Activity-Based Profiling of Proteases

Laura E. Sanman[1] and Matthew Bogyo[1,2,3]

Departments of [1]Chemical and Systems Biology, [2]Microbiology and Immunology, and [3]Pathology, Stanford University School of Medicine, Stanford, California 94305-5324; email: mbogyo@stanford.edu

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Abstract

Proteolytic enzymes are key signaling molecules in both normal physiological processes and various diseases. After synthesis, protease activity is tightly controlled. Consequently, levels of protease messenger RNA and protein often are not good indicators of total protease activity. To more accurately assign function to new proteases, investigators require methods that can be used to detect and quantify proteolysis. In this review, we describe basic principles, recent advances, and applications of biochemical methods to track protease activity, with an emphasis on the use of activity-based probes (ABPs) to detect protease activity. We describe ABP design principles and use case studies to illustrate the application of ABPs to protease enzymology, discovery and development of protease-targeted drugs, and detection and validation of proteases as biomarkers.

Contents

INTRODUCTION

The basic definition of a protease is an enzyme that hydrolyzes peptide bonds in proteins. Approximately 700 enzymes, or roughly 2% of the human genome, have known or putative proteolytic activity (1). Although all share the ability to catabolize proteins, these enzymes vary in almost every other parameter, including size, localization, and quaternary structure. For simplification, proteases are grouped into families on the basis of catalytic mechanism. In humans, there are five protease families: aspartyl, cysteine, metallo-, serine, and threonine proteases. Cysteine, serine, and threonine proteases use nucleophilic active-site residues to hydrolyze peptide bonds, whereas aspartyl and metalloproteases use active-site residues to activate water molecules for nucleophilic attack (2).

Proteolytic activity is central to many necessary biological processes, including development, differentiation, and the immune response (3). However, due to the irreversible nature of proteolysis, proteolytic activity must be highly regulated within cellular systems. Most proteases are translated as inactive zymogens that contain inhibitory prodomains that must be removed for the protease to become active. Proteases that are not synthesized with inhibitory prodomains often require cofactor binding or posttranslational modification for activation. After activation, endogenous protein inhibitors, changes in subcellular localization, and degradation can limit protease activity (**Figure 1**). Given the high level of posttranslational regulation, it is not surprising that measures of protease activity, rather than traditional transcriptomic or proteomic analyses, are often necessary to understand biological function (4). The purpose of this review is to highlight recent methods that allow dynamic measurement of protease activity in complex biological systems. We emphasize the relatively new, but rapidly growing, field of activity-based profiling, which has proven valuable for detailed analyses of proteases and other enzyme classes.

STRATEGIES FOR PROFILING PROTEASE ACTIVITY

The most direct method of tracking protease activity is monitoring hydrolysis of a substrate. Turnover of both natural protein substrates and substrate-based probes can provide a direct measure of protease activation events (5). The quantity of peptide fragments that are produced by substrate proteolysis reflects levels of protease activity, but these substrate fragments must be isolated from their native environment for identification and quantification. In contrast, substrate-based probes are reagents that are designed to directly generate a signal upon cleavage (6). Protease activity can also be indirectly monitored using activity-based probes (ABPs), which contain a substrate-like region but, rather than being processed into fragments, covalently bind to the active protease in an enzyme-catalyzed reaction (6–8). Thus, the extent of target protease modification reports levels of activity. Each of these strategies for determining protease activity has been applied in diverse biological systems, and each has its own strengths and weaknesses.

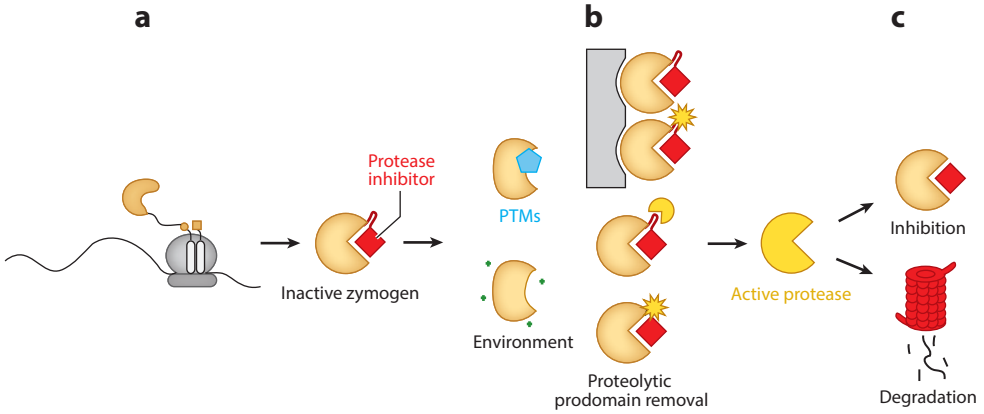

Figure 1

Proteases are often synthesized as inactive zymogens (*left*) that are activated by several mechanisms, including posttranslational modifications (PTMs), cofactor binding, pH change, and proteolytic prodomain removal (*center*). Prodomain hydrolysis (*yellow stars*) can occur at a macromolecular signaling complex (*top*), by the action of another individual protease (*middle*), or by autocatalysis (*bottom*). After activation, protease activity can be limited by endogenous inhibitors or by degradation by the proteasome (*right*).

Below, we discuss examples of applications and the relative merits of each strategy.

Natural Substrate Turnover

Active proteases cleave protein substrates. The most direct example of this process is autoproteolysis, during which a protease catalyzes its own processing to become active (9, 10). The simplest measure of turnover of individual substrates of interest uses sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to resolve proteins and fragments, followed by Coomassie staining or immunoblotting for protein visualization. However, this procedure requires a priori knowledge of the substrates to be measured. Alternatively, turnover of all protease substrates can be measured simultaneously by mass spectrometry (**Figure 2**). For example, in a technique named PROTOMAP, a proteome sample is prepared before and after addition of a protease enzyme. The samples are then resolved by SDS-PAGE, and the locations of all proteins and protein fragments are mapped in the gel by mass spectrometry. Changes in the migration of protein fragments indicate substrate proteolysis (11). Through the use of this method, many proteins can be simultaneously monitored. Gel-free enrichment methods for proteolytic peptides have also been improved, allowing for simultaneous identification and quantification of the majority of substrate hydrolysis events in a given sample (5, 12). These enrichments capitalize on the unique α-amino group exposed by substrate proteolysis. Proteolytic peptides can be enriched for by negative selection, as in the COFRADIC (13) and TAILS (14) methods, or by positive selection, as with selective α-amine biotinylation by subtiligase (15) or selective lysine capping using *O*-methylisourea followed by biotinylation of α-amines (16).

Increasing coverage of the proteolytic "peptidome" by use of gel-based and gel-free enrichment methods has facilitated systems-level monitoring of protease activity. The biological pathways into which protein substrates fall suggest protease function, and common cleavage sequences indicate substrate recognition motifs. Both have been useful for separating the biological functions of closely related proteases and advancing the design of selective substrate-based probes and ABPs. The following examples illustrate how methods

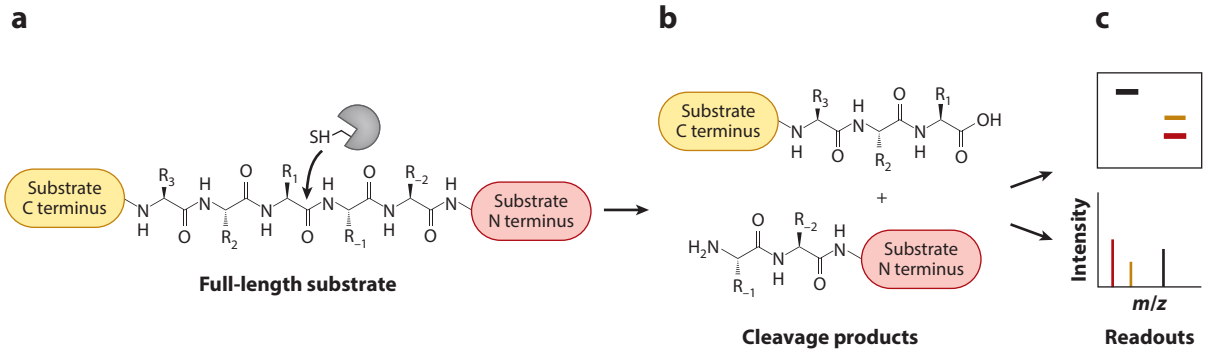

Figure 2

Cleavage of substrate proteins reports protease activation. (*Left*) A cysteine protease (gray; active-site sulfhydryl group represented by –SH) hydrolyzes an amide bond, releasing two polypeptides (*middle*). (*Right*) Potential measures of substrate truncation include (*top right*) immunoblotting or Coomassie stain as well as (*bottom right*) mass spectrometry. Abbreviation: *m/z*, mass-to-charge ratio.

of assessing natural substrate turnover have been used to characterize global proteolysis by a single family of proteases, the caspases.

