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Next Generation Chemical Proteomic Tools for Rapid Enzyme Profiling

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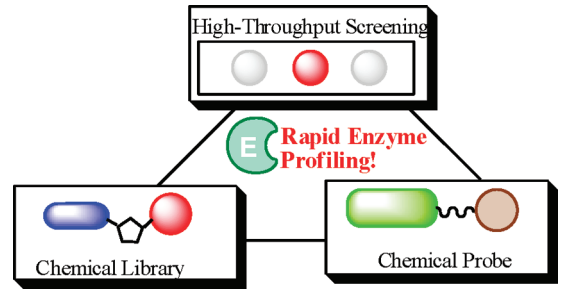
CONSPECTUS

Sequencing of the human genome provided a wealth of information about the genomic blueprint of a cell. But genes do not tell the entire story of life and living processes; identifying the roles of enzymes and mapping out their interactions is also crucial. Enzymes catalyze virtually every cellular process and metabolic exchange. They not only are instrumental in sustaining life but also are required for its regulation and diversification. Diseases such as cancer can be caused by minor changes in enzyme activities. In addition, the unique enzymes of pathogenic organisms are ripe targets for combating infections. Consequently, nearly one-third of all current drug targets are enzymes.



An estimated 18−29% of eukaryotic genes encode enzymes, but only a limited proportion of enzymes have thus far been characterized. Therefore, little is understood about the physiological roles, substrate specificity, and downstream targets of the vast majority of these important proteins. A key step toward the biological characterization of enzymes, as well as their adoption as drug targets, is the development of global solutions that bridge the gap in understanding these proteins and their interactions. We herein present technological advances that facilitate the study of enzymes and their properties in a high-throughput manner.

Over the years, our group has introduced and developed a variety of such enabling platforms for many classes of enzymes, including kinases, phosphatases, and proteases. For each of these different types of enzymes, specific design considerations are required to develop the appropriate chemical tools to characterize each class. These tools include activity-based probes and chemical compound libraries, which are rapidly assembled using efficient combinatorial synthesis or "click chemistry" strategies. The resulting molecular assortments may then be screened against the target enzymes in high-throughput using microplates or microarrays. These techniques offer powerful means to study, profile, and discover potent small molecules that can modulate enzyme activity. This Account will describe the concepts involved in designing chemical probes and libraries for comparative enzyme screening and drug discovery applications, as well as highlight how these technologies are changing the way in which enzymes may be rapidly profiled and characterized.

Introduction

Sequencing of the human genome in 2004 is widely regarded as an important milestone in our journey toward self-discovery.[1] Genomic blueprints have begun to provide the vocabulary but not the language by which to interpret life and living processes. This functional gap may be bridged in part through identifying the roles of enzymes and mapping out their interactions. Enzymes are biocatalysts intimately involved with virtually every cellular process and metabolic exchange. They hence represent key proteins that are not only instrumental in sustaining life but also implicit to its regulation and diversification. Minor

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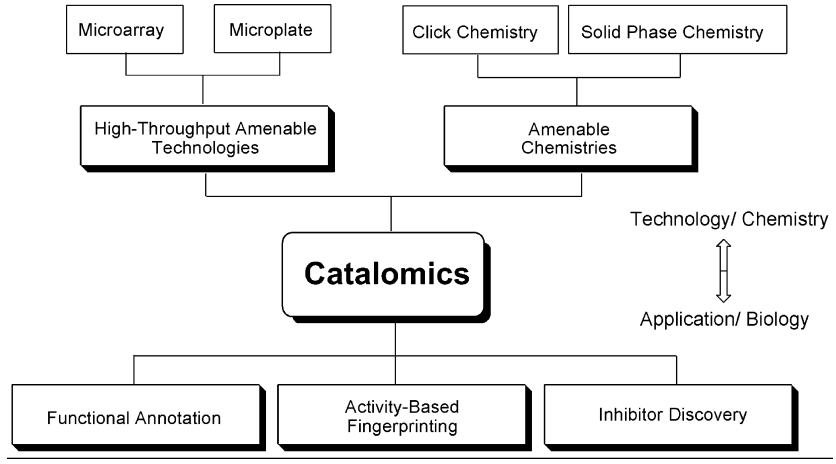

FIGURE 1. Overview of catalomics.

imbalances in enzyme activities, either through mutations, expression changes, or regulatory dysfunction, are known to cause debilitating diseases and even promote cancer or tumor metastasis.[2] Similarly, pathogenic organisms may be targeted by exploiting the uniqueness of their enzymes and metabolic processes. Consequently, it comes as no surprise that nearly one-third of all current drug targets are enzymes.[3]

There is hence a pressing need to elucidate the subtle differences that make each enzyme unique. A detailed understanding of the molecular configurations that are recognized and accepted by an enzyme active site facilitates not only the design of potent and selective inhibitors but also discovery of its biological function and downstream targets.[4] The long-term challenge to perform this task in an efficient and high-throughput manner is enormous, especially when considering the hundreds and thousands of enzymes across different genomes that still remain to be characterized and annotated.

