Chemical Strategies for Activity-Based Proteomics

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The assignment of molecular and cellular functions to the numerous protein products encoded by prokaryotic and eukaryotic genomes presents a major challenge for the field of proteomics. To address this problem, chemical approaches have been introduced that utilize small-molecule probes to profile dynamics in enzyme activity in complex proteomes. These strategies for activity-based protein profiling enable both the discovery and functional analysis of enzymes associated with human disease.

Introduction

The availability of complete genome sequences for numerous prokaryotic and eukaryotic organisms has laid the groundwork for understanding the molecular basis of life in its many forms. However, the information content of genome sequences is limited and alone cannot explain most complex physiological and pathological processes, which are, in general, controlled by protein and RNA molecules, the products of gene expression. Thus, to convert the unprecedented flood of molecular information supplied by genome sequencing efforts into a deeper appreciation of cell and organismal biology, new strategies for the systematic analysis of gene products are needed. Toward this end, the field of proteomics seeks to develop and apply methods for the global analysis of protein expression and protein function.[1, 2] The most mature strategy for comparative proteomics (the analysis of two or more proteomes to identify differentially expressed proteins) is two-dimensional gel electrophoresis (2DE) coupled with protein staining and mass-spectrometry (MS) analysis for the separation, quantification, and identification of proteins.[3] The recent advent of fluorescent dyes such as SYPRO reagents[4] for protein detection has increased the sensitivity and dynamic range of 2DE. However, 2DE-MS methods suffer from an inherent lack of resolving power, and therefore several important classes of proteins, including membrane-associated and low-abundance proteins, are difficult to analyze by this technique.[3, 5–7] Additionally, since 2DE-MS methods measure changes in protein abundance, these approaches offer only an indirect estimate of dynamics in protein activity and may fail to detect important post-translational events that regulate protein function, such as protein – protein or protein – small-molecule interactions.[8]

To address the limitations of 2DE-MS methods, several complementary strategies for protein analysis have been introduced, many of which implement chemical tools to augment the quantity and quality of information obtained in comparative proteomic experiments.[9, 10] Here, we review a subset of these chemical proteomic approaches that aim to develop and utilize active site-directed probes for the quantitative analysis of enzyme activities, including membrane-associated and low-abundance proteins, in samples of high complexity.

Activity-Based Protein Profiling (ABPP)—the Design of Chemical Probes for Functional Proteomics

As mentioned above, the activity of proteins is regulated by myriad post-translational events in vivo. For example, proteases and related enzymes are often produced by cells as inactive precursors (zymogens) that must be processed to gain catalytic power. Likewise, the functions of many enzymes, including kinases, phosphatases, and proteases, are controlled by autoinhibitory domains and/or endogenous protein inhibitors.[8] Such widespread post-translational regulation of proteins indicates that, for much of the proteome, protein abundance may not directly correlate with protein activity. Accordingly, methods for activity-based protein profiling (ABPP) may serve as a valuable complement to conventional genomic and proteomic approaches, which are restricted to recording variations in mRNA and protein abundance, respectively.

In the appraisal of potential strategies for ABPP, it is important to consider how the cell regulates protein activity. For enzymes, most post-translational regulatory mechanisms share a common feature: they perturb, either structurally or sterically, the active sites of these proteins.[8] Accordingly, it was hypothesized that chemical probes capable of directly reporting on the integrity of enzyme active sites in complex proteomes might serve as effective activity-based profiling tools.[9, 10] These ABPP probes were designed to contain at least two molecular elements: 1) a reactive group (RG) for binding and covalently modifying the active sites of many members of a given enzyme class or classes, and 2) one or more reporter groups (tags), like biotin and/or a fluorophore, for the rapid detection and isolation of probe-labeled enzymes (Scheme 1). The RG elements were selected to

