

Functional profiling of the proteome with affinity labels

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The analysis of proteomic samples with affinity labels has been firmly established as a tool for the post-genomic researcher. Recent examples highlight the advantages of profiling functionally active members of specific protein families to identify therapeutically relevant protein targets that have escaped normal physiological regulation leading to increased or decreased activity. This dysregulation may result from any number of biological changes that modulate a protein's activity; for example, post-translational modifications of the protein or an imbalance between the protein and its endogenous inhibitor(s). By providing a direct measure of a protein's functional activity, affinity probe analysis identifies these changes and allows investigators to focus their research efforts upon those proteins that are most likely to be responsible for the biological changes under evaluation.

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Abbreviations

PBP penicillin-binding protein

PTP protein tyrosine phosphatase

Introduction

With the human genome project completed, researchers have turned their attention to an even greater challenge: evaluating protein abundance, interactions and function in biological systems. Numerous strategies have been employed in this quest, including two-dimensional gel electrophoresis followed by mass spectrometry [1], direct mass-spectrometric analysis [2], yeast two-hybrid screens to catalogue protein–protein interactions [3], protein microarrays [4], and abundance-based chemical approaches [5**]. A relatively new approach is to use affinity labels to profile proteins on the basis of their function in biological systems. In the simplest sense, an affinity label consists of a recognition element that is capable of forming a reversible complex with a protein, and a properly positioned reactive group that reacts with the protein converting the reversible complex into an irreversible protein–ligand complex.

Historically, affinity labels have been designed to label a specific protein in a biological sample to profile the specificity of the probe and to determine the localization of a protein target in tissue samples. With the advent of improved mass-spectrometric instrumentation and computational algorithms that utilize genomic databases to identify proteins, investigators are now beginning to use affinity probes to interrogate protein families rather than individual proteins in biological samples. This review summarizes recent developments in proteome evaluation with affinity probes and highlights applications and information provided by the technique that is unique to this approach.

Hydrolase family

Hydrolases represent one of the largest and most diverse enzyme families, as evidenced by the fact that it comprises approximately 4% of the human genome [6] with the majority distributed amongst serine (32%), cysteine (23%) and metalloproteases, with aspartyl making up the remaining 3–5%. Of the known human proteases, approximately 14% were under active investigation as drug targets between 1998 and 2000 [7] with individual enzymes in these classes implicated in numerous disease states including emphysema [8], diabetes [9] and cancer [10]. A common mechanistic feature of cysteine and serine hydrolases, exemplified by the serine hydrolase family, is a catalytic triad consisting of aspartic acid, histidine and serine that modulates the reactivity of the serine hydroxyl group by shuttling a proton between the aspartic acid and serine residues via the imidazole/imidazolium group of the histidine residue. Substrate ester or amide hydrolysis occurs with transfer of the substrate acyl group to the activated serine followed by deacylation of the protein and transfer of the acyl group to a water molecule. Cysteine proteases utilize an analogous reaction mechanism with cysteine in place of the active-site serine. Numerous affinity labels have been developed to capitalize on this enhanced nucleophilicity to preferentially label serine and cysteine hydrolases in biological samples.

Serine hydrolases

Peptidyl chloromethyl ketone affinity probes (1, Table 1) have been used to discriminate between active and inactive serine proteases [11], to label serine proteases involved in blood coagulation [12] and to monitor serine protease activities during apoptosis [13]. In all of these examples, subsite recognition elements were incorporated into the affinity label to yield probes that were specific for the protease under investigation. Researchers in the Cravatt laboratory developed affinity probes that would label the entire serine hydrolase family rather than