Cohesive strategies that combine approaches in synthetic chemistry and biological screening offer new solutions that are quickly changing the way in which enzymes may be rapidly profiled and characterized. We describe such strategies within a unifying framework (we call it "Catalomics"), which involves the systematic use of chemical proteomic approaches in high-throughput enzyme characterization.[5] This emerging field draws on advances in enabling chemistries (including combinatorial chemistry,[6] fragment-based assembly,[7] and click chemistry[8]) and high-throughput screening (through the use of microplates[7] and microarrays[9]), which are geared toward applications in drug discovery and functional proteomics (Figure 1). The well-conceived application of such platforms can be quite informative, especially in unraveling the minute functional differences across members of a closely related enzyme class. These approaches hence strongly complement efforts in structural proteomics, *in silico* drug discovery, and the use of quantitative mass spectrometry in enzymology.[10−12]

The overall strategy is heavily influenced by the class of enzymes being tested and their associated catalytic function. Kinases, for example, add a phosphate group onto a hydroxyl-containing side chain (namely, that of serine, threonine, or tyrosine) within a cognate amino acid sequence of a target protein. Phosphatases reverse this process by removing the phosphate group. The interplay between these two groups of enzymes gives rise to the dynamic signal transduction pathways in eukaryotic systems used for inter- and intracellular communications. Proteases on the other hand hydrolyze the peptide bond linking amino acids in proteins. This may serve several functions, including protein localization, trafficking, and export or roles in digestion and protein turnover. There are over 518 kinases in the human kinome,[13] 1036 proteases in the degradome,[14] and 180 phosphatases in the phosphatome.[15] It has been a significant challenge to globally study the intricacies among these, as well as the many other, classes of enzymes.

In this Account, we will describe the design and application of catalomic approaches for studying kinases, phosphatases, and proteases and examine the unique solutions that such approaches can provide. We will first describe the enabling chemistries that facilitate rapid molecular assembly. We will then highlight the key technologies developed to accelerate the biochemical screening of various classes of enzymes. Finally we will discuss some important biological findings we have recently uncovered, as well as some future directions of this young and exciting field.

Enabling Chemical Strategies

Of the various methods available to screen enzymes, microarrays have emerged as a powerful and versatile tool that is