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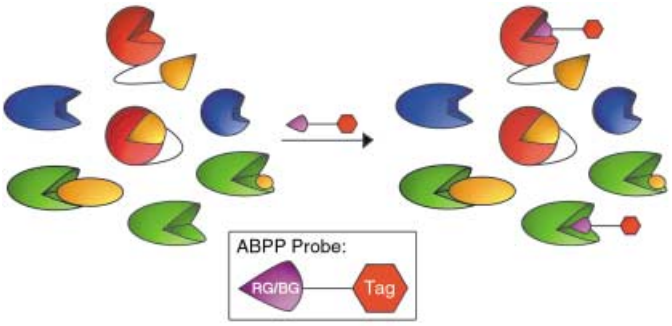
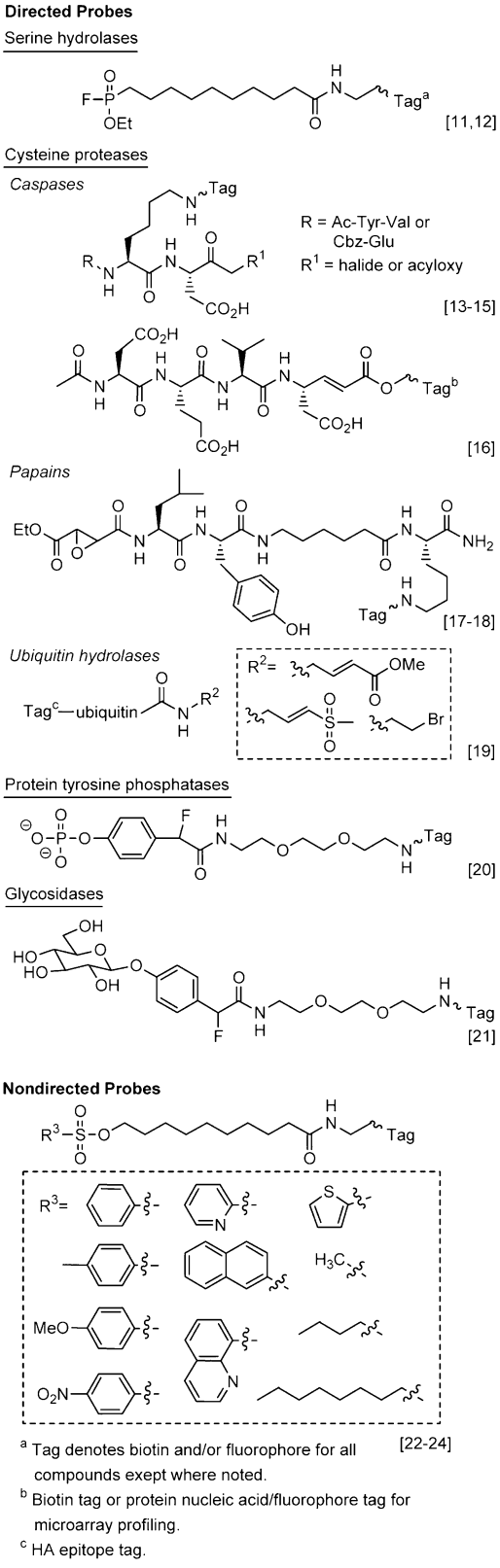


Scheme 1. *General strategy for activity-based protein profiling (ABPP). Proteomes are treated with chemical probes that label active enzymes, but not enzymes inhibited by intra- or intermolecular regulators (orange) or those lacking complementary binding sites (blue). (RG = reactive group, BG = binding group, tag = biotin and/or fluorophore).*

be of moderate reactivity (e.g., electrophilicity), thereby priming them to preferentially modify enzyme active sites, which offer a binding pocket enriched in nucleophilic residues important for catalysis. Finally, a third element may also be introduced into ABPP probes, a binding group (BG) that is intended to direct RGs to different enzyme active sites in the proteome.

