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Applications of Small Molecule Probes in Dissecting Mechanisms of Bacterial Virulence and Host Responses

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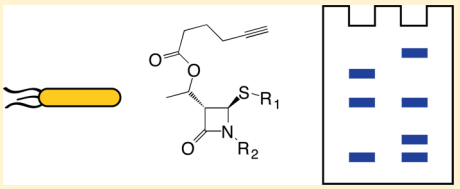
ABSTRACT: Elucidating the molecular and biochemical details of bacterial infections can be challenging because of the many complex interactions that exist between a pathogen and its host. Consequently, many tools have been developed to aid the study of bacterial pathogenesis. Small molecules are a valuable complement to traditional genetic techniques because they can be used to rapidly perturb genetically intractable systems and to monitor post-translationally regulated processes. Activity-based probes are a subset of small molecules that covalently label an enzyme of interest based on its catalytic mechanism. These tools allow monitoring of enzyme activation within the context of a native biological system and can be used to dissect the biochemical details of enzyme function. This review describes the development and application of activity-based probes for examining aspects of bacterial infection on both sides of the host−pathogen interface.



Small molecules have long been applied to the study of bacteria and other microbes; however, they have historically been used in the context of discovering antibiotics and other therapeutics capable of destroying the organism of interest. More recently, chemical tools have found use in creating and monitoring specific phenotypes in an effort to dissect the molecular events underlying pathogenesis. This is especially valuable for monitoring processes that are regulated post-translationally, such as enzymatic cascades that activate host immunity or remodel bacterial surfaces during infection. These events are difficult to study by measuring transcript or protein expression levels alone and only sometimes can be monitored indirectly by traditional biochemical approaches such as Western blotting. Methods employing small molecules that can rapidly and directly interact with enzymes of interest in a broadly applicable manner therefore provide a useful approach for dissecting host−pathogen interactions.

AN OVERVIEW OF THE GENERAL APPLICATIONS OF ACTIVITY-BASED PROBES

Activity-based probes (ABPs) are chemical tools that can be used to profile changes in enzyme activity levels (Figure 1).[1] ABPs function by covalently modifying target enzymes via a specific chemical reaction that is dependent on the enzyme's catalytic mechanism. ABPs generally contain three components: an electrophilic trap or "warhead", a specificity region, and a tag. The warhead portion of the probe is critical as it forms the covalent bond with the enzyme and, together with the specificity region, serves to direct the probe selectivity within the proteome. A wide variety of tags exist depending on the intended application, which can include direct visualization of enzyme activation or target isolation for further analysis. A number of comprehensive reviews have addressed the many possible components of ABPs and the benefits and applications of each type.[1−3]

Because ABP labeling is dependent on an enzyme's catalytic mechanism and takes place within the active site, these tools can often be used to functionally dissect the biochemical details of enzyme structure and function. Although any enzyme target with a nucleophilic residue involved in catalysis is amenable to study using ABPs, and the diversity of ABPs being developed has dramatically increased over the past decade, a majority of examples in which ABPs have been used to study protein function have focused on hydrolase targets. Proteases and other hydrolases often play critical roles in bacterial infection and host responses, making ABPs particularly valuable tools for studying these events.[4−8]

ABPs can be used to monitor enzymatic activation within a cellular setting, thus providing relevant information about target regulation in the context of its native environment. This also alleviates the need to purify or recombinantly express each protein of interest. Some ABP tags including fluorophores can be used to visualize localized enzymatic activity within a cell.[9,10] The rapid readout of these probes makes them useful tools for studying the activation kinetics of an enzyme target, including real-time monitoring using ABPs containing quenched fluorophores.[11,12]

ABPs can also assess the biological targets and selectivity of small molecule inhibitors using a method known as competitive activity-based protein profiling (competitive ABPP) (Figure 2). In this strategy, a compound (or set of compounds) that produces a phenotype of interest is added to a sample followed by labeling with a broad-specificity ABP.[13,14] Using this method, it is possible to monitor the changes in activity of many related targets in response to inhibitor treatment. Furthermore, it is possible to begin to link