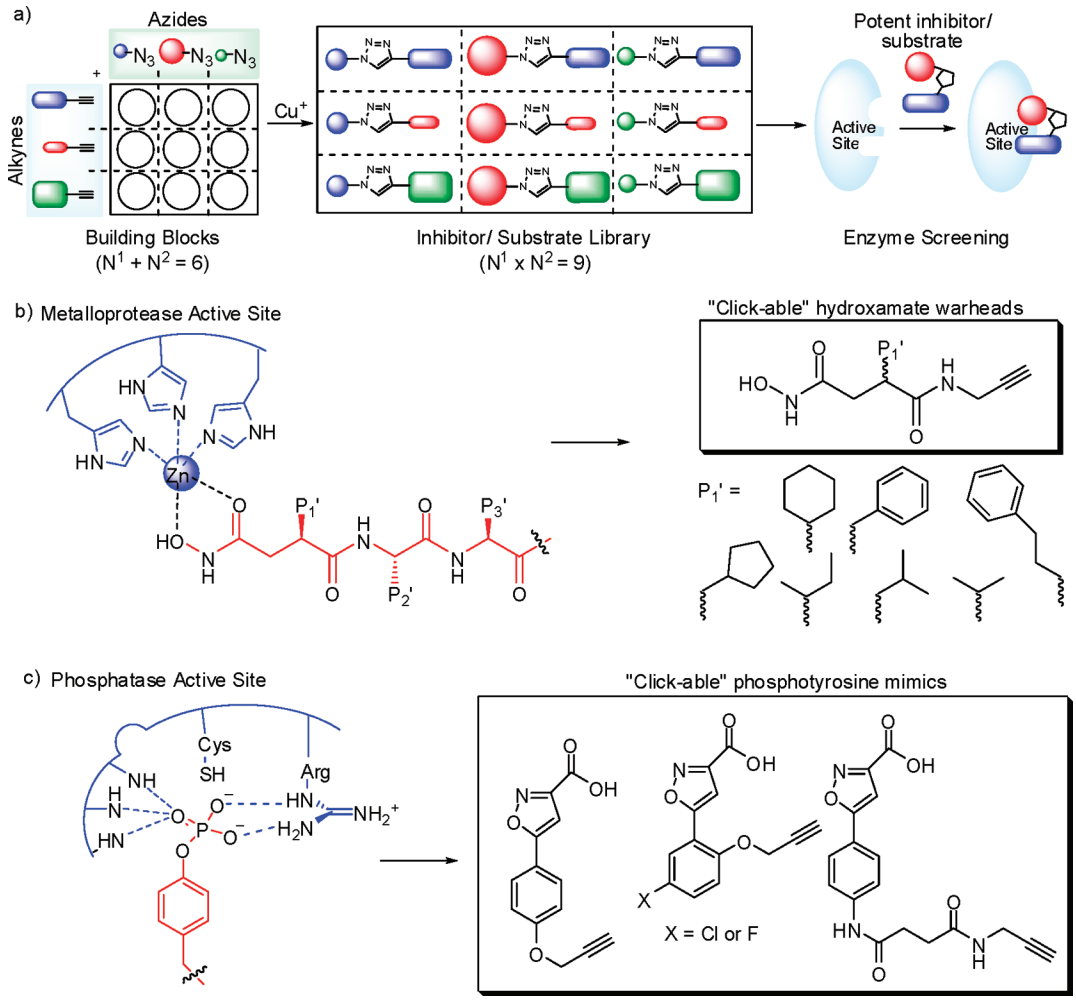

FIGURE 2. Overview of high-throughput amenable chemistries from the fragment-based assembly concept: (a) rapid assembly of bidentate substrate or inhibitors using "click" chemistry; (b) hydroxamate-based peptide inhibitors bound to the active site of a metalloprotease (left), and alkyne-containing core groups used in "click-based" metalloprotease inhibitors (right); (c) phosphotyrosine (*p*Tyr)-bound phosphatase active site (left), and cell-permeable and "click-able" *N*-phenyloxamic acid analogues that mimic *p*Tyr (right).

compatible with many of the traditional solution-based enzyme bioassays. Microarray technology also offers a variety of advantages over conventional screening platforms, in its ability to miniaturize, parallelize, and automate screening, making it possible to perform thousands of enzyme assays simultaneously.[5] The challenge hence is to create high-quality diversity-oriented or target-oriented compound libraries for rapid enzyme profiling. One of the key considerations in the fabrication process is to ensure that compounds produced are sufficiently pure (generally >80−90% purity is desirable) for direct *in situ* screening.[8] It would impose too great a burden (in terms of time, labor, and cost) to purify every product from a combinatorial library before the actual screening process. Suitable chemistries, including those that make use of fragment-based approaches (including click chemistry and amide-forming reactions) and solid-phase synthesis, therefore need to be carefully designed and optimized. For the majority of applications, the identities of library members should also be known *a priori* before the screening process. This may be achieved through the use of an encoding strategy or a systematic approach for compound identification. The fabrication of microarray-based libraries also requires the introduction of compatible tags and linkers for immobilization onto solid support. Such synthetic considerations and associated examples are discussed below.

Fragment-based approaches facilitate the assembly of large libraries of diverse molecules (totaling $N^1 \times N^2$) from smaller numbers of building blocks (totaling $N^1 + N^2$), where N is the number of each type of building block (Figure 2a).[16] "Click chemistry", a term coined by Sharpless et al., is one such fragment-based tool that is becoming increasingly popular because of its ease of use and high efficiency.[8] One important "click" reaction is the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction between azides and terminal alkynes.[8] This

makes use of a biologically orthogonal reaction, which is both efficient and specific, meaning that the reaction may proceed even in the presence of complex biological specimens or extracts. High yields (of up to ~100%) are frequently achievable using this type of click reaction. The Cu(I)-catalyzed "click" reaction between an alkyne and an azide was applied in the synthesis of both affinity-based probes and inhibitors for metalloproteases (Figure 2b) by our group,[17,18] as well as by Cravatt and co-workers.[19] Using the hydroxamate warheads as a zinc binding group (ZBG), we were able to conveniently install a rhodamine reporter and a benzophenone moiety in a modular manner to generate a metalloprotease-targeting probe library that was tested *in vitro*.[17] Cravatt et al. developed mixture-based hydroxamate probe libraries for the identification of metalloproteases within complex cell lysates.[19] Besides the hydroxamic acid ZBG, click chemistry was applied to incorporate rhodamine-based ZBG inhibitors.[20] The 1,3-dipolar cycloaddition chemistry has also applied been applied in the synthesis of inhibitors against many other enzymes, including caspases and phosphatases (Figure 2c).[16,21−23]