Apoptosis, an immunologically silent form of programmed cell death, is an event initiated and executed by the caspase family of cysteine proteases (17). Because apoptosis is tightly regulated by proteolysis, many methods of dynamically monitoring protease activity, including measuring hydrolysis of natural substrates, have been used to study this process. Cleavage of Bid, an important regulatory protein that is cleaved by caspases during apoptosis, is a common measure of caspase activation (18). Although Bid is a useful reporter of cell death, it can be cleaved by multiple proteases (19). In addition, Bid cleavage occurs in only one of the many pathways that are regulated by caspases during apoptosis (17–19). To profile caspase activation comprehensively, a series of recent studies used subtiligase-mediated proteolytic peptide enrichment and PROTOMAP to identify and quantify ~1,300 putative caspase substrates in cells and cell lysates treated with recombinant caspases or caspase-activating agents (11, 20–23). These studies established that, during apoptosis, caspases cleave predominantly proteins involved in transcriptional regulation, chromosomal structure, and cytoskeletal maintenance (11, 20). Quantifying hydrolysis over time in cell lysates treated with recombinant caspases-3, -7, -8, and -9 also generated catalytic efficiency values (k_{cat}/K_D) for ~500 protein substrates. Small but important differences in substrate specificity among the caspases were determined (21) and used to identify substrates specific for caspases-3, -7, and -8. These studies demonstrate the value of systems-level analysis of proteolysis for providing information about the types of pathways that may be regulated by proteases and about protease substrate specificity. However, note that although such global methods produce many valuable leads, it is often difficult to validate an extensive list of substrates.

A major advantage of measuring protease activation by monitoring natural substrate turnover is that the methods are highly adaptable. In fact, mass spectrometry–based identification of proteolytic peptides has been used as a measure of protease activity in the clinic (24–26). For example, a recent proof-of-concept comparison of sera from prostate, bladder, and breast cancer patients showed that proteolytic peptide signatures can predict cancer type (24). A disadvantage of using substrate cleavage as a proxy for protease activity is that it relies on the assumption that only a single protease is capable of cleaving each substrate protein. This assumption is often not true, especially in large, closely related protease families such as the matrix metalloproteases (MMPs) (27),

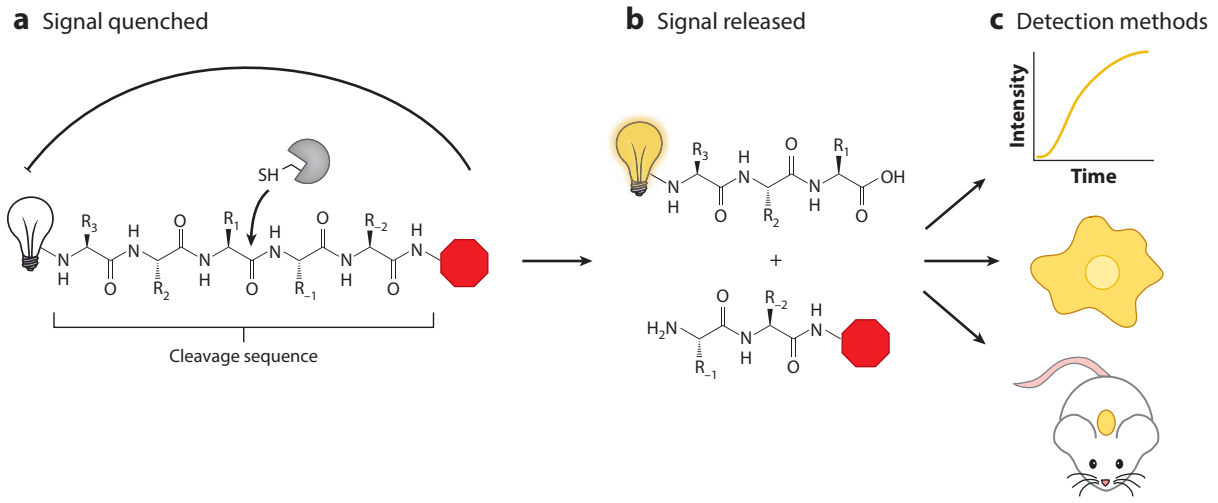

Figure 3

A sample substrate-based probe in which a fluorophore (*lightbulb*) and a quencher (*red octagon*) flank the substrate region. When intact, the probe is not fluorescent (*left*). When the protease is activated, the substrate is cleaved and fluorescence is released (*middle*). Detection of signal from substrate-based probes occurs by in vitro fluorescence measurements and by cell-based or noninvasive animal imaging (*right*).

cysteine cathepsins (9), and caspases (17), in which multiple proteases have highly similar substrate specificity profiles.

Substrate-Based Probes

To measure turnover of natural substrates, proteolytic peptides must be isolated and identified. Substrate-based probes also report on substrate hydrolysis, but without the need for isolation and characterization of the peptide products (6). Most synthetic substrate-based probes have two main components: a recognition sequence that binds in the active site of the protease of interest and a reporter that produces a measurable signal when the probe is cleaved. The design of the recognition sequence is critical because it is the main component of the probe that confers selectivity toward the target protease. This sequence is often designed on the basis of natural cleavage sequences or knowledge of substrate specificity obtained from cleavage patterns of artificial peptide libraries. The recognition sequence is coupled to a reporter of protease activity. The simplest example of a substrate-based probe is a fluorogenic probe in which a peptide is coupled to a fluorophore such as 7-amino-4-carbamoylmethylcoumarin (ACC) or *p*-nitroaniline (pNA). ACC and pNA are minimally fluorescent when attached to the probe but become fluorescent when cleaved from the substrate region (28, 29). Although fluorogenic probes are simple in design, probe selectivity is limited because the recognition sequence occupies only the nonprime side of the active site. Other types of substrate-based probes do not have this limitation and can have recognition sequences spanning both the nonprime and prime sides of the substrate-binding site (6, 28). For example, substrate-based probes can also be designed to produce a signal when a fluorescence resonance energy transfer (FRET) acceptor or fluorescence quencher (6) is cleaved from the substrate. This optical signal can be analyzed by plate-reader assay, fluorescence microscopy, and in vivo imaging (**Figure 3**). Recognition sequences flanked by bioluminescence resonance energy transfer pairs have also been described (30), as have gold nanoparticle–linked probes that change color upon processing by a protease (31).

Substrate-based probes have also been functionalized to increase their clinical applicability. For example, in a recently constructed caspase-targeted probe, the substrate region is flanked by a Gd^{3+} chelator and ^{19}F trifluoromethoxybenzylamide. This probe functions as a magnetic resonance imaging (MRI) contrast agent; the intact probe has a low ^{19}F MRI signal due to the paramagnetic effects of Gd^{3+}. Upon substrate cleavage, this quenching effect is lost and the probe provides MRI contrast (34). Substrate-based probes have also been tethered to so-called nanoworms that accumulate in diseased tissues. Protease-mediated cleavage releases a mass reporter that is secreted into the urine, which can be collected and detected by mass spectrometry (35).