Received: July 1, 2013
Revised: August 7, 2013
Published: August 12, 2013

   dx.doi.org/10.1021/bi400854d | *Biochemistry* 2013, 52, 5985−5996

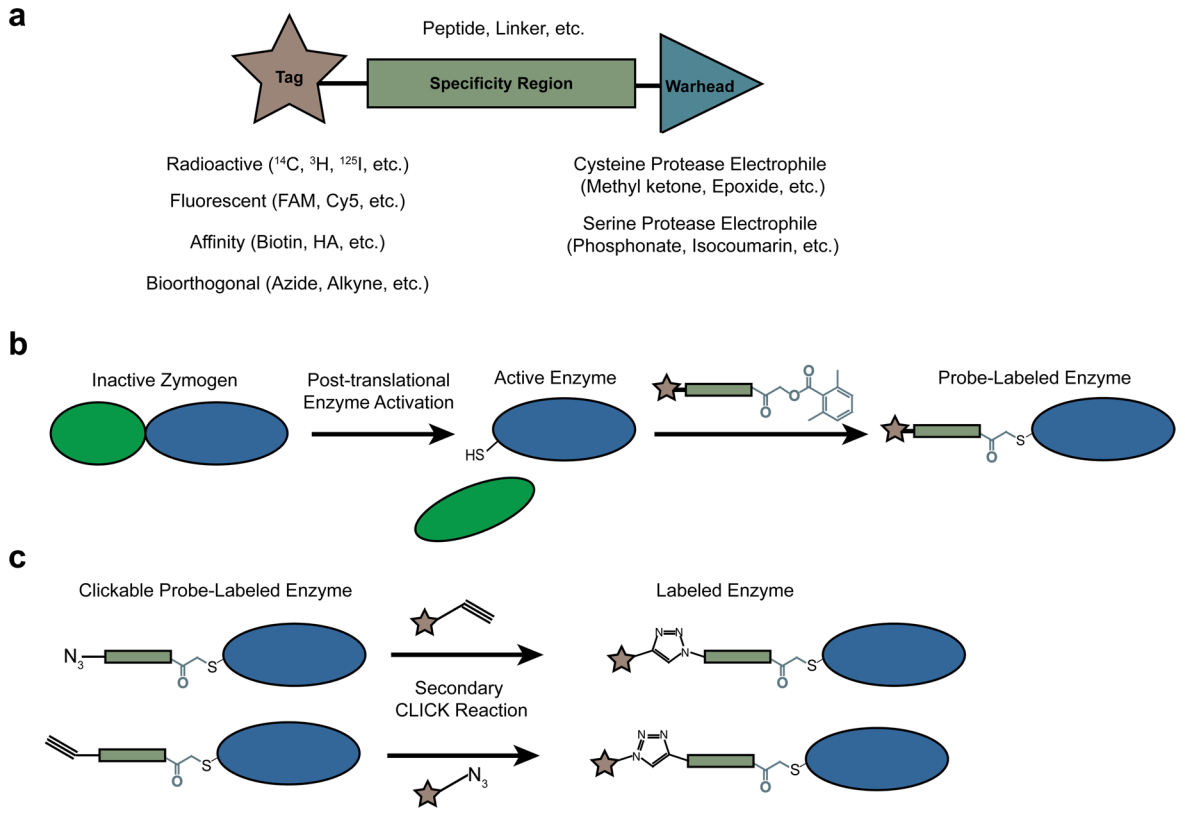


Figure 1. (a) Basic components of an activity-based probe (ABP). (b) General labeling mechanism of a target enzyme by an activity-based probe following post-translational enzyme activation. The green oval represents the zymogen pro-domain and the blue oval the enzyme catalytic domain. (c) Schematic of labeling target enzymes with clickable ABPs.

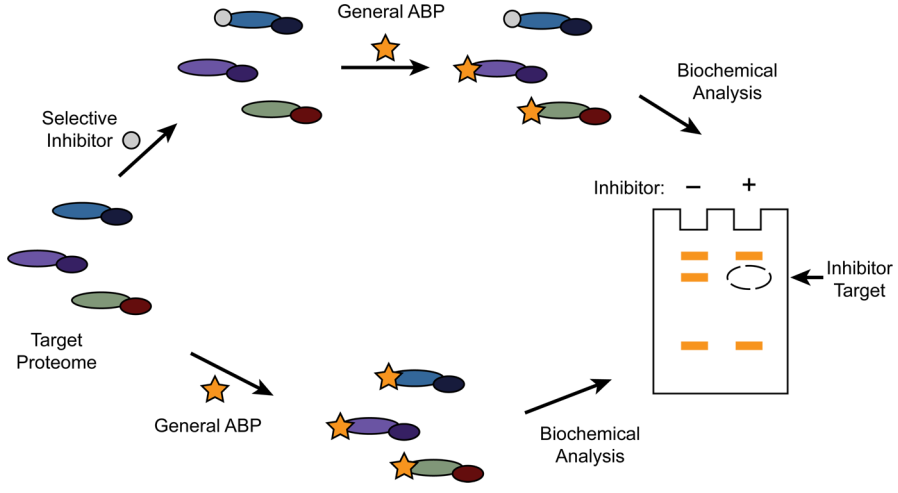


Figure 2. Scheme of the competitive activity-based protein profiling (competitive ABPP) method. A total proteome is preincubated with a specific small molecule (top route) followed by labeling of residual enzyme activities using a broad-spectrum ABP and analysis by gel electrophoresis. This sample is compared to a sample from the same proteome in which the ABP is directly used to label all active targets (bottom route). This method allows direct assessment of target specificity across many related enzyme targets and can be used to develop and characterize enzyme inhibitors.

a specific phenotype of a compound with inhibition of specific enzymatic targets. This approach also allows assessment of the overall selectivity of an inhibitor within a related class of enzymes and can be used to assess the pharmacodynamic properties of a drug.[13,14]

ABPs have broad utility in a number of areas of research. This review will give an overview of how ABPs have been applied to shed light on both bacterial infection mechanisms and host responses.

APPLYING ACTIVITY-BASED PROBES IN CHARACTERIZING BACTERIAL FACTORS ASSOCIATED WITH PATHOGENESIS

Using ABPs To Classify Hydrolases Involved in Bacterial Pathogenesis. The availability of genomic sequence information for many bacterial species has greatly improved the process of identifying new components of important biological processes such as pathogenesis mechanisms. However, biochemical and functional characterization of

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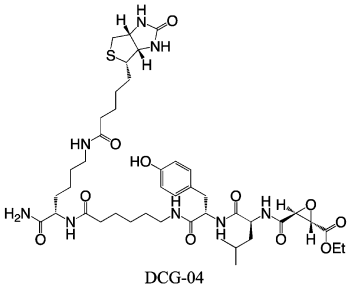
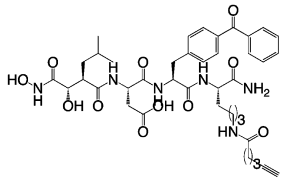
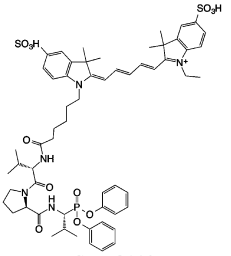
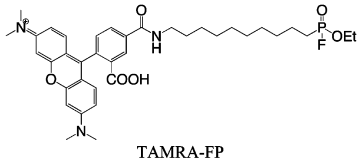
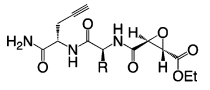
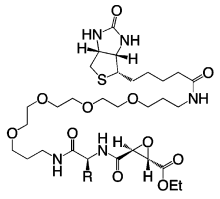
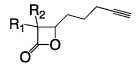
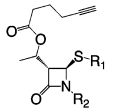
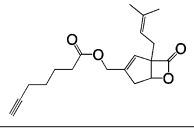
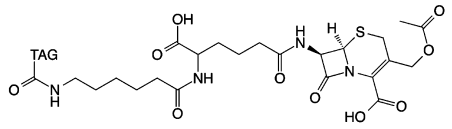
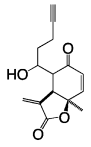
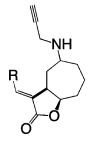
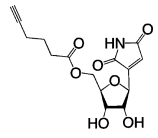
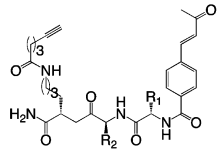
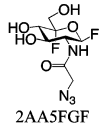
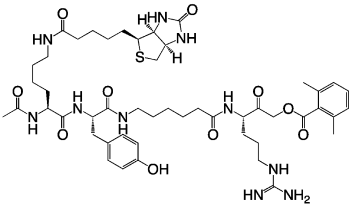
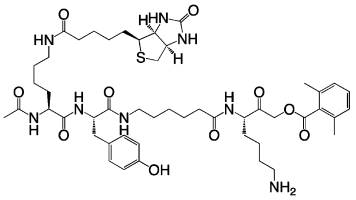
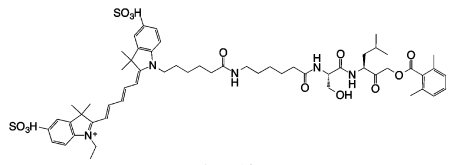
Table 1. Activity-Based Probes Used To Profile Bacterial Factors Associated with Pathogenesis