Initial strategies for ABPP focused on the design and application of chemical probes that target specific classes of enzymes. In these efforts, well-characterized affinity labels that direct probe reactivity toward enzymes sharing a similar catalytic mechanism and/or substrate selectivity were selected as RGs. For example, the following proteomics probes have been developed by using this directed version of ABPP: 1) biotinylated/fluorophore-tagged fluorophosphonates (FPs) that target the serine hydrolase superfamily,[11, 12] 2) biotinylated electrophilic ketones[13–15] and acrylates[16] that target the caspase class of cysteine proteases, and 3) biotinylated/fluorophore-tagged variants of the epoxide natural product E64 that target the papain class of cysteine proteases[17, 18] (Scheme 2). In each of these cases, the chemical probes have been shown to label their target enzymes in an activity-based manner within complex proteomes, distinguishing, for example, active enzymes from their inactive zymogen[12] or inhibitor-bound forms.[11, 12, 17, 18] These protein-labeling events can be visualized by separation of probe-treated proteomes on 1D or 2D gels followed by detection by avidin blotting or in-gel fluorescence scanning. A complementary array-based strategy for ABPP has also been introduced: proteomes are treated with probe–nucleic acid conjugates, so that the labeled proteins, in this case caspases, can be captured and detected on glass slides bearing complementary oligonucleotide sequences.[16] Advantages of this approach should include increased throughput and miniaturization. However, it remains unclear whether such an array-based strategy is suitable for most ABPP probes, which typically label multiple enzymes in a given proteome. FP probes, for example, would lead to a fluorescent signal on a microarray spot that corresponds to a complicated sum of several hydrolase activities. More recently, directed strategies for ABPP have yielded probes for the ubiquitin hydrolase subclass of cysteine hydrolases,[19] tyrosine phosphatases,[20] and glycosidases[21] (Scheme 2). However, only in the first case have these probes been shown to detect their target enzymes in complex proteomes.



[a] Tag denotes biotin and/or fluorophore for all compounds exept where noted.
[b] Biotin tag or protein nucleic acid/fluorophore tag for microarray profiling.
[c] HA epitope tag.

Scheme 2. *Probe structures ordered by the enzyme classes they target (Table 1).*

From these examples of directed approaches for ABPP, it may be extrapolated that, for enzyme classes with known covalent inhibitors, the design of activity-based proteomics probes is, at least in concept, straightforward. However, covalent inhibitors do not yet exist for the majority of proteins. Therefore, an alternative strategy is needed to discover active site-directed profiling reagents for proteins lacking cognate affinity labels. With this goal in mind, a combinatorial or nondirected strategy for ABPP has been introduced in which libraries of candidate probes are synthesized and screened against complex proteomes to identify "specific" protein-labeling events, which were defined as those that occurred in native, but not in heat-denatured proteomes.[22] Heat-sensitive probe – protein reactions were predicted to occur in structured, small-molecule-binding sites that would often determine the biological activity of the proteins, such as the active site of an enzyme or ligand-binding pocket of a receptor. In contrast, proteins reacting with probes in a heat-insensitive manner were considered nonspecific targets, as these labeling events could occur with either native or denatured proteins. This type of general screen to distinguish specific from nonspecific labeling was deemed particularly important for nondirected ABPP, which utilizes probes that lack well-established selectivity for a given class of enzymes. Screening libraries of probes against individual proteomes also provided a complementary method to detect specifically labeled proteins, which were expected to react with a select number of probes based on the structure of their respective binding groups (BGs). Therefore, it should be possible to differentiate them from nonspecific proteins that react indiscriminately with the probe library.

To evaluate nondirected methods for ABPP, a modest-sized library of sulfonate ester probes bearing different alkyl or aryl BGs (Scheme 2) was generated and screened against a collection of tissue and cell line proteomes.[22–24] The sulfonate ester was selected as the library's RG based on a survey of the literature of protein-modifying agents, which revealed that a large range of enzyme classes, including proteases,[25] kinases,[26] and phosphatases,[27] are susceptible to covalent inactivation by natural products or synthetic inhibitors that possess carbon electrophiles. Accordingly, it was hypothesized that ABPP probes incorporating a carbon electrophile RG may prove capable of profiling enzymes not only within, but also across mechanistically distinct classes. Consistent with this premise, several heat-sensitive protein targets of the sulfonate library were identified and found to represent members of at least nine different enzyme classes (Table 1). Interestingly, each enzyme target displayed a unique reactivity profile with the sulfonate probe library; this indicated that the structure of the variable BG strongly influenced probe – protein interactions. Several lines of evidence supported the idea that the sulfonate probes labeled the active sites of their enzyme targets. For example, the addition of cofactors or substrates was found to inhibit the labeling of several enzymes, while the reactivity of others was either positively or negatively affected by known allosteric regulators of catalytic activity.[22–24] Notably, for aldehyde dehydrogenase-1 (ALDH-1) sulfonate probes were shown to act as time-dependent inactivators of catalytic activity.[22]