Another important reaction is the amide-bond formation between an amine and a carboxylic acid. The reaction is highly efficient and generates desired products with quantitative yields, hence facilitating modular fragment based assembly and *in situ* screening.[7] Wong et al. have successfully applied this in solution phase for enzyme inhibitor discovery.[24,25] The *in situ* solution-phase approach is convenient and easy to implement but may introduce byproduct that affects the biological screening, is not amenable to multistep reactions without purification, and cannot always be driven to completion through use of excess starting materials. To overcome these issues, we have recently developed an alternative solid-phase approach.[26,27] It was successfully applied in the development of a panel of phosphatase inhibitors. Commercially available 4-formyl-3-methoxyphenoxy (FMP) resin was used to capture various amine-containing inhibitor fragments by reductive amination, followed by attachment of the phosphatase targeting warheads, in this case, the isoxazole moiety that targets phosphatases like PTP1B, which is implicated in diabetes, obesity, and cancer.[28] This traceless method, coupled with directed sorting, was adopted in the synthesis a 249-member library of azidomethylene cysteine protease inhibitors.[27]

The design of chemical libraries for microarrays demands unique design considerations. With few exceptions, the bulk of peptide/small molecule microarray libraries are synthesized cleanly before attachment onto the microarray, and immobilized through the use of linkers and tags (*vide infra*).[29] Optimized and efficient chemistries, frequently on solid support, are in general used in library synthesis in order to ensure high product purity. It is thus not advisible to create libraries through extensively long synthetic routes that may compromise final product quality. The Schreiber group has developed and pioneered various approaches of combinatorial library design and synthesis for microarray fabrication.[9,30] This has included diversity-oriented synthesis to generate structurally unbiased libraries as well as one-bead−one-compound split−pool approaches.[31] In these early approaches, the identity of the molecules was not known in advance, and only upon hit identification at the end of biological assay was the molecule identified (usually through mass-spectrometry of the stock solution). We adopted a combinatorial synthetic strategy in the fabrication of a library of 2688 triazines that allowed the identification of all library members prior to the immobilization process on microarrays.[32] More recently, radio frequency (R_f)-tagged microreactors and other encoding systems have made it more convenient to synthesize large libraries for microarrays where all the molecules may be identified and known before the spotting process.[33−35]

Developing Technology Platforms

Drawing away from classical hypothesis-driven approaches, where investigators dedicate their efforts to studying individual genes and proteins, catalomics and other systems biology approaches have opened up avenues for comprehensive investigations across wide protein/enzyme classes. To a large extent, this has been facilitated through high-throughput screening platforms like the microarray that has opened up inroads for rapid enzyme profiling and inhibitor discovery. The success of automating organic synthesis and combinatorial chemistry has relieved the bottleneck in probe and library creation and shifted the impetus to the development of effective methods to screen the hordes of molecules now becoming available.[36] A variety of hurdles have been overcome, both in the design of linkers and chemistries to attach proteins, peptides, and other small molecules onto treated glass surfaces to generate high-density microarrays and in the development of efficient methodologies for systematic profiling of enzymes.[36,37] Here we describe strategies to illustrate how microarrays may be applied to elucidate enzyme activity spectra *en masse*.

Microarray Fabrication. One of the most critical steps in any microarray project is perhaps the decision over the way in which the molecules will be immobilized on the array. This would govern the types of tags and linkers that would have to be incorporated in the library during synthesis and the type