Probe	Target Enzyme
 DCG-04	*Staphylococcus epidermidis* Cysteine protease Ecp[16]
 AspR1	*Chlamydia trachomatis* Peptide deformylase (PDF)[17]
 Cy5-JO146	*Chlamydia trachomatis* Serine protease CtHtrA[18]
 TAMRA-FP	*Mycobacterium tuberculosis* Serine protease Chp1[19]
	*Clostridium difficile* Cysteine protease Cwp84[20]
	
	*Staphylococcus aureus* Serine protease ClpP, Various bacterial enzymes (hydrolases, ligases, transferases, oxidoreductases, etc.)[27-30]
	*Staphylococcus aureus* ClpP, β-Lactamase, MecR1, PBP2[32,33]
	*Listeria monocytogenes* Serine proteases ClpP1, ClpP2[34]

Probe	Target Enzyme
	*Streptococcus pneumoniae* Penicillin-binding proteins (PBPs)[35]
	*Escherichia coli* Polyketide/non-ribosomal peptide synthase c2450[36]
	
	*Staphylococcus aureus* Transferases MurA1, MurA2, PEP protein phosphotransferase (PtsI), Alkylhydroperoxide reductase C (AhpC)[37]
	*Staphylococcus aureus* Protein tyrosine phosphotases PTPA and PTPB, Secretory antigen SsaA2[38]
 2AA5FGF	*Vibrio cholerae*, *Pseudomonas aeruginosa* Family 3 β-D-glucosaminidase NagZ[41]
 BiRK	*Porphyromonas gingivalis* Arg-gingipain RgpB[25,44]
	*Porphyromonas gingivalis* Lys-gingipain Kgp[45]
 AWP19	*Clostridium difficile* toxin TcdB[47]

these gene products has remained difficult, yet is critical to fully understand these processes. Because ABPs label enzymes on the basis of their catalytic mode of action, they are ideal tools for biochemically classifying unknown proteins.[14]

dx.doi.org/10.1021/bi400854d | *Biochemistry* 2013, 52, 5985−5996

This includes enzymes with activities associated with bacterial pathogenesis.

The Gram-positive bacterium *Staphylococcus epidermidis* is often considered a commensal organism associated with the skin and mucous membranes of the host. However, *S. epidermidis* also causes nosocomial infections at surgery sites and in immunocompromised patients. The broad-spectrum papain family cysteine protease ABP DCG-04 was used monitor the activity of a putative protease with elastase activity termed Ecp in *S. epidermidis* cultures (Table 1).[15,16] Probe labeling showed that Ecp activity was both associated with the cell wall and released into the growth media. By correlating labeling intensity with culture density, the authors were able to show that the activity of the enzyme was growth phase-dependent and did not begin until midlog phase. Because the protease was labeled by DCG-04 and inhibited by other small molecules specific for cysteine but not serine proteases, the authors classified Ecp as a cysteine protease. N-Terminal sequencing identified Ecp as being homologous to the staphopains A and B, which are known virulence factors in *Staphylococcus aureus*. It is therefore possible that *S. epidermidis* virulence mechanisms are similar to those of its relative *S. aureus*.

An ABP was also used to help identify and classify a factor critical for the growth of *Chlamydia trachomatis* within host cells.[17] Two matrix metalloprotease (MMP) inhibitors were found to block chlamydial growth, and subsequent gene sequencing of mutants resistant to the compounds revealed a point mutation in the promoter of peptide deformylase (PDF). PDF is an enzyme that uses zinc to remove an N-terminal formyl group from bacterial proteins following translation. A hydroxamate-based ABP termed AspR1 was used to confirm that these inhibitors were indeed targeting PDF, as incubation with the inhibitors competed with activity-based labeling of the enzyme. This study identifies a new target with clinical potential for battling *C. trachomatis* infection.

Recently, a small molecule protease inhibitor library was screened against a purified serine protease, CtHtrA, from *C. trachomatis*. This screen identified a peptide diphenyl phosphonate inhibitor JO146 that was then used to characterize the protease function in the bacteria.[18] Inhibition of CtHtrA by JO146 resulted in the destruction of *Chlamydia* morphology and elimination of bacterium-containing inclusion bodies from infected host cells. This compound was also efficacious in mouse models of *Chlamydia* infection. The original inhibitor hit was then converted into an ABP by addition of Cy5 and biotin tags. These ABPs based on JO146 were used to confirm labeling of CtHtrA by both gel competition and proteomics analysis. This study defines an essential role for HtrA in *Chlamydia* pathogenesis and highlights a possible new drug target for *Chlamydia* treatment.

