the
phosphorylation state of tyrosine residues on proteins. In contrast, a second chemical
method for phosphoproteome analysis developed by Zhou and colleagues is applicable to
phospho-seryl, -threonyl, and –tyrosyl residues (28). In this approach, proteins were first alkylated with iodoacetamide to block cysteine residues and then enzymatically digested with trypsin (Fig. 2). Following protection of the amino groups of the resulting peptide mixture with tBOC chemistry, the carboxyl/phosphoryl groups were modified with ethanolamine in a carbodiimide-catalyzed reaction. Treatment with acid promoted the hydrolysis of the less stable phosphoramidate bonds, which were then reacted with a cystamine disulfide-bonded dimer. Reduction of the cystamine substituent resulted in the exposure of a free thiol group at each site of phosphorylation in the peptide sample. Thiol-modified phosphopeptides were then captured on the solid phase by reaction with iodoacetyl groups immobilized on glass beads. After stringent washing, phosphopeptides were released from the solid phase by phosphoramidate bond cleavage with trifluoroacetic acid and analyzed by ESI-LC-MS/MS.

A comparison of the phosphate elimination and phosphoramidate modification methods suggests that these approaches offer complementary advantages for phosphoproteome analysis. The phosphate elimination method requires fewer modification steps and results in a chemically modified peptide suitable for tandem MS analysis to identify the specific site of phosphorylation (Table 1). However, this strategy is only applicable to phospho-serine and phospho-threonine peptides. In contrast, the reversible phosphoramidate modification protocol can analyze any type of phosphorylated peptide, but involves numerous derivatization steps and results in the recovery of unmodified phosphate groups, which typically disassociate during tandem MS analysis, confounding efforts to determine sites of phosphorylation. Importantly,
however, both methods reduce sample complexity, while at the same time enriching for phosphorylated proteins, and therefore should provide access to low abundance constituents of the phosphoproteome. Additionally, because these chemical strategies offer a means to quantify changes in the phosphoproteome by isotope tagging, they should facilitate the discovery of molecular changes in signal transduction cascades associated with particular physiological and/or pathological processes.

Chemical approaches for determining the activity of proteins in complex proteomes

Conventional proteomics methods record variations in protein abundance and therefore provide only an indirect estimate of changes in protein activity. Accordingly, these approaches may fail to detect important post-translational forms of protein regulation such as those mediated by protein-protein and/or protein-small molecule interactions (7). To address these limitations, chemical strategies have been developed for activity-based protein profiling (ABPP) that employ active site-directed probes to determine the functional state of enzymes in complex proteomes (12). Chemical probes for ABPP consist of at least two molecular elements: 1) a reactive group for binding to and covalently modifying the active sites of many members of a given enzyme class (or classes), and 2) a chemical tag for the rapid detection and isolation of reactive enzymes (Figure 3A). Because these probes possess moderately reactive electrophilic groups, they are poised to selectively modify enzyme active sites, which are often enriched in nucleophilic amino acid residues important for catalysis.
To date, two general strategies for ABPP have been devised: 1) directed approaches that target specific classes of enzymes, and 2) non-directed approaches that profile enzymes from several different classes. The chemical foundation for each of these methods, as well as examples of their biological application will be reviewed below.

**Directed ABPP – the design and application of activity-based chemical probes that target specific classes of enzymes.** Directed ABPP approaches have capitalized on a rich history of mechanistic studies associated with particular classes of enzymes to create chemical probes with predictable proteome reactivities. By incorporating as the probe reactive group well-known affinity labeling reagents, researchers have succeeded in creating ABPP probes that target, for example, serine hydrolases (29, 30) and subclasses of cysteine proteases (31, 32).