There are several advantages to using substrate-based probes to profile protease activity. Various design possibilities exist due to the simplicity of the scaffold; anything that can be chemically linked to an amide or a carboxylic acid can be appended to the substrate. Substrate-based probes have also been genetically encoded through expression of a protease cleavage sequence flanked by a FRET pair of fluorescent proteins. This technique has been highly successful for the dynamic study of caspase activation during apoptosis (32). Signal amplification is also a potential advantage of substrate-based probes because multiple substrate molecules can be processed by a single active protease. Expanding upon this idea, investigators have applied macromolecular complexes containing many substrate sequences to studies of protease function (31, 33). A final advantage of substrate-based probes is their utility in high-throughput screening. The frequent use of fluorogenic probes to profile protease substrate specificity and screen for inhibitors has served as the basis for the design of selective inhibitors, substrate-based probes, and ABPs (6, 28). The major disadvantage of substrate-based probes is that it remains difficult to assign substrate cleavage to activation of a particular protease. Activation can be inferred only from knowledge about the substrate specificity of the protease of interest. An additional limitation of substrate-based probes is that after cleavage, the substrate diffuses away from the target protease, preventing isolation of the protease or identification of the precise subcellular localization of the activity being reported (6).

Activity-Based Probes

In contrast to substrate-based probes, ABPs covalently attach to active proteases. The covalent bond between a protease and an ABP is formed between an electrophile on the ABP and the active-site nucleophile of the protease. Alternatively, a photocrosslinker on the ABP can be used to covalently label a noncatalytic residue within the active site of the protease. Only catalytically competent proteases can irreversibly bind to ABPs. As with substrate-based probes, a recognition sequence confers specificity on the target protease. Finally, a reporter tag is appended to the probe, the nature of which depends on the application of interest but is typically either a fluorophore or an affinity handle (6–8). ABP tags can be detected using various analytical platforms, including mass spectrometry, SDS-PAGE, fluorescence microscopy, and in vivo imaging (**Figure 4**).

The covalent linkage of a probe to the enzyme is the major advantage of activity-based profiling because it enables direct isolation and identification of the target protease. This ability has facilitated application of ABPs to protease discovery, prediction of potential off-targets of drugs, and assignment of the functional roles of closely related proteases (6–8), all of which we discuss further below. A disadvantage of ABPs is that design is somewhat limited by the requirement for attachment of an electrophile or photocrosslinker. Another potential disadvantage is that, by covalently modifying the protease active site, the target is irreversibly inhibited. However, in many cases it is possible to use concentrations of ABP such that only a small percentage of the active enzyme pool is modified (6, 36).

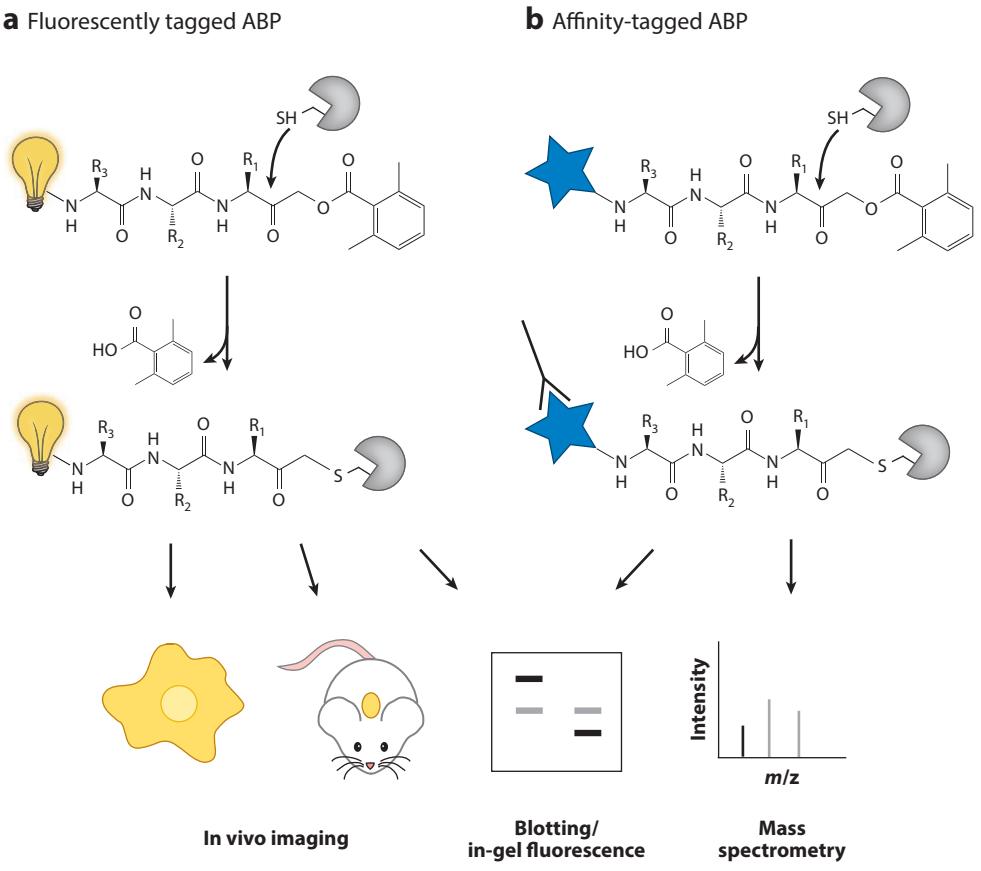

Figure 4

An example of cysteine protease–targeted activity-based probes (ABPs) with acyloxymethylketone electrophiles. (*a*) Fluorescently tagged ABPs can be used for in vivo imaging of protease activity and identification of active protease species by in-gel fluorescence. (*b*) Affinity-tagged (*blue star*) ABPs can be used to isolate active protease species for identification by immunoblotting or by mass spectrometry. Abbreviation: *m/z*, mass-to-charge ratio.

DESIGNING ACTIVITY-BASED PROBES FOR PROTEASES

ABPs have three essential components. The first is a reactive functional group that covalently binds to the protease. The second is a linker or recognition sequence that increases selectivity toward target proteases. The third and final essential component is a reporter tag by which the protease-tethered ABP can be visualized or isolated and identified (6–8, 37). Each of these components is selected to target the protease of interest and to be detected by an optimized analytical platform. This section outlines the options for each of the three ABP components; we focus on design challenges and how they have been successfully addressed.

Reactive Functional Groups

ABPs contain a reactive functional group that binds covalently to active proteases. For most cysteine, serine, and threonine protease ABPs, the reactive functional group is a directly reactive electrophile, whereas for aspartyl protease and metalloprotease ABPs, it is a latent reactive cross-linker that is activated by light.

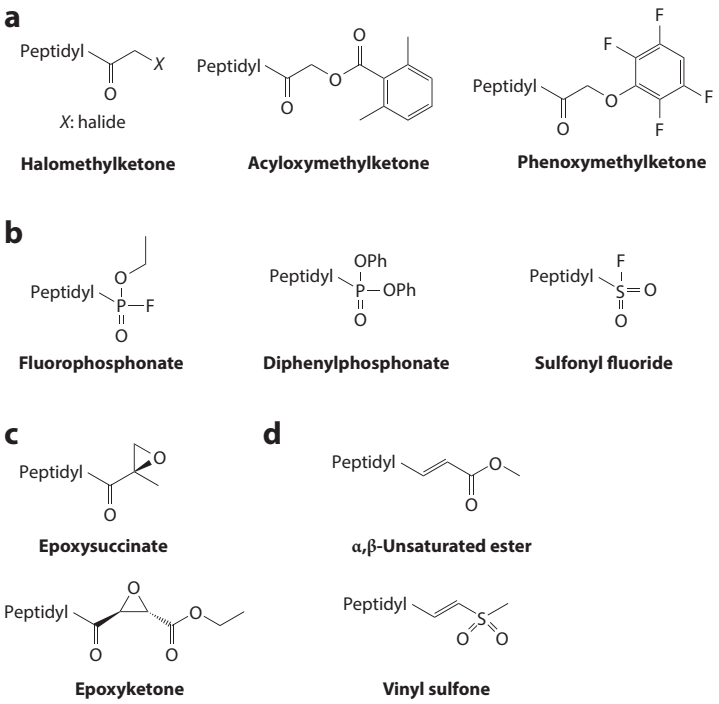

Figure 5

Representative chemical structures of different classes of electrophiles used in activity-based probes. (*a*) Activated ketones. (*b*) Phosphonylating and sulfonylating agents. (*c*) Epoxides. (*d*) Michael acceptors.