Mycobacterium tuberculosis synthesizes a sulfatide known as sulfolipid-1 (SL-1) that is implicated in virulence mechanisms including manipulation of the host immune response. Biosynthesis of SL-1 by the pathogen is therefore of great interest; however, the last steps of the synthetic pathway and subsequent transport have remained unknown. A recent study used bioinformatics to identify a putative membrane-anchored glycolipid acyltransferase termed Chp1 and then showed that this enzyme catalyzes sequential steps at the end of SL-1 biosynthesis.[19] The authors used the broad-spectrum serine hydrolase probe ABP TAMRA-fluorophosphonate to show that Chp1 possesses serine hydrolase activity. On the basis of this information, the authors hypothesized that Chp1 could be a target of the FDA-approved lipase inhibitor tetrahydrolipstatin (THL), which has been shown to be bacteriocidal. Chp1 was identified as a target of THL in *M. tuberculosis* culture, making pharmacological inhibition of Chp1 and SL-1 biosynthesis a promising approach for future studies of Chp1.

Clostridium difficile is a Gram-positive anaerobe that is highly antibiotic-resistant and the leading cause of hospital-acquired infections. The composition of *C. difficile*'s surface includes a robust S-layer, which is critical for both the integrity of the bacterium and its interactions with the host. The S-layer is composed of a low-molecular weight protein and a high-molecular weight protein, both of which are expressed as a single pro-peptide called SlpA. SlpA is then processed by a previously unidentified protease. To identify this enzyme, researchers tested several different classes of protease inhibitors and found that the cysteine protease inhibitor E-64 weakly inhibited SlpA processing.[20] On the basis of this information, the authors then made more potent, alkyne-tagged ABPs and used them to show that processing of SlpA most likely occurs following translocation into the S-layer, because incubation with the compounds led to an accumulation of unprocessed SlpA in the media. Because the labeled target appeared to be extracellular, the authors used biotin-labeled versions of the probes to label, affinity isolate, and identify the protease Cwp84. Subsequent coexpression experiments in *Escherichia coli* confirmed that Cwp84 processes SlpA, and a knockout has confirmed this protease is solely responsible for SlpA cleavage.[21] The authors have also recently improved upon the chemical tools initially used in their study by changing the reactive electrophilic warhead to a C-terminal Michael acceptor.[22]

Novel ABP Scaffolds for Dissecting Virulence and Antibiotic Resistance Mechanisms. While antibiotics are critical for combating bacterial infection, they are also valuable resources for understanding the host–pathogen interface. Some of the first chemical tools that can be characterized as activity-based probes were radiolabeled and resin-linked versions of the β-lactam antibiotic penicillin G, which were used to identify serine hydrolases termed penicillin-binding proteins (PBPs) in bacterial membranes (Figure 3).[23,24] This strategy has since been enhanced upon and has proven to be valuable for understanding mechanisms of antibiotic resistance and also for creating new classes of ABPs. These compounds have also been used to dissect the regulation and activity of virulence mechanisms in several species of pathogens (Figure 4). It is important to understand both the targets of antibiotic classes and the mechanisms

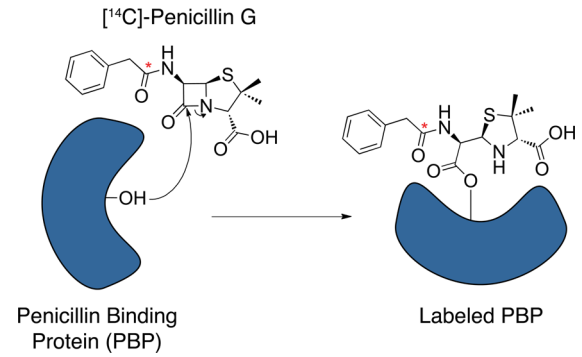


Figure 3. Labeling mechanism of the β-lactam antibiotic penicillin G. The red asterisk indicates the location of the ^{14}C radiolabel on the probe used in the original identification studies.

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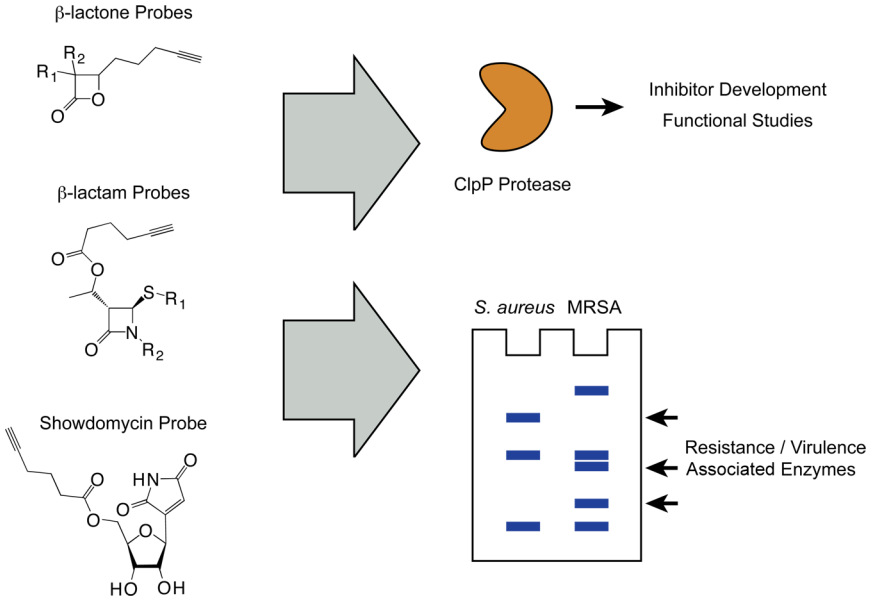


Figure 4. New antibiotic-based ABP scaffolds applied in studying mechanisms of bacterial virulence and resistance. As for the top arrow, β-lactone probes label the widely conserved, virulence-associated protease ClpP. Probes of this type were used to identify inhibitors of ClpP using competitive activity-based protein profiling (ABPP). As for the bottom arrow, probes containing antibiotic scaffolds are valuable for identifying differences in bacterial strains associated with antibiotic resistance, virulence, or other phenotypes of interest. MRSA denotes multiresistant *S. aureus*.

bacterial pathogens use to evade antimicrobial treatments in order to maximize the efficacy of these compounds.