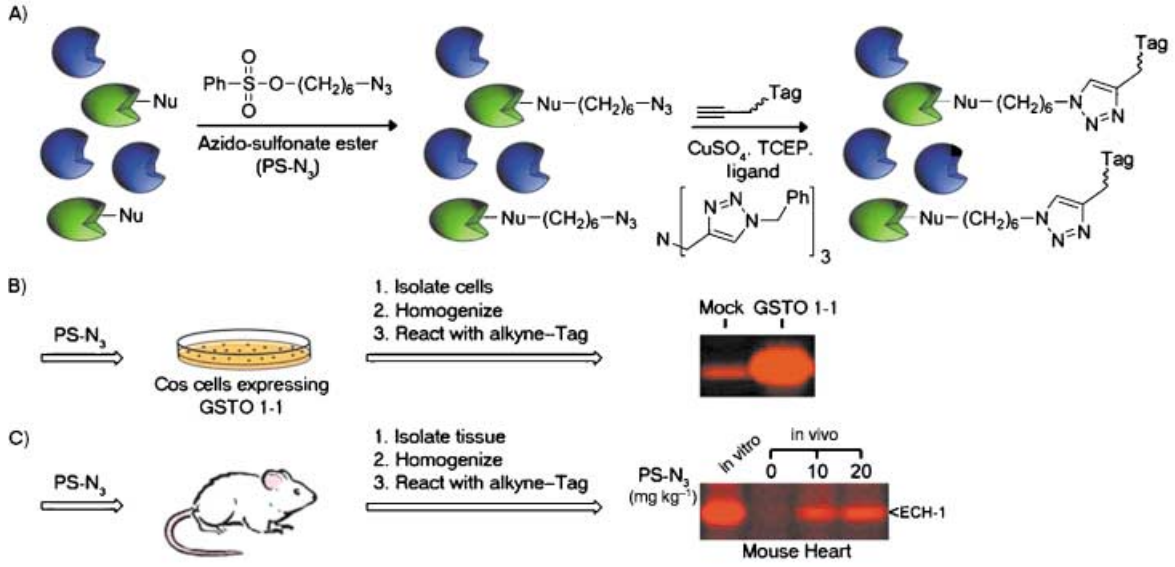
Table 1. *Enzyme classes targeted by directed and nondirected probes*

Directed	Nondirected
serine hydrolase	aldehyde dehydrogenase
cysteine protease	enoyl-CoA hydratase
protein tyrosine phosphatase	epoxide hydrolase
glycosidase	glutathione *S*-transferase
	3β-hydroxysteroid dehydrogenase
	NAD/NADP-dependent oxidoreductase
	phosphofructokinase
	thiolase
	transglutaminase

Collectively, these studies reveal that, through the use of both directed and nondirected strategies, activity-based probes compatible with whole proteome analysis can be generated for numerous enzyme classes. In comparing directed and nondirected approaches for ABPP, it is perhaps most interesting to note the striking lack of overlap in enzyme targets profiled by each method (Table 1). Indeed, none of the sulfonate-labeled enzymes identified to date represent known targets of directed ABPP probes. This finding suggests that the amount of "active-site space" in the proteome accessible to chemical profiling is still far from saturation.