Electrophiles for cysteine, serine, and threonine protease ABPs have been chosen according to the reactivity of the target protease. For example, the thiol group in the active site of cysteine proteases is more polarizable or "soft" than the hydroxyl group in the active site of serine and threonine proteases (38). Therefore, electrophiles in cysteine protease ABPs are correspondingly softer than those used for serine and threonine ABPs (39). Various electrophile classes are reported to irreversibly inhibit cysteine, serine, and threonine proteases (39); a handful of them have been used in ABPs (**Figure 5**) (**Table 1**) (40–67).

Considerations for electrophile selection. Electrophiles are often chosen on the basis of their ease of synthesis, bioavailability, and desired reactivity (39). Electrophile reactivity is particularly relevant when tuning the specificity of an ABP. Probe labeling is a two-step process in which the probe binds in the protease active-site cleft and then covalently attaches to the active-site nucleophile. The binding step is reversible and is driven by affinity of the probe for the protease, which is measured as the equilibrium constant, K_D. The covalent attachment step is driven by the reactivity between the nucleophile and the electrophile, which is measured as the rate constant, k_{cat}. Greater electrophile reactivity increases the k_{cat} value and lowers the impact of the K_D on probe selectivity. In contrast, the rate of modification by less-reactive electrophiles is controlled primarily by the K_D, increasing the impact of the substrate-like region on the specificity of the ABP. However, if the rate of reaction is too slow, overall low labeling efficiency may result, hindering efforts to visualize or isolate targets (**Figure 6**). A comparison between two caspase-1-selective ABPs, FLICA (60) and AWP28 (36), illustrates this point. Because AWP28 has a lower k_{cat} as well as a lower K_D for caspase-1 than does FLICA (36, 68), it displays greater selectivity and lower background labeling (36). This finding demonstrates that, although various electrophiles covalently react with the active-site nucleophile of a protease, electrophiles should be carefully chosen to maintain ABP specificity.

Challenges in reactive functional group design: aspartyl and metalloproteases. Metalloproteases and aspartyl proteases differ from cysteine, serine, and threonine proteases in that they use water as a nucleophile for amide bond hydrolysis. In the few reported examples of ABPs for these classes of proteases, covalent attachment occurs through a benzophenone or diazirine photocrosslinker (47–50). When irradiated with UV light, photocrosslinkers produce radical intermediates that react with nearby atoms to form covalent bonds. The higher the binding affinity of the ABP for the protease is, the more likely that covalent linkage will occur upon UV irradiation. Importantly, active proteases normally have higher ABP binding efficiency than do inactive

Table 1 Earliest and/or notable applications of electrophiles in activity-based probes (ABPs) for various protease classes

Electrophile	Target protease type	ABP designed for	Clan	Family	Reference(s)
Acyloxymethylketone	Cysteine	Cysteine cathepsins	CA	C1	40
		Legumain	CD	C13	40
		Caspases	CD	C14	40, 41
		Separin	CD	C50	42
		SUMO protease 8	CE	C48	43
Alkyne	Cysteine	Deubiquitinating enzymes	CA	—	44
		Caspases	CD	C14	44
α,β-Unsaturated ketone	Cysteine	Cysteine cathepsins	CA	C1	45
		Caspases	CD	C14	46
Aza-epoxide	Cysteine	SUMO proteases	CE	C48	43
Benzophenone	Aspartyl, metallo-	Metalloproteases	—	—	47
		Presenilin-1	AD	A22	48
Diazirine	Aspartyl, metallo-	Metalloproteases	—	—	49
		Signal peptide peptidase	AD	A22	50
Diazomethylketone	Cysteine	Cysteine cathepsins	CA	—	51
Diphenylphosphonate	Serine	Serine proteases	—	—	52
		Granzymes A and B	PA	S1	53
		Serine cathepsins	PA	S1	54
		Urokinase-type plasminogen activator	PA	S1	54
Epoxyketone	Threonine	Proteasome β-subunits	PB	T1	55
Epoxysuccinate	Cysteine	Cysteine cathepsins	CA	C1	56
		Calpain	CA	C1	57
		Caspases	CD	C14	46
Fluorophosphonate	Serine	Pan-serine protease	—	—	58
Halomethylketone	Cysteine	Cysteine cathepsins	CA	C1	59
		Caspases	CD	C14	60
		SUMO proteases	CE	C48	61
Phenoxymethylketone	Cysteine	Papain-fold cysteine proteases	CA	C1	62
Vinylsulfone	Cysteine, threonine	Papain-fold cysteine proteases	CA	C1	63
		Deubiquitinating enzymes	CA	—	64
		Proteasome β-subunits	PB	T1	65
4-Chloroisocoumarin	Serine	PfSUB1	SB	S8	66
		Escherichia coli rhomboids	ST	S54	67

proteases (69). However, photocrosslinking ABPs are still capable of binding to inactive proteases because covalent attachment does not require active-site competency. Therefore, specific binding to active protease forms is a challenge, which has been partially addressed by adding functional groups to the ABP. For example, active metalloproteases chelate a metal ion in their active site. Through the addition of a metal-chelating residue, it is

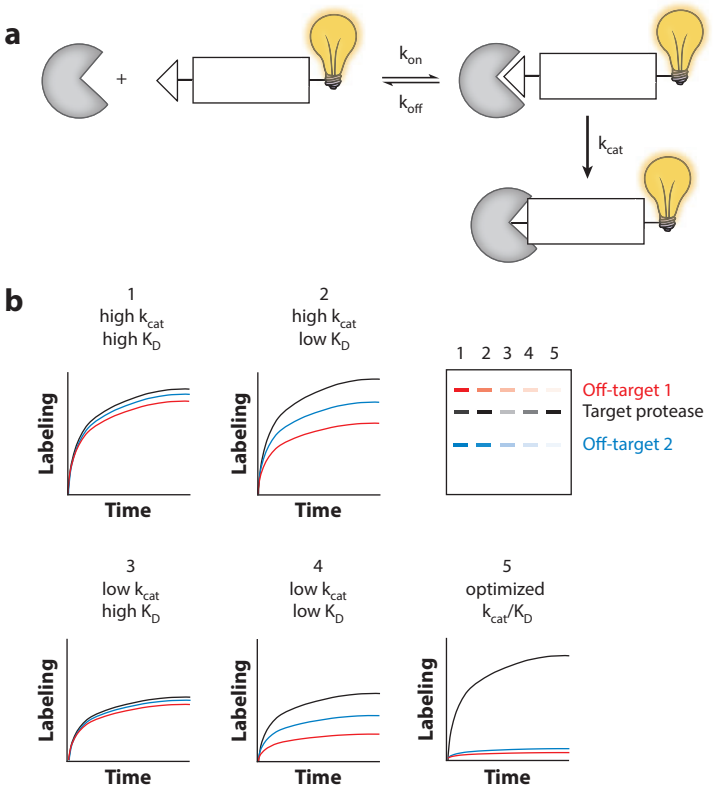

Figure 6

Kinetic binding and reactivity parameters dictate activity-based probe (ABP) specificity. (*a*) ABP–protease interactions have two phases: a reversible binding step determined by the binding affinity (k_{off}/k_{on}, or K_D) of the substrate-like specificity region and an irreversible covalent attachment step determined by the reactivity of the electrophile (k_{cat}). (*b*) Modulation of ABP K_D and k_{cat} to achieve specificity. ABPs with low protease affinity have high K_D values. Highly reactive ABP electrophiles have high k_{cat} values. Numbers 1–5 represent ABPs with various combinations of k_{cat} and K_D. For each ABP, there is a rate plot of reaction between the ABP and both target and off-target proteases. Lanes 1–5 on the gel (*top right*) correspond to the sample ABPs; the bands indicate the labeling intensity of protease species.

possible to generate ABPs that will selectively label only catalytically active metalloprotease forms (47, 49). This strategy has been validated in cell-culture studies of MMPs and in *Xenopus laevis* embryos overexpressing MMP-2 (70).