In addition to commonly used electrophilic warheads such as acyloxymethyl ketones (AOMKs),[25] epoxides,[15] and fluorophosphonates,[26] the strained β-lactone ring has more recently been shown to be a viable electrophilic moiety for use in ABPs. Probes based on the β-lactone scaffold were successfully used to label a number of hydrolases in bacterial cultures, including the widely conserved serine protease ClpP (caseinolytic protein protease).[27,28] ClpP has been associated with virulence in species such as the Gram-positive facultative anaerobes *S. aureus* and *Listeria monocytogenes*. Competitive ABPP was subsequently used to create inhibitors for ClpP, and a refined inhibitor was shown to block activity of the enzyme in *S. aureus* cultures (Figure 4). Using this inhibitor, invasive proteolytic and hemolytic activities were confirmed to be regulated directly by ClpP in wild-type and methicillin-resistant (MRSA) strains.[29] In addition, these techniques were used to demonstrate that ClpP is upstream of several critical virulence factors in pyrogenic toxin superantigen (PTSA)-producing strains of *S. aureus*. These virulence factors included enterotoxins SEB and SEC3 as well as toxic shock syndrome toxin (TSST-1). Similar experiments were also performed in *L. monocytogenes* cultures and led to attenuation of virulence of this intracellular pathogen.[30] The authors also showed that ClpP inhibition downregulates the activity of the pore-forming toxin LLO as well as the virulence factor phosphatidylinositol-specific phospholipase C (PI-PLC).

"Clickable" ABPs contain a latent reactive group that allows conjugation of any desired tag to the probe using highly bioorthogonal CLICK chemistry following target labeling (Figure 1c).[3,31] ABPs of this type have been developed using common antibiotic and synthetic derivatives of the β-lactam scaffold as the primary recognition elements. These probes were used to label both lysates and cultures for several bacterial species.[32] Probes with modifications found in natural antibiotics primarily labeled PBPs, while those with novel substituents labeled other enzymes, including ClpP and the virulence-implicated β-lactamase. These probes were also used to profile differences between antibiotic-sensitive and methicillin-resistant strains of *S. aureus* (Figure 4).[33] This work identified several enzymes that were active in only MRSA cultures, including the known resistance-associated pair MecR1 and PBP2, as well as two novel hydrolases that could contribute to β-lactam resistance mechanisms in MRSA.

More recently, additional ABPs based on β-lactone and β-lactam scaffolds with improved specificity have been developed. Some bacterial species, including *L. monocytogenes*, have two ClpP isoforms known as ClpP1 and -2. However, first-generation ABPs based on the β-lactone moiety were able to label only ClpP2. Therefore, a more strained and therefore more reactive vibralactone-based probe was synthesized.[34] This ABP was able to label both ClpP isoforms and was used in *E. coli* coexpression studies to show that ClpP2 activates ClpP1 and that ClpP1 and ClpP2 hetero-oligomerize with each other. A probe based on the β-lactam antibiotic cephalosporin C that exhibits enhanced selectivity toward a subset of PBPs was also developed.[35] This probe was used to visualize active PBPs in a live unencapsulated derivative of the human pathogen *Streptococcus pneumoniae*.

In another study, a panel of α-alkylidene-γ-butyrolactones was synthesized and used to compare the activity profiles of enzymes in nonpathogenic and uropathogenic *E. coli*.[36] The probes identified the enzyme c2450 as being active in only the pathogenic strain. c2450 is a hybrid polyketide/nonribosomal peptide synthase involved in the synthesis of the virulence factor colibactin that activates the DNA checkpoint damage response in host cells, causing them to enter cell cycle arrest. This new insight into the structure and regulation of these important virulence factors highlights the power of activity-based protein profiling.

The antibiotic showdomycin strongly resembles the nucleoside uridine, but with an electrophilic maleimide moiety conjugated to the pentose ring instead of a base. To analyze the mode of action of this potent class of compounds, one study described an alkyne-tagged clickable version of the parent drug and used it to probe multiple strains of different bacterial species

dx.doi.org/10.1021/bi400854d | *Biochemistry* 2013, 52, 5985−5996

in culture (Figure 4).[37] After lysis and analysis of the labeled proteins, the authors identified several families of enzymes critical for pathogen colonization and survival. This included transferases MurA1 and MurA2 in *S. aureus*, which are essential for cell wall biosynthesis. Subsequently, the authors showed that while MurA2 is transcribed in four different *S. aureus* strains, it is active in only MRSA, highlighting the utility of a readout of activity levels. The MRSA strain was the only one resistant to higher concentrations of fosfomycin, a MurA inhibitor. These results provide a possible mechanism for the mode of action of showdomycin. The showdomycin-based ABP also more generally was able to probe pathogenesis mechanisms, by showing that phosphoenolpyruvate-protein phosphotransferase (PtsI) is active in only virulent toxin-producing *S. aureus* strains, including clinical isolates. In contrast, alkylhydroperoxide reductase C (AhpC) was labeled in only less pathogenic lab strain NCTC 8325. This work resulted in a highly novel ABP scaffold based on a naturally occurring maleimide electrophile that has already proven to be useful for gaining insight into differences between virulent and avirulent bacterial strains.

In theory, any compound containing an electrophilic moiety that has biological activity can be used to generate an activity-based probe. Cinnamic acid is a key flavor component that contains a Michael acceptor known to label cysteines. A library of peptides conjugated to cinnamic acid was used to make ABPs for detecting the activity of recombinantly expressed protein tyrosine phosphatases PTPA and PTPB from MRSA.[38] These probes were then used to profile the MRSA membrane proteome. Although the authors were unable to detect endogenous levels of PTPs in the samples, the probes did label the staphylococcal secretory antigen SsaA2. The function of this enzyme is unknown, but it has been implicated in *S. aureus* pathogenesis mechanisms, including sepsis and endocarditis. This unique class of probes may be useful for further studying this potentially important virulence factor.