**ABPP probes that target the serine hydrolase superfamily.** Serine hydrolases are one of the largest and most diverse classes of enzymes in higher eukaryotes, representing approximately 1% of the predicted protein products encoded by the human genome (33, 34). Representative serine hydrolases include proteases like thrombin (35), trypsin (36), and urokinase plasminogen activator (37), lipid hydrolases like phospholipase A₂ (38), esterases like acetylcholinesterase (39), and amidases like fatty acid amide hydrolase (FAAH) (40). Due to shared features of their catalytic mechanism, nearly all members of the serine hydrolase superfamily are irreversibly inactivated by fluorophosphonate reagents (41). Accordingly, for the design of ABPP probes that target serine hydrolases,
Liu and colleagues synthesized compounds consisting of: 1) a fluorophosphonate (FP) reactive group, 2) an alkyl or polyethylene glycol chain linker, and 3) a biotin tag (Fig. 3B; see refs. 29, 30). These FP probes were found to label numerous members of the serine hydrolase superfamily directly in complex proteomes. Additionally, FP probes were shown to read out the functional state of serine hydrolases, labeling, for example, active proteases, but not their inactive zymogen and/or inhibitor-bound forms. Kidd and colleagues exploited the activity-based nature of FP-proteome reactions to detect multiple brain serine hydrolases sensitive to trifluoromethyl ketone inhibitors of FAAH, demonstrating that ABPP can serve as a screen to determine the potency and selectivity of enzyme inhibitors (30). Notably, these inhibitor selectivity screens were conducted directly in complex proteomes, thus alleviating the need to recombinantly express or purify the enzymes under investigation. Finally, by establishing a covalent link between the labeled enzymes and a biotin tag, FP probes provided a straightforward route for the affinity purification and molecular identification of targeted proteins by avidin chromatography and mass spectrometry procedures, respectively (30).

Although valuable for the affinity purification of probe-reactive proteins, biotin-conjugated ABPP probes displayed several shortcomings for the systematic detection of enzyme activities in complex proteomes. In particular, biotin labeling events must be visualized indirectly, typically with avidin-horseradish peroxidase complexes and chemiluminescent substrates. These assays are limited in sensitivity, throughput, and dynamic range, thus hindering efforts to rapidly and quantitatively compare large numbers of proteomic samples. To address these limitations, the FP reactive group has
been conjugated to fluorescent tags (either rhodamine or fluorescein), permitting the use of direct in-gel fluorescence scanning as a rapid, sensitive, and quantitative screen for activity-based protein labeling events (42). Notably, Patricelli and colleagues have estimated that in-gel fluorescence scanning can detect on the order of 100 amol of FP-rhodamine-labeled enzyme (42), a value nearly two orders of magnitude more sensitive than the detection limit of biotin-conjugated probes (29). Thus, a two-tiered strategy for ABPP has since been adopted in which first, fluorescent probes are used for rapid and quantitative comparative proteome analysis, and second, biotin probes are applied to affinity enrich and identify differentially expressed enzyme activities.

Capitalizing on the technical advantages afforded by both fluorescent and biotin-avidin ABPP methods, Jessani and colleagues set out to comparatively profile a panel of human cancer cell lines to determine if a global analysis of serine hydrolase activities would yield proteomic information of sufficient quantity and quality to depict higher-order cellular properties (43). In this study, cancer cell proteomes were split into three fractions prior to characterization: the secreted, membrane, and cytosolic fractions. Profiling of these proteomic fractions from eleven breast and melanoma cancer lines identified a cluster of serine hydrolase activities that distinguished these lines based on tissue of origin. Interestingly however, nearly all of these enzymes were downregulated in the most invasive cancer lines examined, which instead upregulated a distinct set of serine hydrolase activities that included the protease urokinase and a novel membrane-associated enzyme, KIAA1363. A more detailed analysis revealed that most of the serine hydrolase activities responsible for classifying cancer cells into subtypes based on tissue
of origin and/or state of invasiveness resided in the secreted and membrane proteome, suggesting that these proteomic fractions were particularly enriched in enzyme markers of cellular behavior. Collectively, these studies demonstrate that ABPP can generate molecular profiles that accurately depict higher-order cellular properties, and in the process, identify uncharacterized enzyme activities, like KIAA1363, that may represent new biomarkers and/or therapeutic targets for the diagnosis and treatment of human disease.