An important design consideration for ABPs of metalloproteases and aspartyl proteases is the location of the photocrosslinker. Unlike electrophiles in cysteine, serine, and threonine protease ABPs, in which the electrophiles must be positioned directly at the substrate cleavage site, photocrosslinkers can be located anywhere along the probe scaffold. It is important to test multiple attachment points because an incorrectly placed photocrosslinker can disrupt interactions with the target protease or indiscriminately react with both inactive and active protease forms (69). A recent study of the aspartyl protease presenilin-1 demonstrated that optimization of photocrosslinking ABPs can be used as an opportunity to generate information about the active-site cleft of a protease. In a strategy termed photophore walking, investigators synthesized a panel of ABPs in which a benzophenone photocrosslinker was attached to three positions of a presenilin-1 inhibitor (48). Each position had previously been shown to interact with a different region of the presenilin-1 active-site cleft (71). Protease mutants were expressed that lack activity due to conformational changes in the S2 subsite of the active site. Of the three ABPs, only the ABP containing a photocrosslinker interacting with the S2 subsite selectively labeled the wild-type presenilin-1 and not the inactive mutants. The other two ABPs bound to all protease mutants or did not bind to the wild-type protease (48). This observation supports the principle that photocrosslinker attachment either can hinder interaction with the target protease or can enable labeling of inactive protease forms. It also highlights the need to test different attachment locations.

In summary, photocrosslinking ABPs have some limitations compared with ABPs for cysteine, serine, and threonine proteases because covalent linkage is not a direct readout of active-site competency. However, careful optimization and validation have generated successful ABPs for several aspartyl proteases and metalloproteases (47–50).

Recognition Sequence Design

The linker region between the reactive functional group and the reporter tag is often used to confer target specificity to an ABP.

Potential recognition motifs. In some cases, this linker can be a protein domain or even an entire substrate protein. For example, a

recently described calpain-targeted ABP contains a helical calpain recognition domain (57), and ABPs for deubiquitinating enzymes use the full-length ubiquitin as a recognition sequence (72). For most protease-targeted ABPs, the linker between the reactive group and the tag is a peptide sequence that corresponds to the sequence of an optimal protease substrate (6).

Although optimal recognition sequences can be determined, a major challenge for the design of the recognition sequence is overlapping substrate specificity of closely related proteases, which is problematic for imaging applications, the goal of which is to monitor the localization and relative activity of a single protease (6–8). Several recent advances in substrate screening, protease inhibitor discovery, and probe design have addressed this problem.

Optimizing target specificity. It is often difficult to choose a recognition sequence that is specific for a single protease. However, increases in the chemical diversity of substrate screening libraries have facilitated discovery of recognition sequences that discriminate between closely related proteases (73). For instance, recently designed fluorogenic substrate and peptide inhibitor libraries are more chemically diverse due to inclusion of both nonnatural and natural amino acids (74). Such a library was recently used to identify a recognition sequence that has 30-fold selectivity for caspase-3 over the closely related caspase-7 protease and an ABP with 50-fold selectivity for caspase-3 (75). Notably, the pentapeptide recognition sequence contains three nonnatural amino acids, illustrating the utility of a chemically diverse screening library.

Phage display has also been used to generate and screen chemically diverse protease inhibitor libraries (76–78). Using phage display, researchers encoded peptides with constant cysteine residues and variable spacer regions containing diverse residues. Bicyclic structures reminiscent of peptide macrocyclic drugs were generated by cross-linking the cysteine residues through a *tris*-(1,3,5-bromomethyl)benzene linker. The resulting millions of candidate peptide bicycles could be simultaneously screened for protease binding and inhibition. This strategy generated potent and selective inhibitors of plasma kallikrein (PK) and factor XII (76–78). Notably, the final optimized PK inhibitor had >1,000-fold selectivity for human PK over murine PK. This selectivity is remarkable, considering the 79% sequence identity and 92% sequence similarity between the two enzymes (78).

Although increasing peptide structural diversity will aid in the design of ABPs with exquisite specificity, both of the above approaches have limitations. Bicyclic peptides are bulky and cell impermeable (76–78), and although chemical diversity in linear peptide structure is increasing, finding inhibitors with absolute selectivity for a native protease remains a major challenge (28). To address these limitations, researchers recently developed a protease engineering strategy in which a specific protease target is engineered to bind with absolute specificity to an ABP. This task was accomplished by introducing an engineered cysteine nucleophile near the protease active site. A latent electrophile could then be added to the ABP to make a unique reactive pair (**Figure 7**). Using this strategy, experimenters designed ABPs that exclusively target caspase-1, caspase-8, MMP-12, and MMP-14 (79, 80). Taken together, these recent advances show that it is possible to harness small differences between even highly similar proteases to generate ABPs with exquisite specificity.

Reporter Tags

A main advantage of using activity-based profiling to assess protease activity is the ability to isolate and identify targets using various reporter tags. The choice of reporter tag depends on the analytical platform. For instance, ABP imaging requires fluorescent or radioactive tags, and ABP-assisted proteomic identification of active proteases requires affinity handles such as biotin (6–8). However, the choice of tag within the broad categories of fluorophore and affinity handle is not trivial, because the tag often

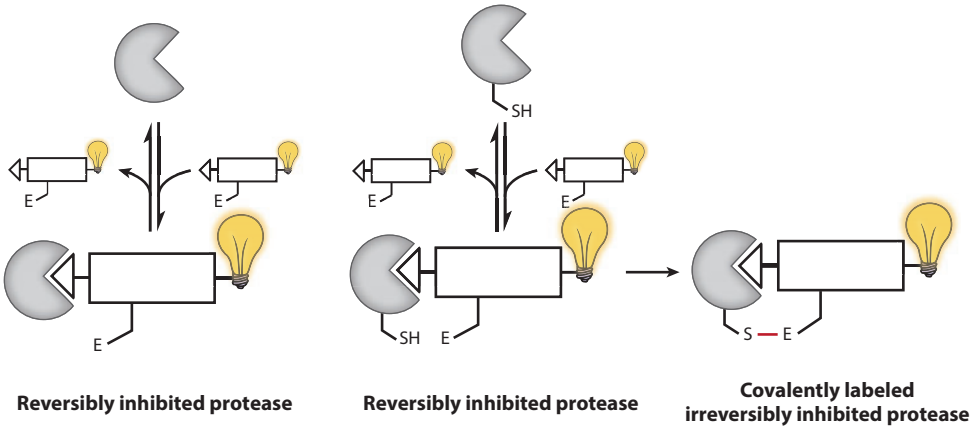

Figure 7

Engineering activity-based probes (ABPs) and proteases to achieve absolute specificity. Engineered ABPs contain reversible and irreversible electrophiles. The reversible electrophile allows positioning within the active site (*left*, *middle*). The addition of an irreversible electrophile (E) allows specific covalent linkage to a non-active-site cysteine (SH) on the engineered protease (*middle*, *right*).

contributes to the physicochemical properties of the ABP. Although ABP electrophiles and recognition sequences can be carefully selected to target a protease of interest, they are ineffective if they do not encounter that protease in vivo. Incorrect localization can also lead to off-target labeling. For example, improper biodistribution has been a major challenge when designing ABPs for cytoplasmic proteases because many ABPs are hydrophobic, charged, or bulky and therefore are not cell permeable. Cell-impermeable ABPs are often endocytosed and label predominantly endosomal and lysosomal proteases (83). ABPs can also suffer from lack of solubility, which limits their biodistribution. Fortunately, the increasing diversity of reporter tags has correspondingly allowed for optimization of ABP efficiency. Considerations for tag selection are discussed in detail below.

Considerations for selection of an imaging tag. Although ABPs are known as small-molecule probes, they often are not classically defined as small molecules but rather can have masses approaching 10 kDa (82, 83). Much of this mass is often the reporter tag, especially when the tag is a dye. Small-molecule dyes have long been employed in biological research (84), and a wide variety of fluorescent dyes have been synthesized. Among these, BODIPY (85), fluorescein and rhodamine (86), cyanine dyes (49), dansyl (87), and NBD (nitrobenz-2-*oxa*-1,3-diazole) (88) have been used in ABPs. The choice of dye usually depends on the desired chemical properties of the resulting probe. For example, fluorescein and rhodamine have poor photostability and should not be used for experiments that require extended imaging time (86, 89), but they are inexpensive and thus an economical choice for other applications.