The glycoside hydrolase NagZ is involved in peptidoglycan recycling in Gram-negative bacteria and has also been shown to regulate AmpC, a β-lactamase that confers resistance to several types of β-lactam-based antibiotics.[39,40] NagZ is an exoglycosidase that uses a catalytic nucleophile to hydrolyze *N*-acetylglucosamine residues during cell wall recycling. This nucleophile creates a covalent intermediate with its substrate, making it amenable to ABP profiling. An azide-labeled probe termed 2-azidoacetamido-2-deoxy-5-fluoro-β-D-glucopyranosyl fluoride (2AA5FGF) that stabilizes this covalent intermediate under acidic conditions and allows activity to be monitored after click chemistry attachment of a label was created.[41] This tool was used to identify a presumed 3β-D-glucosaminidase in *Pseudomonas aeruginosa* lysate. On the basis of molecular weight, the authors hypothesized this enzyme was the putatively annotated PA3005. This was confirmed following cloning, expression, and biochemical characterization of PA3005. More recently, it has been shown that chemical or genetic inactivation of NagZ in *P. aeruginosa* results in increased susceptibility to antipseudomonal β-lactam antibiotics,[42] highlighting the importance of this target in resistance mechanisms.

Using ABPs To Analyze Mechanisms Regulating Enzymes Involved in Bacterial Pathogenesis. *Porphyromonas gingivalis* is an anaerobic, Gram-negative pathogen that causes the widespread oral inflammatory disease periodontitis. A major component of *P. gingivalis* virulence involves the secretion of cysteine endoproteases called gingipains. Gingipains include the Arg-gingipains RgpA and RgpB and the Lys-gingipain Kgp. These proteases cleave their substrates after arginine or lysine residues and are involved in all phases of pathogenesis, from colonization and nutrient acquisition to neutralization of host immune responses.[43]

Like most proteases, gingipains are synthesized as inactive zymogens. Their trafficking and activation is regulated by post-translational proteolytic processing. Gingipain activation has been studied using ABPs containing the cysteine protease-specific acyloxymethyl ketone (AOMK) warhead.[25] In one study, a biotin-labeled Arg-Lys-AOMK called BiRK was used to dissect the proteolytic activation sequence of pro-RgpB expressed in *Saccharomyces cerevisiae*.[44] The authors used the probe at a subsaturating concentration to determine the relative activity levels of different forms of the enzyme during processing. They then compared this with the total amounts of each form present as determined by saturated probe labeling. Using this information, it was possible to demonstrate that RgpB gains activity during three discrete processing steps, including truncation of both the N- and C-termini, resulting in an overall 80-fold increase in activity. This finding was surprising because proteolytic activation of protease zymogens usually results in an activity increase of several orders of magnitude. This led the authors to conclude that the N-terminus of the zymogen may actually serve as a chaperone for folding of the catalytic domain, rather than as an inhibitor of the enzyme.

The gingipains Kgp and RgpA both function as large, noncovalent complexes on the surface of *P. gingivalis*. In a recent study, ABPs were used to examine the formation of these complexes by the Lys-gingipain Kgp.[45] The authors identified an oligomerization sequence motif present in both the catalytic and hemagglutinin-adhesin domains of Kgp thought to be essential for complex processing and assembly. To test this hypothesis, they genetically disrupted this motif within the catalytic domain of Kgp. The authors used an ABP to show that the mutant Kgp was no longer properly processed and assembled on the surface of the bacteria; instead, the active catalytic domain was secreted into the growth media. The probes also did not label the full-length zymogen, indicating that the prodomain is inhibitory. These two studies are examples of how ABPs can provide insight into the processing and activation mechanisms of critical *P. gingivalis* virulence factors.

C. difficile pathogenesis is also highly regulated by post-translational events. *C. difficile* secretes multidomain protein toxins, including the large glucosylating toxins TcdA and TcdB, that are responsible for a majority of the pathology associated with *C. difficile* infection (CDI).[46] These toxins contain an internal cysteine protease domain (CPD) that autoproteolytically activates the toxin upon binding the eukaryote-specific small molecule inositol hexakisphosphate ($InsP_6$) inside host epithelial cells. Our laboratory developed a set of covalent inhibitors and activity-based probes to study the substrate recognition and activation mechanisms of the TcdB CPD.[47] As with other proteases, the library of peptidic inhibitors provided a way to rapidly gain biochemical insight into the specificity of the CPD active site that was not obvious from structural analysis alone.[20,33]

The probe AWP19 was designed from the inhibitor library screening information and has subsequently been used to biochemically dissect the details of an allosteric circuit in the TcdB CPD that regulates activation of this toxin protease domain upon infection of a host cell.[48] The sensitivity of AWP19 allowed small differences in the activity levels of various CPD mutants to be quantified, allowing functional roles to be attributed to individual residues in the protein. Additionally, incubation of the

CPD with saturating concentrations of AWP19 revealed that basal amounts of the enzyme are labeled even in the absence of the activator $InsP_6$. This labeling was dependent on the presence of the CPD active site cysteine and was not due to contaminating $InsP_6$ from recombinant production of the CPD in *E. coli*. These data suggest that the CPD transiently adopts an active conformation without $InsP_6$, and this active conformation is stabilized by $InsP_6$.

AWP19 has also been used to compare the activation characteristics of the TcdB cysteine protease domain (CPD) from the historical *C. difficile* strain and the hypervirulent NAP1 variant within the context of the full-length holotoxin.[49] This study revealed that there is significantly more processing of the TcdB toxin from the hypervirulent strain ($TcdB_{HV}$) than the historical one. The authors also found that the active site of the full-length $TcdB_{HV}$ is less accessible to small molecule probes, leading them to hypothesize that the toxin CPD may have an increased affinity for its intramolecular substrate. These findings, together with studies that show $TcdB_{HV}$ is able to undergo conformational changes at a higher pH during endosome acidification, support the notion that the augmented activation characteristics of $TcdB_{HV}$ play a role in the hypervirulence of the NAP1 strain.[50]