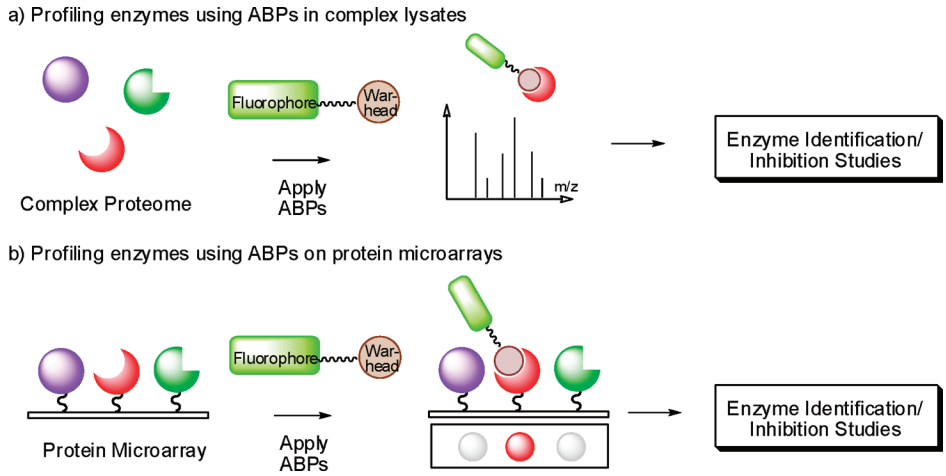

FIGURE 3. Activity-based probes (ABPs) for functional annotation and inhibition studies of enzymes: (a) application of ABPs in complex cellular lysates; (b) profiling enzymatic activity on protein microarrays using ABPs.

of microarray slide coating required. Efforts over the past decade have introduced a plethora of options for the immobilization of biomolecules onto microarrays. Covalent immobilization is usually preferred, and again there are a variety of options now available. Coated slides (*N*-hydroxysuccinimide, epoxy, aldehyde) are available from commercial vendors that immobilize molecules containing primary amines. By this method, peptides, proteins, or molecules that contain more than one amine group (for example, side chain lysine residues) may hence be heterogeneously displayed on the array, which is not always ideal. There is hence a need for tags and complementary surfaces that facilitate site-specific immobilization of proteins and peptides. This has involved the use of complementary tag pairs on molecules and arrays, for example, through Cu(I)-catalyzed 1,3-dipolar cycloaddition (alkyne/azide), Staudinger ligation (phosphane/azide),[38] and native chemical ligation (amino-terminal cysteine/thioester).[39] Noncovalent interactions have also been applied through the use of avidin/biotin interactions,[40] hexahistidine tags/Ni-NTA, and GST/anti-GST antibody interactions,[41] though these strategies are more commonly applied in the fabrication of protein/proteome microarrays. The ideal tag should facilitate stable, irreversible immobilization on the microarray surface, be easily introduced into the protein/peptide/small molecule, and not jeopardize its biological activity. These strategies have been reviewed extensively elsewhere, and more detailed interest is referred therein.[36,37,39]

High-Throughput Functional Annotation. Microarray platforms are beginning to fuel exciting opportunities for characterizing enzyme activities and facilitating protein annotation. With whole proteome microarrays reaching the consumer markets,[42] several groups have developed a variety of tools and methods by which a proteome's enzymatic activity may be queried in high throughput. Among these are tools based on activity-based probes (ABPs), which have been extensively developed and applied by the groups of Cravatt and Bogyo in gel-based proteomics.[43,44] Activity-based probes are chemical tools that are able to bind a distinct enzyme class in an activity-dependent manner within a complex biological milieu, even in the presence of other proteins (Figure 3a). This is achieved using a specially designed warhead that is only responsive to active enzymes and not the inactive or zymogen (precursor) forms.[45] The enzyme acts on the warhead and triggers a series of chemical conversions, which results in the probe being covalently and irreversibly bound to the enzyme. Such probes are hence also considered mechanism-based suicide inhibitors of enzymatic activity. A reporter group like a fluorescent label or a biotin handle is frequently included in the probe design to facilitate visualization of positive labeling. One unique application of ABPs was to probe enzymatic activities in high throughput on a protein microarray platform.[46] We first demonstrated this concept against a panel of phosphates, cysteine proteases, and serine proteases (Figure 3b).[46] More recently Miyake et al. have applied ABPs to quantitatively characterize the probe/inhibitor binding affinities of enzymes on microarrays.[47] A similar strategy was adopted to profile cysteine proteases on microarrays using a panel of 20 vinyl sulfone-based probes.[48] Compared with the solution-phase approach where enzymes within diverse proteomes may be labeled with ABPs and later separated using gels, activity based profiling on microarrays requires first the immobilization of purified enzymes on the microarrays and subsequent screening using ABPs.