Although many dyes may lack optimal chemical or biological properties, their characteristics can be improved by derivatization. For example, esterification of fluorescein quenches its fluorescence until it enters the cell and is liberated by cytosolic esterases (89). If a dye has high in vivo background, it can be paired with a quencher that absorbs its fluorescence until the ABP is bound to the target protease. This method has been used successfully for in vivo imaging of protease activity (**Figure 8**). Quenchers can also be optimized; for example, sulfonation of the quencher Qsy21 increases its solubility and biodistribution. Applying this modification to a pan-cathepsin ABP resulted in significant improvements in tumor contrast

in an orthotopic mouse model of breast cancer (90).

Considerations for selection of an affinity handle. Many factors should be taken into account when choosing an appropriate ABP affinity handle. Typical choices include biotin, hemagglutinin (HA), azides, and alkynes (6–8, 37, 72). Biotin- and HA-tagged ABPs can be used to isolate labeled targets by affinity purification. Azides and alkynes react with one another in a copper-catalyzed click reaction to form a triazole (91). These two functional groups can be used as latent reporter tags on ABPs that can be converted into affinity probes by use of the corresponding azide or alkyne tags.

Direct biotin or HA tagging is advantageous because it allows direct isolation of a labeled target. However, these tags generally reduce cell permeability. A "clickable" handle often does not hinder the permeability of an ABP, but isolation depends on efficiency of the click reaction and subsequent isolation steps (92). Both methods of affinity purification have been successfully used to identify drug targets and discover new enzymes (72). In general, HA or biotin tags are useful if protein isolation is performed in cell lysates and if these relatively significant modifications to the ABP do not prohibit binding to the target protease. In other cases, a clickable handle may be more effective (37, 72).

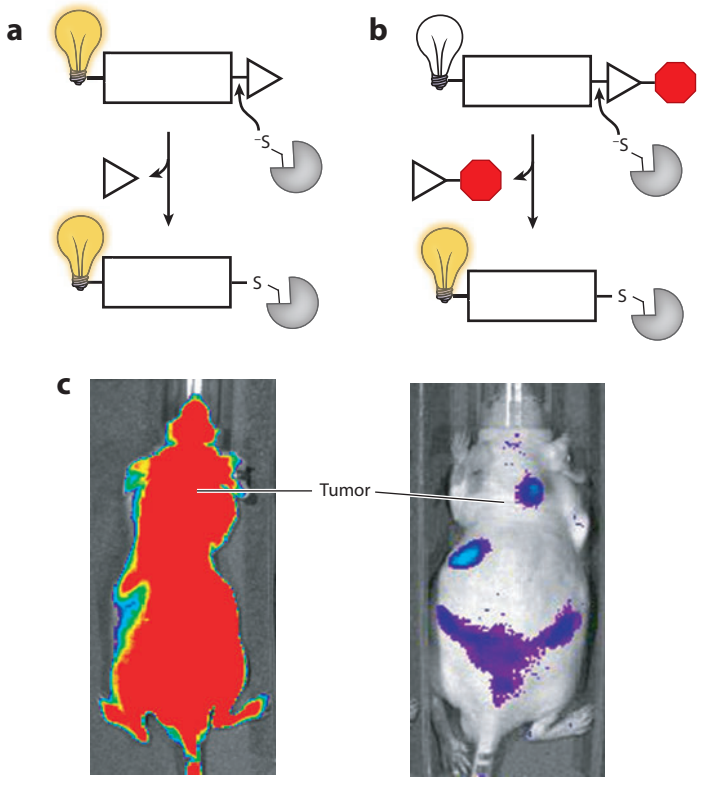

Figure 8

Quenched activity-based probes (ABPs) have improved contrast relative to unquenched ABPs in noninvasive imaging experiments. (*a*) Unquenched probes are constitutively fluorescent. (*b*) Quenched probes are not fluorescent until cleavage of the fluorescence quencher (*red octagon*) occurs upon binding to protease. (*c*) Quenched ABPs have lower background fluorescence than unquenched ABPs, as demonstrated by this comparison of in vivo ABP labeling of tumor-associated cathepsins by an unquenched ABP (*left*) and a quenched ABP (*right*). Modified with permission from Reference 83.

APPLICATIONS OF ACTIVITY-BASED PROFILING TO PROTEASES

Advances in probe design have facilitated applications of ABPs to the study of fundamental protease enzymology, to the discovery and development of drugs, and to the diagnosis of disease (6–8). In the following sections, we discuss these ABP applications by highlighting case studies that best illustrate each application.

Protease Enzymology

Proteolytic processing, cofactor binding, post-translational modification, and pH change are all capable of inducing protease activation by altering the conformation of or access to the active site (2). Of the methods of profiling protease activity, substrate-based probes are the most commonly used to determine the requirements for protease activation. For example, pNA-linked peptides were used to establish the effect of amino acid point mutations on binding of the serine protease factor VIIa to its allosteric activator, tissue factor (93, 94).

ABPs have also been successfully used to study mechanisms of protease activation. The main advantage of using ABPs is that the protease forms that are active can be identified.

In contrast, measurements of substrate hydrolysis only designate whether a protease is active under the conditions of interest; they do not provide insight into which protease forms are capable of hydrolyzing substrates. ABPs can identify all active protease forms, an ability that has been particularly advantageous for the identification of transiently active protease forms (96, 97). Another advantage of using ABPs to study protease enzymology is that covalent attachment of ABP to protease depends on the catalytic competency of the active site and not its substrate-binding ability. This advantage is useful for identifying protease mutations that restrict access to the active-site cleft without altering its conformation (95, 96). The following case studies expand on these issues and illustrate how ABPs can be used to determine details of protease activation that are difficult to identify using other techniques.

Intermediates of caspase-7 activation. Caspase-7 is a cysteine protease that is activated during the programmed cell death process, apoptosis (12, 17). Its activation was originally thought to occur by limited proteolysis coordinated by related family members caspase-3 and caspase-9. Furthermore, only the mature, processed form of caspase-7 was thought to have proteolytic activity. Caspase-7 activation had previously been measured by immunoblotting, which allowed direct observation of the conversion of the 36-kDa precursor enzyme to the 20-kDa mature form, and by fluorogenic substrate assay. Alternatively, an ABP was successfully used to identify incompletely processed forms of caspase-7 that have transient activity. In this study, the apoptotic caspase cascade was induced in a cell-free system by cytochrome *c* and dATP. Activated caspases were labeled by a pan-caspase ABP. Upon stimulation, the mature form of caspase-7 and the full-length, uncleaved "zymogen" form were labeled by the ABP (96). A further analysis indicated that the full-length species is part of a heterodimeric complex composed of mature, processed caspase-7 and full-length, zymogen caspase-7 in which the mature caspase-7 allosterically activates the full-length caspase-7 prior to completion of proteolytic processing. This finding was more recently supported by structural studies of caspase-7 mutants bound to covalent active-site inhibitors (98). Importantly, monitoring caspase-7 activation by immunoblotting or fluorogenic substrate assay would not have provided this insight, supporting the concept that ABPs are useful for trapping and identifying transiently present active protease forms.

Profiling allosteric activation of the *Clostridium difficile* TcdB cysteine protease domain. *Clostridium difficile* is a gram-positive bacterium that is a major cause of hospital-acquired diarrhea. The glucosylating toxins TcdA and TcdB are the primary virulence factors of this pathogen (99). TcdB contains a cysteine protease domain (CPD) that is activated by allosteric binding of the host cell cofactor inositol hexakisphosphate ($InsP_6$) (100). Because the primary function of the CPD is autoprocessing of the full-length toxin, it does not efficiently bind substrate in *trans* and therefore does not process short peptide fluorogenic substrates (101). Therefore, an ABP specific for TcdB was required to detect activation of the CPD in response to $InsP_6$. Surprisingly, the recombinant TcdB CPD could be labeled by ABP in the absence of $InsP_6$. This finding supports a dynamic structure of the CPD that samples active conformations even without the activating cofactor bound. The authors of this study proposed that the ABP served as a substrate mimic that stabilized the active conformation of the protease, which was supported by limited proteolysis experiments (97). This intermediate could not have been measured by fluorogenic substrate assays due to lack of sensitivity and the transient nature of the interaction between a substrate and the enzyme, yet it could be readily isolated and identified using a covalently bound ABP (**Figure 9**).