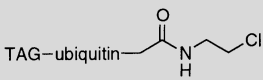
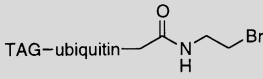
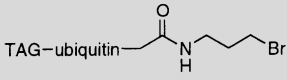
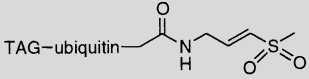
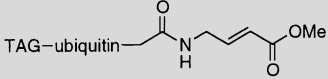
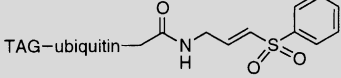
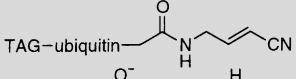
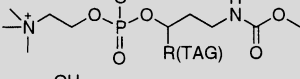
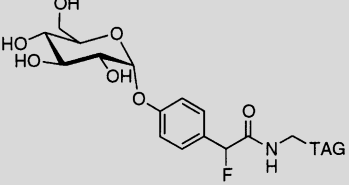
specific subsets. Taking advantage of the ability of fluorophosphonates to label serine hydrolases by phosphorylating the active site serine hydroxyl group (Figure 1)[14] they prepared an affinity probe consisting of a fluorophosphonate and biotin linked together by a polyethylene glycol or alkyl chain (2) and evaluated its ability to label serine hydrolases in biological samples [15].

Labeling profiles of various rat tissues demonstrated that the probe labeled serine hydrolases down to subnanomolar concentrations in complex biological samples and that significant differences in labeling profiles were observed depending upon tissue origin. Furthermore, labeling was dependent upon the functional state of the enzyme as heat-denatured enzymes (disrupts the

Table 1
Examples of affinity labels.

	Affinity probe	Protein family	References
1		Serine protease	[11–13]
2		Serine hydrolase	[15,16,17**,18**]
3		Cysteine protease	[23–25]
4		Cysteine protease	[26**,27]
5		Tyrosine phosphatases	[31*,32]
6		Aldehyde dehydrogenase	[35]
7		Thiolase NAD/NADP-dependent oxidoreductase Enoyl CoA hydratase Epoxide hydrolase Glutathione S-transferase	[36**]
8		Penicillin-binding proteins	[37]
9		Kinases (IKKβ)	[38]
10		NF-κB and unknown target	[39]
11		Unknown	[40]

Table 1 Continued

	Affinity probe	Protein family	References
	TAG-ubiquitin 		
	TAG-ubiquitin 		
	TAG-ubiquitin 		
12	TAG-ubiquitin 	Deubiquitinase	[41]
	TAG-ubiquitin 		
	TAG-ubiquitin 		
	TAG-ubiquitin 		
13		PAF-acetylhydrolase	[42]
14		Glucosidase	[43]