ABPP Strategies for the In Vivo Analysis of Enzyme Activities

Until recently, ABPP experiments have been conducted almost exclusively with proteomic material prepared in vitro, for example, cell and tissue homogenates. Since the physical disruption of cells and tissues may alter the concentrations of endogenous activators and inactivators of enzymes as well as their respective subcellular distributions, in vitro proteomic preparations can only, at best, approximate the functional state of proteins in the living cell or organism. Accordingly, a need existed to advance ABPP so that this strategy could be generally applied in vivo. However, activity-based probes in the form shown in Scheme 1 are quite large with a molecular weight of about 700 – 1000 Da; this limits their cellular uptake and distribution in vivo. A major portion of the probe mass derives from the reporter group (fluorophore, biotin, or both), which, being a constant element of probe libraries, may unduly bias the properties of these reagents in vivo. With these considerations in mind, a "tag-free" version of ABPP was introduced in which the reporter group could be attached to activity-based probes after the covalent labeling of protein targets.[28] Conjugation of the reporter group to the probe following proteome labeling was accomplished by engineering into these reagents a pair of biologically inert coupling partners, the alkyne and azide, which can react to form a stable triazole product by the Huisgen's 1,3-dipolar cycloaddition reaction (Scheme 3). Key to the success of this strategy was the recent description by Sharpless and colleagues[29] of a Cu^{I}-catalyzed, stepwise version of the azide – alkyne cycloaddition reaction that can be carried out under mild conditions to produce high yields of product in rapid reaction times. The general biocompatibility of this click-chemistry



Scheme 3. *A "tag-free" strategy for ABPP based on the azide-alkyne cycloaddition reaction (click chemistry). A) Proteins are first labeled by an azido-sulfonate ester probe and then treated with an alkyne-tag under click-chemistry conditions (tag = Rhodamine, TCEP = tris(carboxyethyl)phosphine). B and C) In vivo labeling of cells and organisms by click chemistry-based ABPP.[28] Gel fluorescence of representative targets of sulfonate ester probes labeled in living cells (GSTO 1-1, B) and mice (ECH-1, C) is shown. In the mock transfected lane (B), the low level signal corresponds to endogenous protein. In (C), mice were administered 0, 10, and 20 mg kg^{-1} intraperitoneal injections of the PS-N_3 probe. Reprinted with permission from A. E. Speers, G. C. Adam, B. F. Cravatt,* J. Am. Chem. Soc. ***2003***, 125, *4686 – 4687. Copyright 2003 American Chemical Society.*

reaction was demonstrated by Finn and colleagues,[30] who showed that azide-modified, purified virus particles could be labeled with alkyne-coupled fluorescent dyes.

The suitability of the Cu^{I}-catalyzed azide-alkyne cycloaddition reaction for profiling enzyme activities in whole cell and tissue extracts was demonstrated by comparing the proteome reactivity profiles of an azide-derivatized ("tag-free") phenylsulfonate ester (PS-N_3) probe to those generated with a standard rhodamine-conjugated variant of this probe.[28] The labeling profile of the PS-N_3 probe was visualized by subsequent reaction of treated proteomes with an alkyne-rhodamine reporter group (Rh-alkyne) under click-chemistry conditions. Notably, for at least three enzyme targets, glutathione *S*-transferase omega class (GSTO 1-1), enoyl CoA hydratase-1 (ECH-1), and ALDH-1, equivalent signal intensities were observed with either the azide- or rhodamine-coupled sulfonate ester probes; this indicated that click chemistry-based strategies for ABPP could profile enzyme activities in whole proteomes with a sensitivity that rivals standard ABPP methods.

Once shown to be operational in vitro, click chemistry-based ABPP was then evaluated for its ability to profile enzyme activities in vivo by using two complementary model systems (Scheme 3B,C).[28] First, cells in culture expressing a known sulfonate target GSTO 1-1 were treated with the PS-N_3 probe for 1 h, washed, and then homogenized prior to the treatment with Rh-alkyne under click-chemistry reaction conditions. Gel analysis revealed a strong fluorescent signal for GSTO 1-1, indicating that the PS-N_3 probe labeled this enzyme in living cells (Scheme 3B). Encouraged by these findings, the PS-N_3 probe was then administered to living mice and, after 1 h, these animals were sacrificed and their tissues removed, homogenized, and treated with Rh-alkyne. Clear fluorescent signals coinciding with the mass of known enzyme targets of PS-N_3 probe, as for example ECH-1, were observed in these samples (Scheme 3C). Collectively, these results indicate that azide-alkyne cycloaddition chemistry may form the basis for a general "tag-free" strategy to profile enzyme activities in living cells and organisms.