Activity-based profiling of the *Escherichia coli* rhomboid protease GlpG. The rhomboid proteases are a family of intermembrane

serine proteases with proposed roles in cell signaling, cell death, and host–pathogen interactions. These proteases cannot be easily purified for functional studies, which makes biochemical characterization challenging. The structure of the *Escherichia coli* rhomboid protease GlpG was recently determined (102). However, the structures did not include the cytoplasmic domain of the protease. To circumvent purification difficulties, the activities of mutants of the cytoplasmic domain were determined in crude membrane extracts. Substrate-based probe screening revealed an α-helix in the cytoplasmic domain that is important for GlpG activity. Specific mutations were made in this α-helix and screened for activity by use of a substrate-based probe and an ABP. Interestingly, a mutation was identified that showed complete loss of fluorogenic substrate processing but was labeled by the ABP. The ABP did not have a peptidic specificity region but instead had a small alkyl linker, which led to the hypothesis that the protease mutant could no longer bind to native substrates but still had a catalytically competent active-site nucleophile (95). This finding demonstrates that ABPs can distinguish mutations that alter substrate-binding ability from those that alter catalytic activity. It also illustrates the value of using complementary methods to measure protease activity.

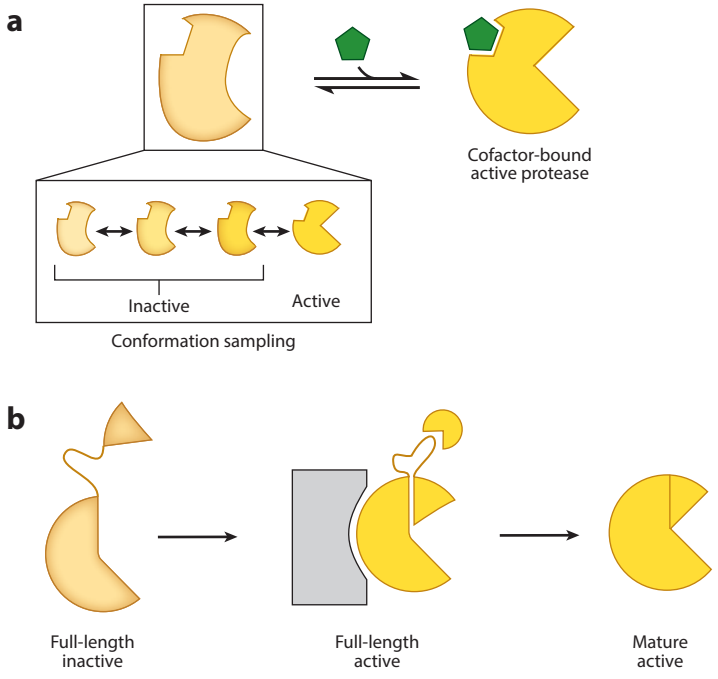

Figure 9

Activity-based probes can be used to capture latent active forms of proteases. (*a*) A protease that requires cofactor binding (*green pentagon*) for full activity can also transiently assume an active conformation when unbound. (*b*) Many proteases must undergo proteolytic processing to become active and pass through a series of intermediate truncation steps. Here, processing is promoted by allostery. Allosteric binding supports an active conformation of the incompletely processed protease (*middle*).

Drug Discovery and Development

Dissecting the activation mechanism of a protease can provide insight into potential mechanisms by which it can be artificially modulated. The ability to manipulate protease activity is useful for mapping biological function. It is also clinically useful because proteases are aberrantly active in many disease pathologies, and inhibition is often a viable method of treatment (4).

Protease inhibitors and activators are often identified by screening compounds against enzymes purified from a native source or enzymes isolated from recombinant expression in bacteria. Fluorogenic substrates are commonly used to assess target inhibition due to their suitability for high-throughput screening. As an alternative, investigators developed a method termed fluopol-ABPP that uses fluorescent ABPs to enable high-throughput screens (103). This technique uses fluorescence polarization to detect binding of a fluorescent ABP to an active protease. When an ABP is bound to the target, the fluorophore tag "tumbles" more slowly in solution, resulting in enhanced fluorescence polarization. However, when binding of an inhibitor blocks labeling, the unbound ABP rotates quickly in solution and fluorescence polarization is reduced. By measuring the change in the fluorescence polarization value, one can assess the ability of a compound to bind and inhibit a target protease. The benefit of this method is that it does not require a substrate, is highly amenable to high-throughput screening, and is less affected

by artifacts that result from intrinsic fluorescence and poor solubility of the compounds being screened. Fluopol-ABPP has been used to identify selective and potent inhibitors of the serine protease PREPL, as well as several other serine hydrolases (SHs) (104, 105).

Although useful, such screens require that the protease of interest be isolated in pure form. In addition, these types of in vitro screens do not provide any information about the in vivo potency or selectivity of the identified lead compounds. However, such characteristics can be assessed by competitive ABPP, in which a complex proteome or intact cell is first treated with a drug candidate and then labeled by an ABP. This approach is advantageous because screens can be performed in a physiologically relevant setting. Potency is measured by assessing loss of ABP labeling of the target protease over a titration of the lead compound (7, 8). An estimate of selectivity can also be determined if the ABP can label related enzymes (8). The following examples demonstrate the applicability of ABPs to drug discovery and development.

Discovering inhibitors of the *Clostridium difficile* protease Cwp84. In addition to killing host cells through the CPD-containing toxins TcdA and TcdB, *C. difficile* infection is promoted by another protease, Cwp84. Cwp84 is a recently identified papain family cysteine protease that is responsible for generating the polypeptide surface layer that coats *C. difficile*. This surface layer protects *C. difficile* from host defenses and aids in the invasion process (106). Cwp84 is a potential drug target because its inhibition simultaneously facilitates host immune system activation and blocks bacterial invasion of host cells. Because Cwp84 is a papain family cysteine protease, a panel of potential inhibitors was synthesized on the basis of the structure of the well-known papain inhibitor E-64 (107). This library was screened by competitive ABPP in *C. difficile* cell lysates. The top candidate, an E-64 analog with an α,β-unsaturated ester electrophile, was the most potent and displayed little off-target binding in bacterial cell lysates. Notably, this screen was conducted in the native environment of the protease, and covalent attachment of the drugs enabled verification of target engagement and analysis of off-target binding.

Trypanocide development. The trypanosomes *Trypanosoma cruzi* and *Trypanosoma brucei* are human parasites that cause Chagas disease and African sleeping sickness, respectively. Existing treatments for these diseases are toxic and often ineffective, and drug resistance is increasing (108). The cysteine proteases cruzain and rhodesain are essential for the parasites (109), making them promising drug targets. In a recent study, fluorogenic substrates were used to identify a novel class of inhibitors with azanitrile electrophiles. Of the lead compounds, two were converted into ABPs by tagging the carboxybenzyl cap of the inhibitors with an alkyne. Click chemistry–enabled conjugation of azido-rhodamine to the alkyne allowed visualization of probe labeling by in-gel fluorescence and characterization of compound uptake and subcellular distribution by fluorescence microscopy. Reaction with azido-biotin facilitated immunoprecipitation and identification of ABP-bound proteases. In both cases, the ABPs confirmed binding to off-target proteins such as ketoacyl-CoA thiolase, BILBO1, and succinyl-CoA synthetase α, all of which are essential to parasites. Therefore, the authors speculated that the off-target binding increased the lethality of their compounds. Although genetic validation is required to prove this claim (110), this case study shows the complementarity of substrate-based probes and ABPs and demonstrates the value of ABPs for dissecting off-target effects of drugs.