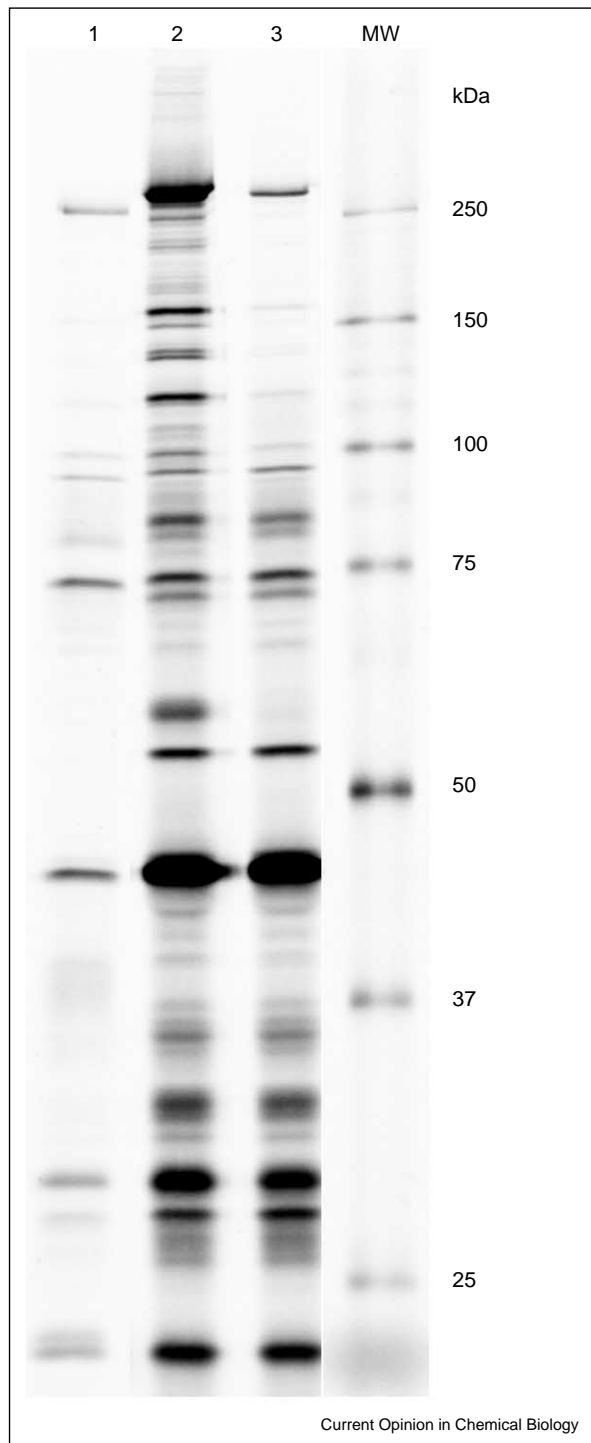
catalytic triad), zymogens and proteins bound to endogenous or small molecule inhibitors were not labeled. Consequently, the researchers were able to directly profile active serine hydrolases in biological samples, an important capability considering that the majority of drugs elicit a therapeutic effect by interacting directly with active proteins. The probe was also used to evaluate the potency and selectivity of trifluoromethylketone-based fatty acid amide hydrolase (FAAH) inhibitors directly in complex biological samples to identify off-target activities and guide the development of more selective inhibitors [16].

Further improvements in probe design were achieved by incorporating a fluorescent tag into the probe [17^{••}]. Although biotin enabled avidin-based isolation of probe-labeled proteins and could be visualized with avidin/horse-radish peroxidase and chemiluminescent substrates, compared with fluorescence-based methods, the sensitivity, dynamic range, analysis throughput, and complications arising from endogenously biotinylated proteins were

limiting. The fluorescently tagged probes are greater than 100-fold more sensitive than biotin-conjugated probes and enable direct in-gel fluorescence analysis. The authors also described affinity columns containing anti-fluorophore antibodies that enable capture and purification of probe-labeled proteins from biological samples to facilitate mass spectroscopic identification.

Jessani and colleagues evaluated serine hydrolase activity profiles of secreted, membrane and soluble fractions from eleven breast and melanoma cancer cell lines [18^{••}]. Activity profiles for the different cell lines varied substantially with respect to both the intensity of labeled proteins and the presence or absence of numerous proteins. Hierarchical clustering of the combined labeling profiles for all three fractions identified a set of serine hydrolase activities that segregated the cancer cell lines into three groups, melanoma, breast and invasive. More detailed analysis of the individual fractions revealed that the secreted and membrane fractions were the major contributors to the observed clusters, with minimal

Figure 1



Serine hydrolase labeling profiles obtained with the fluorophosphonate probe highlighting changes in hydrolase activity levels in the cytosol of pre-adipocytes (lane 1), adipocytes (lane 2) and adipocytes treated with a small-molecule inhibitor (lane 3).

contribution from the soluble fraction. Interestingly, the clusters that correlated with non-invasive melanoma or breast samples were down-regulated in the invasive

melanoma and breast samples and a different set of proteins were up-regulated, including urokinase and a novel membrane-associated enzyme, KIAA1363. This study provides functional proteomic evidence that the primary determinant of invasive cancer clusters may be cellular phenotype rather than tissue origin. The ability to identify dysregulated proteins in a biological sample with these activity-based serine hydrolase probes allows scientists to monitor protein targets that are more likely to be valid drug-discovery targets.