**ABPP probes that target cysteine proteases.** For the design of activity-based chemical probes that label cysteine proteases, researchers have also benefited from a rich history of well characterized active-site directed covalent inhibitors. For example, Thornberry and colleagues appended (acyloxy)methyl ketone inhibitors of caspases with biotin (Fig. 3B; see ref. 44), creating first generation ABPP probes for this subclass of cysteine proteases (approximately 15 predicted caspases are encoded by the human genome). Biotinylated variants of caspase inhibitors have since been applied to several model systems to identify members of this enzyme family associated with cellular events like apoptosis (31, 45). Recently, Winssinger and colleagues coupled caspase-directed reactive groups to peptide nucleic acids (PNAs), permitting the detection of activated caspase-3 on a glass slide microarray bearing complementary oligonucleotide sequences (46). Although this approach may offer a more high-throughput and miniaturized assay platform for the detection of probe-labeled proteins, it remains unclear whether such a strategy is applicable to the majority of ABPP probes which each target multiple enzymes (e.g., FP-
probes, where the fluorescent signal on a given microarray spot would likely represent a complicated sum of the levels of several enzyme activities).

To generate ABPP probes for the papain class of cysteine proteases, Greenbaum and colleagues have synthesized biotinylated variants of the peptide epoxide natural product E-64, a covalent inhibitor of several papain family members (Fig. 3B; see ref. 32). The papain class of proteases includes cathepsins, a family of lysosomal proteases (approximately 15 predicted members encoded by the human genome), and calpains, a group of calcium-dependent cytosolic proteases (approximately 10 predicted members encoded by the human genome). E-64-based ABPP probes have been used to identify cathepsin activities that correlate with skin cancer progression (32) and calpain activities implicated in cataract formation (47). Recently, Greenbaum and colleagues created variants of E-64-based ABPP probes coupled to BODIPY dyes and showed that these fluorescent reagents could be used to visualize cathepsin activities in living cells by fluorescence microscopy (48). Additionally, these probes were applied in combination with libraries of peptide epoxides to identify selective irreversible inhibitors of cathepsin B (48).

ABPP probes that target tyrosine phosphatases. Lo and colleagues have reported the synthesis and application of first generation activity-based probes to profile members of the protein tyrosine phosphatase (PTP) family (49). These probes were comprised of a mechanism-based reactive group [a 4-fluoromethyl-1-phosphophenyl substituent (50)], a diethylene glycol linker, and a biotin or dansyl tag (Fig. 3B). The authors hypothesized
that PTP-catalyzed hydrolysis of the phosphate group would promote a 1,6 elimination of the fluorine atom to form a highly reactive quinone methide that might label phosphatase active sites. Consistent with this notion, the tyrosine phosphatase PTP-1B, but not other proteins like phosphorylase b and albumin, was covalently modified by these mechanism-based probes. Still, high probe concentrations (1 mM) were required to label PTP-1B and further studies will be needed to determine if such conditions are compatible with profiling members of the PTP family in complex proteomes.

*Non-directed ABPP – the design and application of libraries of activity-based chemical probes that target multiple classes of enzymes.* As described above, the creation of activity-based probes for some enzyme classes, like serine and cysteine hydrolases, has been relatively straightforward. Because active site-directed affinity labels were already known for these enzymes, chemical proteomics researchers needed only to couple these “reactive group” elements to an appropriate linker and detection/isolation tag to generate probes for ABPP. For many enzyme classes, however, cognate affinity labels do not yet exist, thus limiting the scope of such directed ABPP efforts. To expand the number of enzyme classes addressable with ABPP methods, Adam and colleagues have introduced a non-directed or combinatorial strategy in which libraries of candidate probes are synthesized and screened against complex proteomes for activity-dependent protein reactivity (51, 52).