Our group pioneered "expression display", a strategy where an ABP was used to probe desired enzymatic activities from protein libraries expressed by ribosome display, followed by

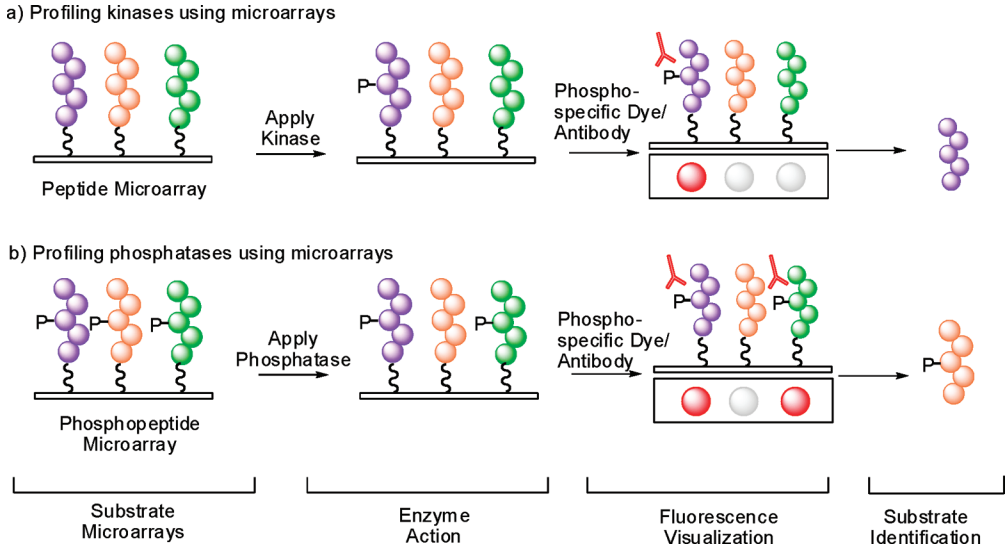

FIGURE 4. Substrate microarrays for high-throughput enzyme screening, detecting the activity of (a) kinases and (b) phosphatases using peptide microarrays. Through the use of diverse peptide libraries on microarrays, the profiles obtained reveal the enzyme−substrate spectrum.

high-throughput decoding using DNA microarray.[49] Ribosome display enables cell-free expression of a large pool of proteins, each tagged with its encoding mRNA through the ribosomal complex. The protein library is then incubated with biotinylated ABPs, followed by selective capture on avidin probes. The identity of the captured probe-bound proteins are revealed upon RT-PCR amplification of the tag mRNA(s), followed by hybridization/decoding on a DNA microarray. The four enzymes of interest, in this case protein tyrosine phosphatases (PTPs), were successfully isolated and identified from a mixture of 400 diverse proteins. This method represents a valuable proteomic tool, by possibly allowing all the proteins in a proteome to be displayed and screened for desired enzymatic activities. Similarly, Sieber et al. have shown that ABPs may be also used to label enzymes within a complex proteome, which may thereafter be identified using an antibody array.[50]

Enzyme Substrate Profiling Using Microarrays. A range of downstream applications have been developed to leverage the throughput of substrate microarrays to profile enzymatic activity. In an early demonstration of this concept, we coated fluorogenic small molecule coumarin derivatives on microarrays as sensors for the rapid characterization of four different classes of hydrolases.[51] Such coumarin-based substrates have also been used to profile the substrate specificity of proteases on peptide microarrays.[52] Diamond et al. also developed a droplet-based method using microarrays to assay the substrate specificity of serine proteases in which fluorogenic substrates and enzymes are sprayed in aerosol form onto the slides.[53] More recently, alternative surfaces have been developed for droplet-based enzyme profiling.[54−56] With use of slides coated with fluorogenic substrates, activity-dependent profiles of proteases and phosphates may be obtained in nanoliter-sized droplets in a time- and concentration-dependent manner.[54] This method was applied to screen for inhibitors of metalloproteases, namely, thermolysin and collagenase, using nearly 150-fold less substrate in comparison to the traditional microplate method.[57] Winssinger et al. developed an alternative approach that applies DNA microarrays for deconvolution of PNA-encoded libraries to profile the activities of proteases.[58]

Apart from hydrolases, methods have been developed to screen the substrate specificity of kinases using peptide microarrays.[59,60] The use of fluorescently labeled anti-phosphoserine and anti-phosphotyrosine antibodies or phospho-specific dyes precluded the need for radioactive methods to detect kinase activity (Figure 4).[61,62] We studied the use of combinatorial peptide libraries to obtain the activities of a kinase p60-Src, demonstrating the possibility of using such peptide libraries to screen for kinase substrate specificity.[62] Phosphopeptide libraries have also been fabricated and immobilized on microarrays to test the activities of phosphatases, in assaying their ability to remove the phosphate group from specific amino acid sequences (Figure 4).[63,64]