Biological Applications of ABPP

Methods for ABPP have matured rapidly since their introduction in the late 1990s, providing a new avenue for identifying novel disease-associated enzymes (target discovery) and chemical inhibitors thereof (inhibitor discovery). A select number of these applications of ABPP are highlighted below.

Target discovery by ABPP

Several enzyme classes profiled by ABPP probes have been implicated in cancer progression, including proteases,[31, 32] lipases,[33] GSTs,[34, 35] and ALDHs.[36] Accordingly, the analysis of human tumors and tumor models by ABPP may identify novel enzyme activities that represent markers or targets for the diagnosis and treatment of cancer. With this goal in mind, Jessani and colleagues utilized ABPP to profile serine hydrolase activities across a panel of human cancer cell lines.[37] A group of secreted and membrane-associated enzyme activities was discovered that could be used to classify cancer cells based on their tumor of origin, like breast carcinoma or melanoma, and even to identify potentially misclassified cancer lines. Notably, a distinct set of serine hydrolase activities was found to be upregulated in invasive cancer cells from several different tumor types. These invasiveness-associated enzyme activities included established markers of cancer progression, such as the protease uroki-

nase,[31, 32] and novel enzymes, like the membrane-associated hydrolase KIAA1363. More recently, nondirected strategies for ABPP have also identified several enzyme activities upregulated in invasive breast-cancer cells, including GSTO 1-1,[23] platelet-type phosphofructokinase, and type II tissue transglutaminase.[24] Collectively, these findings demonstrate that ABPP generates discrete enzyme-activity signatures that can depict the higher-order properties of human cancer cells.

ABPP has also been applied to discover protease activities involved in the life cycle of *Plasmodium falciparum*, the human parasite that causes malaria. Greenbaum and colleagues[38] utilized activity-based probes that target the papain class of cysteine proteases to identify falcipan 1 as a protease that is upregulated in the invasive merozoite stage of *P. falciparum* growth. Interestingly, other proteases, like falcipan 2 and 3, were not up regulated during this phase of the *P. falciparum* life cycle. These findings indicate that falcipan 1 may play a unique and important role in host cell invasion and thus may represent an attractive target for antimalarial drugs. In support of this hypothesis, the authors showed that falcipan 1 inhibitors were able to significantly reduce parasite invasion of cultured human erythrocytes. Notably, these inhibitors were discovered by utilizing ABPP methods as described below.

Inhibitor discovery by ABPP

Because ABPP probes label the active sites of their enzyme targets, these reagents can be used to screen for inhibitors.[12, 17, 18] Inhibitor discovery by ABPP offers several potential advantages over conventional screening methods. First, enzymes can be tested within the confines of their native proteomes, alleviating the need for recombinant expression and purification. Second, probe labeling serves as a surrogate for conventional substrate assays, thereby making novel enzymes that lack known substrates amenable to analysis. Finally, because ABPP tests inhibitors against many enzymes in parallel, the potency and selectivity of these compounds can be concurrently evaluated.

Initial methods for inhibitor screening by ABPP focused on the discovery of irreversible enzyme inhibitors, wherein enzymes or proteomes were first preincubated with a library of candidate inhibitors and then treated with ABPP probes to identify inhibitor-inactivated enzymes. Using these protocols, Greenbaum and colleagues[38] developed selective covalent inhibitors of the *P. falciparum* cysteine protease falcipan 1 and utilized these reagents to define a role for this enzyme in parasite invasion of erythrocytes. Importantly, these inhibitors were identified by ABPP by using parasite extracts that expressed native levels of falcipan 1, thereby circumventing previously described difficulties with the recombinant expression and purification of active forms of this protease.[38]