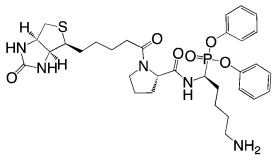
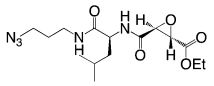
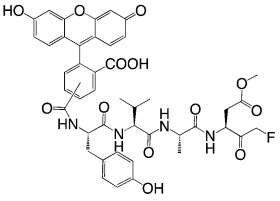
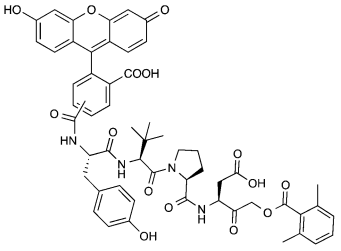
USING ACTIVITY-BASED PROBES TO PROFILE HOST RESPONSES TO BACTERIAL INFECTION

The host response to bacterial infection involves a complex set of protective and destructive programs, many of which are regulated by proteolytic activity. For example, both the caspase and cathepsin protease families play key roles in cell death and inflammatory responses.[51,52] Additionally, in many cases, the damaging effects of the host response contribute significantly to the disease pathology associated with bacterial infection. Because these processes involve hydrolases and are regulated post-translationally, ABPs are useful tools for studying both host defense mechanisms and contributions to bacterial pathogenesis. However, to date, relatively few classes of ABPs have been used for this purpose.

Mice infected with the attaching−effacing enteropathogen *Citrobacter rodentium* serve as a model for disease caused by human enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). *C. rodentium* causes mucosal inflammation in the intestinal tract, and the proteinase-activated receptors (PARs) are known to play a role in controlling intestinal inflammatory responses. One study showed that host proteases capable of activating PAR2 are released into the intestinal lumen upon infection with *C. rodentium*.[53] The authors used a diphenyl-phosphonate-based ABP called Bio-PK to characterize these host factors as serine proteases with trypsin-like activity (Table 2). On the basis of this result, the authors treated mice orally with soybean trypsin inhibitor (STI) and showed that it was possible to decrease the inflammation-based pathology associated with infection. These results provide a possible new target for reducing unproductive host inflammatory responses to enteropathogens.

Salmonella enterica serovar Typhimurium (*Salmonella typhimurium*) is a Gram-negative intracellular pathogen that invades host cells by triggering phagocytosis. One study used an ABP to monitor activation of the lysosomal cysteine protease cathepsin B in endocytic compartments containing *S. typhimurium*.[9] The authors synthesized an ABP with an epoxide warhead and a click tag and used it to image cathepsin B activity by microscopy (Figure 5). The authors discovered that active CatB was absent from vacuoles containing *Salmonella*, suggesting that the bacterium is capable of inhibiting CatB activation. Because the cathepsins are involved in inflammatory responses, the authors surmised that this could be part of *Salmonella*'s method for evading host defense mechanisms.

Table 2. Activity-Based Probes Used To Study Host Responses to Bacterial Infection

Probe	Target Enzyme
 Bio-PK	Trypsin-like serine protease activators of proteinase-activated receptor PAR_2 in response to *Citrobacter rodentium* infection.[53]
 Azido-E-64	Lysosomal cysteine protease cathepsin B in response to *Salmonella enterica* serovar Typhimurium infection.[9]
 Caspase-1 FLICA	Pro-inflammatory cysteine protease caspase-1 activation in response to several bacterial pathogens.[55-60,65-67]
 AWP28	Pro-apoptotic caspases-3, -7 and pro-inflammatory caspase-1 in response to bacterial infection.[10]

Using FLICA To Study Inflammasome-Mediated Caspase-1 Activation and Pyroptosis in Response to Bacterial Infection. The cysteine protease caspase-1 plays a critical role in controlling host inflammatory response to infection. A cytosolic, multiprotein complex called the inflammasome activates the procaspase-1 zymogen upon detection of multiple pro-inflammatory signals, including pathogen-associated molecular patterns (PAMPs) (Figure 5).[4,7] In phagocytes including macrophages, caspase-1 catalyzes the processing and subsequent release of pyrogenic cytokines, including interleukin-1β and interleukin-18. These signals mount an innate immune response to the offending stimulus. Caspase-1 also triggers the pro-inflammatory cell death program termed pyroptosis, which leads to osmotic lysis of the cell in an effort to control infection by destroying the replicative niche of the pathogen.[51,54] Because caspase-1 is activated post-translationally by the inflammasome in response to bacterial pathogens, it is an ideal target for studying using ABPs. The fluorescent inhibitor of caspases (FLICA) probes are commercially available fluoromethyl ketone (FMK)-based ABPs that have been used extensively to study the host response to a variety of bacterial pathogens. This includes species of *Shigella*,[55] *Chlamydia*,[56] *Mycobacterium*,[57]