Cysteine proteases

Numerous irreversible inhibitors of cysteine proteases have been developed as potential therapeutics that target the active site cysteine residue. A wide range of electrophiles have been evaluated, including activated ketones, epoxides and vinyl sulfones, many of which have been utilized as affinity labels [19]. Early designs for affinity labels used inhibitors that were fairly specific, and attempted to determine the selectivity profile of an irreversible cysteine protease inhibitor in biological samples [20,21] or the activation of individual caspases in cells [22]. It is clear from this early work that, unlike the serine hydrolase family where the chemoselective, transition-state analogue fluorophosphonate affinity label provides coverage for the majority of family members, the cysteine protease family will require multiple probes incorporating differing subsite recognition elements to achieve broad coverage.

Recently, Bogoyo and colleagues have utilized probes based upon the peptidyl-epoxide natural product E-64 (3), to profile the cysteine protease papain family, focusing on cathepsins and calpains in particular. Affinity probes containing either biotin [23] or a fluorescent group [24] were prepared and used to profile inhibitor potency and selectivity in biological samples for inhibitor development and classification of cathepsins on the basis of small-molecule affinity fingerprints. The fluorescent E-64 probe was also used to evaluate proteolytic activities during cataract formation [25]. Lens-specific calpain Lp82 and m-calpain immunoblots revealed that protein levels of both are nearly equivalent in wild-type and cataractogenic lenses; however, only Lp82 activity correlated with cataract formation. In lenses undergoing cataract formation, E-64 probe labeling identified 62-kDa and 32-kDa calpains, resulting from proteolytic processing of Lp82. The spatial and temporal correlation of Lp82 and its processed forms with cataractogenesis suggest they are the principal cysteine proteases responsible for cataract progression.

Researchers in the Schultz laboratory have recently described an interesting approach that combines affinity labeling with high-density microarrays [26**]. Affinity probes consisting of peptidyl acrylamide inhibitors tethered to fluorescently labeled peptide nucleic acids

(4) were synthesized to enable the capture of probe-labeled cysteine proteases on glass slides derivatized with complementary oligonucleotide sequences. Caspase-3 activation was monitored in Jurkat cell lysates treated with granzyme B to initiate the apoptotic pathway [27]. After treating the lysates with probes in solution, and removal of free probe by gel filtration, the lysate was hybridized to the oligonucleotide microarray. Fluorescence image analysis correctly identified a known caspase-3 inhibitor and provided a measure of caspase-3 activity in the samples. This approach combines the ease of analysis and low sample volume requirements of high-density microarrays with the ability to generate libraries of small-molecule combinatorial libraries to create a platform that can be used to simultaneously screen multiple protein targets against inhibitor libraries. As described, the approach holds significant promise for inhibitor screening; however, a major challenge will be deconvoluting inhibition profiles when there is minimal inhibitor selectivity between proteins.

Protein phosphatases

The reversible process of protein phosphorylation and dephosphorylation is fundamental to many important cellular functions such as cellular signaling and cell growth. The dynamic interplay of protein kinases and phosphatases, which mediate the phosphorylation state of proteins, are therefore crucial for normal cell function. Amongst the protein phosphatase families, protein tyrosine phosphatases (PTPs) have received considerable interest since they were first identified in 1988. PTPs share a catalytic domain of about 240 amino acids containing the active site signature motif (H/V)C(X)₅R(S/T) [28]. The cysteine residue is activated in the enzyme active site, forming a thiophosphate intermediate during phosphotyrosine hydrolysis, and is an attractive target for active-site labeling. Indeed, the Dixon laboratory [29,30] took advantage of this increased reactivity to label the active site cysteine of rat LAR and *Yersinia* PTP by ¹⁴C-labeled iodoacetate.