Although the identification of irreversible inhibitors by ABPP has provided valuable research tools for certain classes of enzymes like cysteine proteases, reversible inhibitors that lack affinity labeling groups are, in general, more desirable as lead therapeutic agents for the in vivo analysis of enzyme function. To adapt ABPP for the discovery of reversible enzyme inhibitors, it was crucial to take into account the kinetics of probe – proteome reactions. Indeed, reversible inhibitors will only affect probe labeling for a restricted period of time, depending on the affinity of the inhibitor and the rate of probe reactivity. To address this concern, Leung and colleagues, who were interested in evaluating the selectivity of a panel of inhibitors of fatty acid amide hydrolase (FAAH, an endocannabinoid-degrading enzyme),[39] established conditions under which the rates of FP labeling for the majority of serine hydrolases in mouse tissue proteomes could be monitored collectively.[40] Under such kinetically controlled conditions, the binding of competitive reversible inhibitors to enzymes was detected as a reduction in probe labeling (i.e., a decrease in fluorescent signal intensity) (Figure 1) By

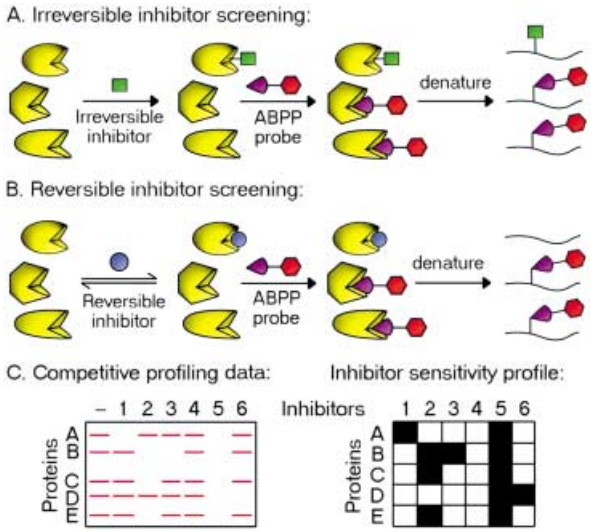


Figure 1. *Comparison of methods for A) irreversible and B) reversible inhibitor screening by ABPP. C) Data from either method can be visualized by 1DE (left cartoon, simplified view right cartoon) to distinguish specific inhibitors (inhibitor **1**, **3**, **6**) from promiscuous agents (inhibitors **2** and **5**).*

varying the concentration of competitive inhibitors, IC_{50} values for these agents were measured by ABPP and found to match closely the K_i values determined by standard substrate assays. Hierarchical clustering analysis of the resulting data sets readily distinguished FAAH-selective inhibitors from agents that showed equal or greater activity toward other serine hydrolases. Notably, none of these additional enzyme targets of the inhibitor library shared any sequence homology with FAAH, highlighting the value of proteome-wide screens like ABPP that can detect unanticipated "off-target" activities of compounds in samples of high complexity. The FAAH-selective inhibitors identified in this study may represent attractive lead compounds for the development of therapeutic agents for the treatment of pain and other neurological disorders.[41, 42]

Conclusions and Future Challenges

The discipline of chemistry is perhaps uniquely suited to provide powerful new tools and methods for the functional analysis of the proteome. As has been highlighted in this review, chemical approaches for activity-based protein profiling (ABPP) have, over