To demonstrate the feasibility of non-directed approaches for ABPP, a relatively small library of candidate probes was synthesized that incorporated the following
elements: 1) a variable alkyl/aryl binding group, 2) a sulfonate ester reactive group, 3) an aliphatic linker, and 4) a rhodamine or biotin tag (for the detection and affinity isolation of protein targets, respectively) (Fig. 4A). By selecting a carbon electrophile (sulfonate ester) as the reactive group element of the probe library, it was hoped that the probes would label the active sites of enzymes from several different mechanistic classes. In support of this hypothesis, natural products bearing carbon electrophiles have been identified that covalently modify the active sites of a diverse number of enzymes, including wortmannin, which targets kinases (53), microcystin, which targets phosphatases (54), and fumagillin, which targets metalloproteases (55). Rhodamine-tagged members of the sulfonate probe library were applied to tissue and cell line proteomes in a screen for specific protein reactivities, which were defined as those that occurred in native, but not heat-denatured proteomes (conversely, proteins showing heat-insensitive reactivity were considered non-specific targets) (Fig. 4B). The authors hypothesized that proteins reacting with sulfonate probes in a heat-sensitive manner would possess structured sites for small molecule interactions, and that these sites would often determine the biological activity of the protein (e.g., the active site of enzyme). In these initial studies, several heat-sensitive sulfonate targets were detected in both soluble and membrane proteomes (51, 52). Interestingly, most proteins showed preferential labeling with specific members of the sulfonate library, indicating that the varied binding group element was at least in part specifying the proteome reactivity of the probes. Biotin-conjugated sulfonate probes were used to affinity isolate several protein targets and these proteins were identified by mass spectrometry methods as enzymes from nine mechanistically distinct classes (Table 2). Each enzyme was recombinantly expressed in
COS-7 cells to confirm its sensitivity to sulfonate labeling. For several enzymes, additional evidence was obtained that sulfonate probes were modifying the active site. For example, the addition of cofactors or substrates was found to reduce the labeling of some enzymes (52, 56), while the sulfonate reactivity of other proteins was either enhanced or inhibited by known allosteric regulators of catalytic activity (56). For one enzyme, aldehyde dehydrogenase, the sulfonate probes were also shown to act as active site-directed irreversible inhibitors (51). Finally, it is notable that several of the sulfonate targets, including glutathione S-transferase GSTO 1-1 (51), tissue transglutaminase (56), and platelet-type phosphofructokinase (56), were found to be upregulated in invasive breast cancer cells, indicating that non-directed methods for ABPP can identify novel protein markers of discrete pathological states.

In summary, through the development and application of non-directed methods for ABPP, Adam and colleagues have shown that activity-based chemical probes compatible with whole-proteome analysis can be generated for numerous enzyme classes. Strikingly, none of the enzymes labeled by the sulfonate library represented targets of previously described proteomics probes. This finding suggests that proteomics researchers are still far away from saturating the amount of “active site space” addressable with chemical probes. Nonetheless, successful attempts to further expand the scope of ABPP will likely require probe libraries of considerable chemical and structural diversity, as well as efficient strategies to screen proteomes for targets of these profiling tools. The future design of ABPP probes would also benefit from a deeper understanding of the parameters that drive the probe-enzyme reactions observed to date with the
sulfonate library. The absence of a shared catalytic mechanism among the sulfonate
targets argues that other features are dictating probe labeling. Efforts to identify the
specific sites of sulfonate modification on the targeted enzymes may help to define the
molecular properties that support active site-directed labeling events. Finally, it is worth
considering how often active site-directed labeling actually equates with an “activity-
based” event. For certain enzymes like tissue transglutaminase, sulfonate labeling
appears to provide an exquisite readout of catalytic activity, as both properties show a
strict requirement for calcium and inhibition by the allosteric regulator GTP (56). On the
other hand, for some enzymes, active site modification may occur on non-catalytic
residues, akin to the manner in which microcystin labels a non-catalytic cysteine residue
in the active sites of serine/threonine-phosphatases (54). Can such labeling events be
considered activity-based? From a pure mechanistic standpoint, the answer would be no;
however, from a more biological perspective, if, as is often the case, enzyme activity is
regulated in vivo by autoinhibitory domains, protein partners, and/or small molecules that
sterically obstruct the active site (7), then any probe that is sensitive to such molecular
interactions would provide an effective readout of the functional state of the enzyme in
the context of the cell biology of the proteome.