High-Throughput Enzyme Fingerprinting. Well-designed ABPs and substrate/inhibitor libraries provide the resources to begin the process of probing the functional differences across a specific class of enzymes. Assays may be designed for microtiter plates and microarrays for inhibitors and substrates, which are frequently optimized to save on the

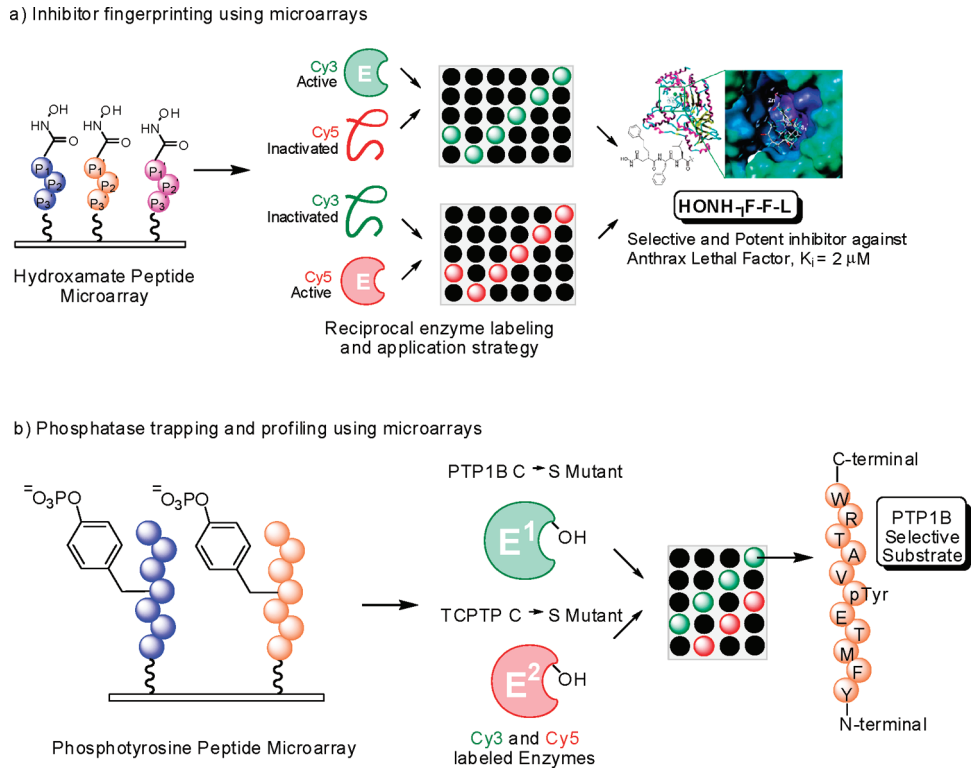

FIGURE 5. Biological applications and discoveries: (a) a 1400-member hydroxamate peptide microarray was used to obtain activity-dependent binding profiles of metalloproteases using a dual-color application method; (b) substrate-trapping mutants of PTPs (with cysteine in the active site replaced with serine) were labeled in two channels and applied on phosphopeptide microarrays to identify selective phosphatase substrates.

quantities of the often costly purified enzymes needed for screening. Fluorescence-based techniques offer a sensitive means of detecting binding and enzyme activities. Once established, these techniques may be applied routinely to test newly discovered enzymes as well as compare the activities of enzyme variants, both within and across species. These initial methodologies have led to the adoption of the concept of protein fingerprinting, which has had significant impact on functional protein differentiation. Such specificity screening should be useful at the outset of drug discovery programs to reduce hit attrition due to nonspecific binding effects. Here we will highlight several strategies that have been developed for this purpose.

Mihara and co-workers were among the earliest to develop protein fingerprinting approaches using immobilized peptides on microarrays.[65,66] A 112-member α-helical peptide microarray was used to distinguish seven proteins.[66] In another example, Kodadek and Reddy developed high-density microarrays comprising 7680 octameric peptoids, which were applied in the large-scale protein fingerprinting of three model proteins.[67] In this case, the library was designed in a manner in which the identities of the library members remain unidentified, preventing functional binding data to be elucidated from the protein binding profiles. We have performed high-throughput analysis of metalloproteases using a library of 1400 hydroxamate inhibitors to elucidate functional enzyme binding fingerprints (Figure 5a).[34] In our case, the identity of each molecule immobilized on the microarray was known *a priori*, as synthesis was carried out using radio-frequency tagging. Similarly, microplate screens were carried out on seven human matrix metalloproteases (MMPs) from all five known MMP classes against this 1400-member hydroxamate library to yield fingerprints of enzyme type.[33] This led to the identification of inhibitors from within the library that were specific to MMP-7 and MMP-13.