Class-selective activity probes (5) for PTPs were developed by Lo and colleagues [31,32] from the mechanism-based inactivator 4-halomethylaryl phosphate, pioneered by Widlanski [33,34]. Phosphate hydrolysis of the 4-halomethylaryl phosphate results in formation of a quinone methide, a highly reactive alkylating agent that can react with nucleophilic amino acids at, or near, the phosphatase active site. The activity probes described by the researchers combined a dansyl fluorophore or a biotin group with 4-fluoromethylaryl phosphate. PTP1B-labeling kinetics was monitored by ¹⁹F NMR followed by gel analysis. Other hydrolases, such as trypsin and β -galactosidase were not labeled, although fairly high probe concentrations (submillimolar to millimolar) were used. The site of alkylation and selectivity of these probes has not been reported.

Chemotype affinity labels

Historically, affinity labels have been developed after a thorough understanding of a protein's mechanism has been achieved. Although this approach continues to be a fertile avenue for affinity label development, there are many protein families for which affinity labels or the mechanistic understanding to design new affinity labels does not exist. In an attempt to circumvent this limitation and recognizing that the active sites of enzymes often contain nucleophiles with enhanced activity that are involved in catalysis, Adam and colleagues evaluated screening combinatorial libraries containing a common chemotype against complex proteomes to identify novel affinity labels [35]. A library of biotinylated sulfonate esters was prepared and evaluated with rat testis to reveal that the probes had different labeling profiles. One concern with this strategy is differentiating protein labeling on the basis of abundance and intrinsic reactivity versus protein labeling on the basis of mechanism of action. To address this issue, the authors compared labeling profiles of heat-denatured and native proteomes, to discriminate between non-specific and specific protein reactivities on the assumption that proteins exhibiting heat-sensitive sulfonate reactivity were specific. Using this criterion, several specific protein reactivities were observed. Aldehyde dehydrogenase-1 was identified by mass spectrometric analysis as one of the proteins labeled by the pyridyl sulfonate probe (6).

An expanded evaluation of this library with additional biological samples identified six mechanistically distinct enzyme classes — thiolase, aldehyde dehydrogenase, NAD/NADP-dependent oxidoreductase, enoyl CoA hydratase, epoxide hydrolase and glutathione S-transferase — that were labeled by the phenyl sulfonate probe (7) [36**]. Breast cancer cell line profiles identified an omega-class glutathione S-transferase whose activity was significantly upregulated in oestrogen-negative cancer lines compared with oestrogen positive cancer lines.

Natural-product-derived affinity labels

An alternative source of affinity labels that is being evaluated by researchers is irreversible natural products. Many natural products have been identified that interact with specific protein families and covalently label nucleophilic residues within the active site. Researchers have begun attaching reporter groups to natural products to identify proteins that are covalently labeled. Utilizing these natural product probes at low concentrations allows researchers to identify the protein(s) that are preferentially labeled while higher concentrations reveal additional targets that are often functionally related.

Penicillin is one of the best-known natural products, the discovery of which led to the development of β -lactam antibiotics, the cornerstone of anti-bacterial treatments for the latter half of the 20th century. Emerging resistance

to β -lactam antibiotics by pathogenic Gram-positive bacteria has limited the usefulness of this important class of antibiotics. One resistance mechanism is the development of altered penicillin-binding proteins (PBPs) that have reduced affinity for the antibiotics. Zhao and colleagues described the application of a fluorescently labeled penicillin probe (**8**) to detect and characterize PBPs in bacterial cell membranes [37]. With this probe they were able to rapidly detect functionally active PBPs present in different bacterial strains and determine relative affinities towards different β -lactam antibiotics.