Conclusions and future directions

In this review, we have highlighted a promising new class of proteomics methods that has
united the fields of synthetic chemistry and protein biochemistry to create powerful tools
and assays for the global analysis of protein expression and function. Chemical
approaches like the isotope-coded affinity tag (ICAT) method offer proteomics researchers the opportunity to compare the expression level of low abundance proteins in samples of high complexity (16, 18). The extension of ICAT methods to chemical probes specific for phosphorylated peptides has engendered assays to monitor changes in the post-translational modification state of proteins in cell and tissue proteomes (26-28). Finally, both directed and non-directed strategies for activity-based protein profiling (ABPP) have produced a menu of chemical probes that can be used either separately or in combination to discover enzyme activities associated with discrete physiological and/or pathological states (29, 32, 43, 52, 56). The value of ABPP as a method for functional proteome analysis has been further highlighted by its application as a screen to evaluate the potency and selectivity of enzyme inhibitors (30, 32). Nonetheless, despite the considerable advances made to date, chemical approaches for proteome analysis still face significant technical challenges. Perhaps most notably, an unsatisfying trade-off seems to exist between the need for high sample throughput and the desire for in-depth analysis of individual proteomes. For example, with a 1D gel format, hundreds of proteomic samples treated with ABPP probes can be readily analyzed in a single day by a given academic lab (43). However, the modest resolution afforded by 1D gels likely will result in some low abundance and/or co-migrating protein targets eluding detection. In contrast, proteomic investigations that utilize LC as a separation method can achieve exceptional resolution of chemically tagged peptides, but with a much lower sample throughput. In the end, the optimal platform with which to analyze probe-labeled proteomes will likely depend on the biological question being addressed. Indeed, if extensive detail is sought on a select number of samples, then one may wish to apply all
of the proteomics methods described above, thereby approaching a complete picture of
dynamics in protein abundance, modification state, and activity.

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Figure Legends

FIG. 1. Isotope-coded affinity tags (ICAT) methods for quantitative proteomics. Both solution-phase (A; see ref. 16) and solid-phase (B; see ref. 18) ICAT methods are outlined. See text for details.

FIG. 2. Chemical methods for measuring the phosphorylation state of proteins in complex proteomes. Both the base-catalyzed phosphate elimination method (left flow chart; see refs. 26 and 27) and the phosphoramidate modification method (right flow chart; see ref. 28) are outlined. See text for details.

FIG. 3. Activity-based protein profiling (ABPP) with chemical proteomics probes. A, General mechanism by which activity-based probes label the active sites of targeted enzymes, where: NU = nucleophilic amino acid residue, RG – reactive group, L = linker, TAG – detection/affinity tag. B, Representative activity-based protein profiling (ABPP) probes directed towards specific classes of enzymes. See text for details.

FIG. 4. A, Representative library of chemical probes for non-directed activity-based protein profiling (ABPP), where a variable alkyl or aryl binding group is attached to a sulfonate ester reactive group (see refs. 51 and 52). B, Cartoon depiction of proteomic data generated with non-directed ABPP, where specific and non-specific (NS) targets are defined as those that show heat-sensitive and heat-insensitive probe labeling, respectively.
Table 1. A comparison of different proteomic strategies for measuring protein phosphorylation.