the past few years, enjoyed an intense phase of technical innovation, in which these strategies have already advanced our understanding of the role that enzymes play in complex physiological and pathological processes. Looking forward, researchers interested in broadening the scope and impact of ABPP are faced with several conceptual and experimental challenges. First, active site-directed chemical probes, which constitute the fundamental currency of ABPP, have to date only been developed for a modest portion of the proteome. The successful generation of proteomics-compatible profiling reagents for additional enzyme (and protein) classes will probably require the synthesis of more structurally diverse libraries of candidate probes, which may be either directed or nondirected in nature. Whether these probe libraries should also be scaled up in terms of molecular complexity remains an interesting question open for debate. Although probes of complex structure should display enhanced affinity for particular enzymes, the ability of these reagents to form productive interactions with many enzymes in the proteome may prove more restricted than for probes of simpler structure. Indeed, given that most of the characterized ABPP probes display only micromolar or weaker binding affinities for their respective targets,[12, 18, 22] the specifications for new probe design may mandate only moderate binding affinity for protein targets coupled with tempered reactivity. A similar idea has been put forth in the field of fragment-based ligand discovery for drug design, where diverse libraries of structurally simple compounds are preferred for initial screens to identify lead ligands.[43–45] Where these endeavors differ, however, is in the degree of target specificity expected of their respective chemical agents. While in drug design the target promiscuity typically displayed by moderate-affinity leads must be eradicated by iterative cycles of medicinal chemistry, broad target selectivity is actually a preferred property of ABPP probes as it enables these reagents to sample greater portions of proteomic space.

In the development of new active site-directed proteomics probes, it is also important to consider the fidelity with which these reagents will report on changes in protein activity. For certain probes, like the FPs, which react with conserved catalytic residues in the active sites of their enzyme targets, probe labeling has been shown to provide a precise readout of catalytic activity.[11, 12] However, it is likely that other probes may be discovered that modify enzyme active sites on noncatalytic residues, akin to the manner in which microcystin labels a noncatalytic cysteine residue in serine/threonine phosphatases.[27] Although such active site-directed labeling events would not be considered purely activity-based in a mechanistic sense, from a biological perspective, if enzyme activity is regulated in vivo by steric blockade of the active site, for example by autoinhibitory domains or protein/small molecule binding partners,[8] then any probe that is sensitive to these molecular interactions should effectively report on the functional state of enzymes in complex proteomes. More generally, these issues highlight the importance of understanding the molecular basis for individual probe–enzyme reactions, especially those originating from nondirected ABPP efforts where the parameters that dictate probe binding and labeling are not always obvious.

Finally, as the proteome coverage of ABPP continues to grow, it is becoming clear that this strategy would benefit from improved methods for the qualitative and quantitative analysis of probe-labeled samples. Currently, most probe-labeled proteomes are analyzed by 1D or 2D electrophoresis, which exhibits limited resolving power especially for large protein families with members of similar molecular mass. Future efforts to merge ABPP with gel-free proteomic platforms like liquid chromatography–mass spectrometry (LC-MS),[46] may provide a complementary strategy for resolving large numbers of probe-labeled enzyme activities. The enhanced resolution offered by gel-free methods may permit the multiplexing of ABPP probes, such that proteomes of limited quantity could be analyzed simultaneously with a collection of probes. Adapting ABPP for direct LC-MS analysis should also permit comparative quantitation of probe-labeled proteomes by isotope-coded mass tagging.[47] Still, it is important to emphasize that, although such LC-MS platforms will surely exhibit superior resolving power compared to 1D gel-based methods for analyzing probe-labeled proteomes, the 1DE approach does possess the advantage of exhibiting much higher throughput (i.e., dozens of proteomes can be compared on a single gel). Thus, the choice of whether to employ gel-based, gel-free, or both strategies for the analysis of ABPP experiments will probably depend on the scientific problem under examination, with the former strategy being more suitable for the rapid comparison of large numbers of proteomes and the latter approach being superior for the in-depth analysis of a restricted set of samples. In either case, continued efforts to advance both the chemical and technical components of ABPP should foster the development of an increasingly robust and sensitive platform for the functional analysis of both the proteome and its individual constituents.

Note added in proof: Since the submission of this manuscript, a second chemical strategy for profiling enzyme activities in living cells has been reported that utilizes the bio-orthogonal Staudinger ligation: H. Ovaa, P. F. van Swieten, B. M. Kessler, M. A. Leeuwenburgh, E. Fiebiger, A. M. van den Nieuwendijk, P. J. Galardy, G. A. van der Marel, H. L. Ploegh, H. S. Overkleeft, *Angew. Chem.* **2003**, *115*, 3754–3757; *Angew. Chem. Int. Ed.* **2003**, *42*, 3626–3629.

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