Biological Applications and Discoveries

One of the greatest challenges with microarray-based experimentation is differentiating true functional interactions from nonspecific binding. Frequently a large number of hits are generated on microarrays, but only a handful of them may be true activity-dependent binders. In order to address this problem, we have become more focused in the design of the libraries that are immobilized on the arrays, ensuring that they would bind or exert activity-dependent interactions with the enzymes of interest. Next in order to ensure that the signals

observed are truly activity-dependent, we have established a two-color microarray system where one channel is active and one channel is denatured, allowing activity-dependent measurements to be elucidated clearly within the same microarray slide. We have applied this method to fingerprint and discern four different metalloproteases: thermolysin, collagenase, carboxypeptidase, and anthrax lethal factor (ALF).[33] This has led to the identification of micromolar inhibitors that are specific to an enzyme of interest (e.g., ALF) and not to the other related metalloproteases (Figure 5a). Taking the two-color strategy one step further, we compared closely related enzymes using microarrays. Across slide comparisons can be a great challenge because of slide-to-slide variations; hence comparing two proteins on the same slide allows actual functional differences to be more readily elucidated. A 1000-member fragment-based phospho-serine/threonine heptapeptide library was generated to screen the activity of the family of seven human 14−3−3 proteins on microarrays, which are important cellular regulators that bind to phosphoserine-containing proteins.[35] The 14−3−3 proteins are very closely related, and it has been a challenge to identify their unique target specificities. The microarray-based two-color screening method revealed a novel sequence that bound 14−3−3 Sigma, which is a protein implicated in tumorigenesis. MacBeath et al. have recently also developed a method for the quantitative determination of binding constants on microarrays through concentration-dependent protein application, which has made it possible to obtain quantitative binding data from microarray experiments.[68] This was applied to study PDZ binding domains and to establish protein interaction networks.

Separate strategies have also been developed for screening the activities of serine and threonine phosphatases (PP2A, Lambda, PP1) using microarrays.[63] A phospho-specific dye was used to detect the signal decrease from the phosphopeptide library as a result of dephosphorylation by phosphatases. Distinct phosphatase profiles were obtained using a panel of 89 different phosphopeptides. Unique profiles were further obtained upon co-incubation of a phosphatase, PP2A, with a potentially regulatory peptidyl-prolyl isomerase Pin1, demonstrating that this strategy is applicable in studying the regulatory role of different proteins within the signal transduction cascade. This led to the discovery that the dephosphorylation of a protein involved in apoptosis (Bcl-2) is regulated by both Pin1 and PP2A.

We recently developed a "substrate trapping" peptide microarray that facilitated the substrate screening of putative protein tyrosine phosphatases (PTPs) and determination of the potent and specific binders of different PTPs.[69] This was achieved by replacing the conserved active site cysteine residue with serine to generate a substrate-trapping mutant that retains the substrate recognition property but loses its dephosphorylation ability. As a result, the enzyme is linked with its substrate of choice. Using dual-channel dye labels, we compared the substrate binding profiles of phosphatase mutants and were able to discern very subtle differences in the substrate binding of two closely related phosphatases (i.e., PTP1B and TCPTP; see Figure 5b). A putative peptide sequence derived from mitogen kinase was found to preferentially bind PTP1B over TCPTP. This may have potential therapeutic implications.

Conclusion

High-throughput screening has been used extensively, but not always as systematically. As showcased herein, purposeful screening initiatives can yield valued insight beyond the conventional "hit" or "lead" for drug discovery, to reveal an in-depth picture of the enzymatic profiles and activity spectra. Using examples of kinases, phosphatases, and proteases, we have described ways in which high-throughput approaches may be applied to chart the intrinsic properties of enzymes. Gradual integration and assimilation of these empirical data sets in public databases could facilitate the future prediction and simulation of the biological effects of molecular interactions *in silico*. We hope such catalomic strategies will inspire future innovation in the development of novel chemical entities and chemical proteomics tools that target various other enzyme classes. This will build the biological understanding of all the enzymes in our genomes and those of other organisms to advance drug discovery, pharmacology, and medicine.

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FOOTNOTES

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