Researchers in the Crews laboratory have evaluated several natural products using this strategy. They developed an affinity probe (**9**) based on the natural product parthenolide, which contains an unsaturated lactone as a Michael acceptor [38]. The molecular target was identified as I κ K kinase β (IKK β) and presumably is responsible for the anti-inflammatory properties of parthenolide. Cys179 was confirmed as the residue modified by parthenolide by mass spectrometric analysis and loss of labeling when this residue was replaced with alanine. More recently, Crews reported biotinylated isopanepoxydone affinity probes (**10**) that target NF- κ B [39] and other unidentified proteins in HeLa cells. The same group also reported the synthesis of eponemycin derivatives as probes (**11**) to study the mode of action of this angiogenic compound in endothelial cell lysates [40]. At least two protein receptors with molecular weights of 23 and 25 kDa were labeled with the probe but have not been identified.

Other enzyme families

Novel members of the deubiquitinating enzyme family were identified in EL4 cells by labeling with specific probes (**12**) followed by SDS gel and mass analysis [41] of the tryptic fragments. In addition, deubiquitinating-like activities of other, novel proteases were identified. The probes contained seven different thiol-reactive groups, including ethyl chloro/bromo and vinyl sulfone/ester groups, attached to ubiquitin and an epitope tag. Labeling was carried out using whole-cell lysates of EL4 mouse thymoma cell line. The selectivity of the probes was greatly influenced by the type of electrophile (thiol reacting group) that was attached.

Phosphatidylcholines containing a carbamoyl ester group and pyrene fluorophore (**13**) were designed to label the plasma enzyme PAF-acetylhydrolase (PAF-AH) and study its cellular distribution in U937 cells [42].

Utilizing the same suicide mechanism that they successfully applied to tyrosine phosphatases, hydrolytic generation of a quinone methide, the Lo group attempted to develop probes for β -glucosidase (**14**) [43]. Although the authors were able to demonstrate that the probe was a substrate for β -glucosidase and labeled the protein, they observed significant cross labeling in proteomic samples.

The authors attributed this to the lack of appropriately positioned nucleophiles within the glucosidase active site and the ability of the reactive quinone methide to diffuse away from the enzyme and label other proteins non-specifically.

Future directions

The majority of affinity probes that have been applied to proteomic analysis target enzymes that have a catalytically activated nucleophile. The development of affinity probes for protein families lacking hyper-nucleophilic residues will continue to be an area of active research as some important therapeutic targets, for example kinases and receptors, fall into this category. Strategies exist for these families, for example, utilizing recognition elements that have a higher affinity for their protein targets; however, this increased binding affinity usually results in increased selectivity, thereby reducing the coverage provided by individual affinity probes. Although there are numerous examples of affinity labels reported in the literature designed to target specific receptors as pharmacological tools and to characterize G-protein-coupled receptors, they are not covered in this review as they provide limited coverage of the proteome [44–46]. It will be interesting to see if the application of these affinity agents can be extended to provide useful proteomic tools.

In addition to ongoing target identification and inhibitor profiling, future applications of affinity probe technology will also have an impact on target validation. By integrating discovery research teams around specific protein families, highly biased screening libraries will be used to generate potent leads. The potency and selectivity of these leads will be optimized directly in biological samples of interest using the probe as substrate, without requiring the expression and purification of the proteins under investigation, saving substantial time and resources. Suitable compounds will then be evaluated in appropriate biological models to validate the protein target.

Summary

In conclusion, the analysis of proteomic samples with affinity labels has been firmly established as a tool for the post-genomic researcher. Examples provided in this review highlight the advantages of profiling functionally active members of specific protein families to identify therapeutically relevant protein targets that have escaped normal physiological regulation leading to increased or decreased activity. This dysregulation may result from any number of biological changes that modulate a protein's activity; for example, post-translational modifications of the protein or an imbalance between the protein and its endogenous inhibitor(s). By providing a direct measure of a protein's functional activity, affinity probe analysis identifies these changes, information that is not provided by genomic or abundance-based proteomic analyses. This allows researchers to focus their research

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