| Analytical Method | Phosphorylated amino acids detected | Quantitative | Site of phosphorylation identified | General comments |
|-------------------|-------------------------------------|--------------|------------------------------------|-----------------|
| **Conventional Methods** | | | | |
| 32P Radio-labeling | Ser, Thr, Tyr | Semi | Difficult | Requires living samples |
| Phosphospecific antibodies | Ser, Thr, Tyr | No | Difficult | High nonspecific binding |
| IMAC | Ser, Thr, Tyr | Yes (d0 or d3 methanol) | Difficult | Methylation of carboxylic acids reduces background*** |
| **Chemical Methods** | | | | |
| []-elimination* | Ser, Thr | Yes (d0 or d4 ethanedithiol) | Yes (tag survives MS/MS) | Potential for []-elimination of O-linked carbohydrates |
| Phosphoramidate** | Ser, Thr, Tyr | Yes (d0 or d4 ethanolamine) | Difficult | Lengthy protocol |

*See refs. 26 and 27; **see ref. 28; ***see ref. 25.
Table 2. Representative enzyme activities identified from mouse and human proteomes by non-directed ABPP methods using a sulfonate ester probe library.

| Enzyme                                             | Enzyme Class                                           | Proteome Source                  |
|----------------------------------------------------|--------------------------------------------------------|----------------------------------|
| Acetyl CoA acetyltransferase*                       | Thiolase                                               | Mouse Heart                      |
| Aldehyde dehydrogenase 1**                         | Aldehyde dehydrogenase                                 | Mouse Heart, Rat Liver           |
| Aldehyde dehydrogenase 7*                          | Aldehyde dehydrogenase                                 | Mouse Heart                      |
| Dihydropdiol dehydrogenase*                        | NAD/NADP-dependent oxidoreductase                      | Mouse Heart                      |
| Enoyl CoA hydratase, peroxisomal*                  | Enoyl CoA hydratase                                    | Mouse Heart, Human Breast Cancer Line|
| Epoxide hydrolase, cytoplasmic*                    | Epoxide hydrolase                                      | Mouse Heart                      |
| GSTO1-1*                                           | Glutathione-S-transferase                              | Human Breast Cancer Line         |
| 3β-Hydroxysteroid dehydrogenase 5-isomerase-1***   | 3β-Hydroxysteroid dehydrogenase                        | Mouse Testis                     |
| Platelet phosphofructokinase***                   | Phosphofructokinase                                    | Human Breast Cancer Line         |
| Type II tissue transglutaminase***                 | Transglutaminase                                       | Human Breast Cancer Line         |

*See ref. 52; **see ref. 51; ***see ref. 56.
Fig. 1

A

Solution Phase ICAT

Cell state 1
Label with light (d0) ICAT
Combine Fractionate Proteolyze
Isolate ICAT-labeled peptides with avidin affinity chromatography
μLC-MS/MS
Quantitate relative protein levels by comparing peak ratios

Cell state 2
Label with heavy (d6) ICAT

B

Solid Phase ICAT

Cell state 1
Digest Reduce
Solid phase capture with d0 beads
Combine and wash
Photocleave peptides from resin
μLC-MS/MS
Quantitate relative protein levels by comparing peak ratios

Cell state 2
Solid phase capture with d7 beads

Isotope tagged linker
X = H (d0) or D (d6)
Cysteine-specific reactive group

Isotope tag
Cysteine-specific reactive group
X = H (d0) or D (d7)

Beads Photocleavable linker
Fig. 3

A

Enzyme + Activity-Based Probe → Labeled Enzyme

B

Serine Hydrolases

Papain Class of Cysteine Proteases

Caspase Class of Cysteine Proteases

Protein Tyrosine Phosphatases

TAG = Biotin or fluorescent group
Fig. 4

A

\[ \text{TAG} = \text{Biotin or fluorescent group} \]

\[
\begin{align*}
X &= \text{Phenyl} & \text{Quinoline} & \text{Octyl} \\
&= \text{Nitrophenyl} & \text{Naphthyl} & \text{Mesyl} \\
&= \text{Pyridyl} & \text{Thiophene} & \text{Butyl} \\
&= \text{Tosyl} & \text{Methoxyphenyl} \\
\end{align*}
\]

B

Activity-Based Gel Profile