Pharmacodynamics of cathepsin K inhibitors. Cathepsin K is a cysteine protease that is involved in bone resorption, the process by which osteoclasts degrade bone. This process is important for increasing calcium levels in the blood, but excessive bone resorption can make bones brittle (111). For this reason, cathepsin K has become an important drug target for treatment of osteoporosis. A recently developed

class of cathepsin K inhibitors containing a piperazine ring failed to progress to the clinic due to toxicity issues. Notably, analogous inhibitors lacking the piperazine functional group were nontoxic (112). An ABP was used to analyze the specificity and distribution of these two classes of compounds, with the aim of explaining the observed differences in toxicity. To analyze the effects of both groups of inhibitors on cysteine cathepsin activity, female rats were treated with the compounds and then treated with a pan-cysteine cathepsin ABP. Tissues were excised and cathepsin inhibition assessed by in-gel fluorescence. In all the tissues analyzed, the cathepsin K inhibitors containing a piperazine ring inhibited the off-target cathepsins B, S, and L, whereas the other inhibitors did not. The authors of this study hypothesized that off-target inhibition was due to protonation of the basic nitrogen in the piperazine ring and accumulation in the lysosome, which increases binding to lysosomal cathepsins B, S, and L. This study was valuable because it demonstrated that nonbasic compounds have increased selectivity for cathepsin K due to better distribution properties, which should improve the safety and efficacy of future cathepsin K–targeted compounds (113). Furthermore, it illustrates an application of ABPs in the drug-development process and emphasizes the importance of being able to monitor drug selectivity and distribution dynamics in vivo.

Profiling Proteases in Disease

In addition to being viable targets for treatment of disease, proteases are also potential biomarkers that can be used for diagnostic purposes. For example, proteases are ideal biomarkers for inflammatory diseases because they are secreted from activated immune cells (114). They are also useful biomarkers for cancer because increased protease activity is associated with many of the hallmark processes of cancer, including angiogenesis, tissue remodeling, and cell death (115). Specific protease biomarkers are often identified from gene-expression profiling or shotgun proteomic data (8). More recently, activity-based profiling coupled with mass spectrometry has proven to be a good source of novel disease biomarkers (116).

Once a biomarker is identified, methods must be developed for reliable clinical detection. Such methods must be able not only to detect protease activity, but also to distinguish healthy tissue from diseased tissue. ABPs have been successfully used to detect aberrant protease activity in various patient samples. These include brain tumor tissue, in which fluorescent ABPs were topically applied and imaged (117), and blood, in which peripheral-blood mononuclear cells were labeled with an ABP, separated by cell type, and analyzed by in-gel fluorescence (118). ABPs have also been used to noninvasively image areas of inflammation and cancer in various animal models (81, 83). These applications and the use of ABP to identify novel biomarkers are discussed in the following case studies.

Identification and validation of myeloblastin as a non-small-cell lung cancer biomarker. The prognosis of non-small-cell lung cancer (NSCLC) is based on the stage of the disease at diagnosis, which often is not predictive of the disease course. Therefore, clinically relevant biomarkers of NSCLC are needed. An ideal biomarker would display significant changes in activity that correlates with disease onset and progression. Because differential SH activity has previously been associated with multiple types of cancer (119), SHs are candidate biomarkers for NSCLC. To profile a wide and relatively unbiased range of potential SH biomarkers, biopsies from both healthy patients and patients with lung adenocarcinoma, the most common type of NSCLC, were labeled with a pan-SH ABP. Labeled proteases were affinity-purified with streptavidin and identified using mass spectrometry. Using this method, investigators identified 33 SHs that had significantly elevated activity in tumor biopsies. Notably, 9 of the 33 SHs were serine proteases (120). In a follow-up study, the authors pursued myeloblastin, a serine protease, as a potential biomarker for KRAS-driven NSCLC

(121). Upregulation of myeloblastin activity in tumors from NSCLC patients with a G12C mutation in KRAS was established by ABP-enabled affinity purification. Although these findings need to be repeated in more patients in order to effectively validate myeloblastin as a biomarker, they demonstrate the applicability of ABPP for biomarker discovery and validation. On the basis of the low sample size of the study, it is also impressive that coherent trends in protease activity could be identified, which illustrates the value of profiling protease activity within a native cellular environment.

Noninvasive imaging of cathepsin S. Cysteine cathepsins are lysosomal proteases that change subcellular distribution and activity in conditions such as inflammation and cancer. Cysteine cathepsins are useful targets for imaging sites of cancer and inflammation because their expression is elevated in the macrophages that infiltrate many types of diseased tissues. Several reported classes of ABPs label cysteine cathepsins B, L, and S (40, 83, 122). However, although the expression of cathepsins B, L, and S is elevated in macrophages, cathepsins B and L are also active in the surrounding tissues. In contrast, cathepsin S is expressed only in antigen-presenting cells (118), suggesting that an ABP that exclusively labels cathepsin S might have improved contrast between diseased tissue and healthy tissue. Researchers designed an ABP containing a cathepsin S–selective nonpeptidic specificity region (122, 123). Importantly, this ABP showed improved tumor contrast compared with pan-cathepsin ABPs. Cathepsin S–selective labeling was verified by in-gel fluorescence (123), again demonstrating one of the major advantages of using ABPs for imaging protease activity, namely that the specific proteases labeled by an ABP can be identified.

Activity-based probe–based methods to determine kallikrein 6 activity in patient samples. Kallikreins (KLKs) are serine proteases that have trypsin- or chymotrypsin-like activity. Within this family, KLK3 is a widely used biomarker for ovarian cancer. KLK6 is also a potential biomarker because it activates proteinase-activated receptors, leading to pathologies such as inflammation, carcinogenesis, and tumor metastasis. KLKs have elevated expression in tumors but differential expression in metastatic and primary tumors, suggesting that they are markers of tumor progression (124). However, diagnostic assays are required to verify that KLK6 can function as a cancer biomarker and to identify KLK6 activity in relevant tissues or fluids. To this end, an ABP-mediated assay was developed to detect KLK6 in biological fluids from cancer patients. In this assay, termed ABRA-ELISA (ABP ratiometric enzyme-linked immunosorbent assay), relevant biological fluids were collected and incubated with a biotinylated ABP. The ELISA for KLK6 reported the total enzyme levels, and the ELISA for biotin reported active enzyme levels. The results of both assays were combined to successfully quantify the percentage of total KLK6 that is active in cerebrospinal fluid, ascites fluid, and cancer cell supernatants. In most samples, only ~5% of the expressed KLK6 was active (125). The biotin ELISA had sufficient sensitivity to identify this low level of activity, but the assay could not have been performed without covalent linkage of the ABP to the protease.

SUMMARY POINTS

1. Levels of protease activity more accurately define function than do protease messenger RNA or protein quantities.
2. Protease activity can be determined by measuring hydrolysis of natural substrates and substrate-based probes or covalent attachment of ABPs.

3. The various methods of profiling protease activity have distinct advantages and are best used complementarily.
4. The three major elements of an ABP—the reactive functional group, specificity region, and reporter tag—are chosen on the basis of the target protease and the desired analytical platform.
5. ABPs can be used to identify intermediate species in protease activation and to distinguish factors altering catalytic activity from those obstructing access to the active site.
6. A major advantage of applying ABPs to drug discovery and development is that the in vivo potency and selectivity of drug candidates can be simultaneously screened.
7. Protease ABPs have also been successfully applied to the discovery, validation, and imaging of disease biomarkers.

FUTURE ISSUES

1. Systems-level substrate profiling should be used to determine fine differences in substrate specificity between closely related enzymes.
2. ABPs usually react with multiple proteases, which is problematic for imaging studies. Chemically diverse inhibitor libraries and phage methods may increase the selectivity of future ABPs.
3. Improvements in synthesis and derivatization of dyes and affinity handles should be applied to further improve ABP biodistribution, target engagement, and selectivity.
4. Incorporation of novel electrophiles into ABPs will enable targeting of broader classes of proteases and allow for better control of specificity of existing probes.
5. The clinical applicability of ABPs can be improved by addition of reporter tags facilitating detection by positron emission tomography, MRI, or other commonly used clinical methods.

DISCLOSURE STATEMENT

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Errata

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