dx.doi.org/10.1021/bi400854d | *Biochemistry* 2013, 52, 5985−5996

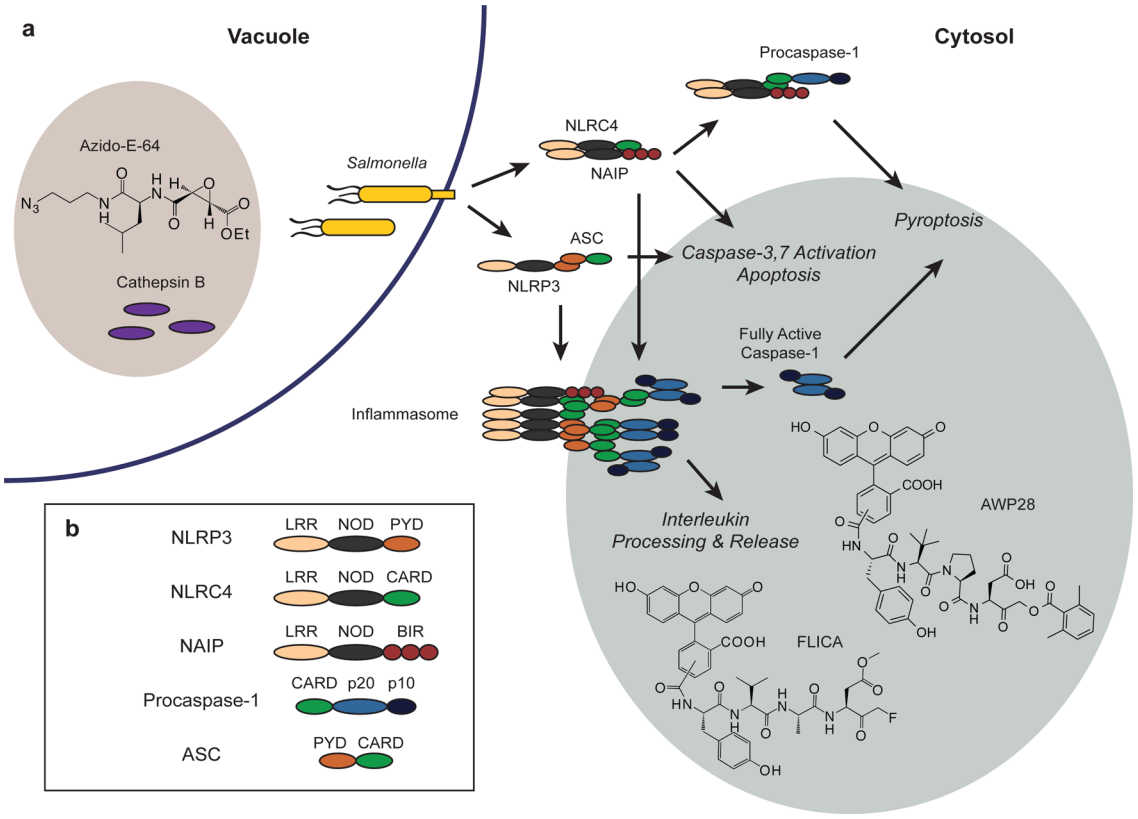


Figure 5. Applications of small molecule probes to the study of pathogen-induced inflammation pathways. (a) Inflammasome-mediated caspase-1 activation by the intracellular pathogen *Salmonella*. Caspase-1 activation leads to pyrogenic cytokine processing and release and also triggers the pro-inflammatory cell death program pyroptosis. The tan oval highlights the compound in which the activity-based probe Azido-E-64 has been used to study cathepsin B activity, and the gray oval highlights the reactions in which FLICA and AWP28 have been used to monitor caspase-1 activation during infection. Caspase-3 and -7 activation and apoptosis is triggered in the absence of inflammasome-mediated caspase-1 activation. (b) Protein domain structures of selected inflammasome components involved in *Salmonella* recognition. These proteins assemble via homotypic domain interactions.

Helicobacter,[58] and others, including some discussed here in more detail. FLICA is primarily used with host cell lines lacking specific response genes to determine whether these genes play a role in caspase-1 activation using microscopy or flow cytometry. These studies are useful for elucidating intracellular pathways involved in pathogen detection.

FLICA has been used to study the spatial organization of active caspase-1 in macrophages that have been infected by intracellular pathogens, including *S. typhimurium* (Figure 5). One study showed that the cytosolic receptors NLRP3 and NLRC4 respond to distinct *Salmonella* PAMPs upon infection, and they integrate these signals into a single, defined macromolecular focus that contains the adapter protein ASC.[59] Formation of this structure was independent of caspase-1, which is subsequently recruited to the focus. Microscopy with FLICA showed that while ~80% of the foci contained caspase-1 in wild-type cells, only ~10% contained active caspase-1. The authors used a caspase-1 inhibitor to determine that this focus is the site of interleukin processing. Subsequent work revealed that receptors containing a caspase recruitment domain (CARD), including NLRC4, trigger pyroptosis by directly associating with caspase-1 in the absence of ASC (Figure 5).[60] In these cases, the basal activity of the procaspase-1 zymogen is sufficient to cause pyroptosis. Microscopy with FLICA showed that no large focus containing active caspase-1 is formed in pyroptotic ASC knockout cells. This work spatially and biochemically decouples the cell death and cytokine processing roles of caspase-1 in response to bacterial infection.

Some bacterial pathogens actively manipulate host responses during infection. FLICA has been used with microscopy to dissect how species of the Gram-negative *Yersinia* genus trigger and manipulate cell death programs in infected macrophages. The *Yersinia* effector protein YopJ is secreted into host cells via the bacterium's type III secretion system (T3SS) and inhibits host survival signals, including mitogen-activated protein (MAP) kinase pathways, which can lead to apoptosis.[61] Other *Yersinia* T3SS effectors have also been implicated in caspase-1 inhibition.[62,63] FLICA was used to show that prestimulation of macrophages with toll-like receptor (TLR) agonists including LPS causes macrophages to activate caspase-1 and undergo pyroptosis in response to infection with *Yersinia pseudotuberculosis* in a YopJ-independent manner.[64] FLICA was also used to show that infecting macrophages with the KIM strain of *Yersinia pestis*, the causative agent of plague, triggers caspase-1 activation.[65] These cells release processed IL-1β and undergo cell death, but the death was found to be independent of caspase-1. Subsequent work showed that this strain harbors a form of YopJ that inhibits the host protein inhibitor of nuclear factor κ-B kinase β (IKKβ), and FLICA experiments in IKKβ knockout macrophages showed that infection of cells lacking IKKβ leads to increased levels of caspase-1 activation.[66] Together, these studies confirm that the interaction between *Yersinia* and its host consists of a balance of both apoptotic and pyroptotic cell death programs.

FLICA has also been used to show that active caspase-1 has a pro-survival role in response to some bacterial virulence factors.

dx.doi.org/10.1021/bi400854d | *Biochemistry* 2013, 52, 5985−5996

Many bacteria, including *Aeromonas* and *Listeria* species, use pore-forming bacterial toxins during infection, leading to the loss of intracellular potassium. One study used FLICA and flow cytometry to show that caspase-1 is activated by pore-forming proteins such as the *Aeromonas* toxin aerolysin.[67] Knockdown experiments confirmed that full caspase-1 activation involved contributions from NLRC4, NLRP3, and ASC. Furthermore, caspase-1 activation was shown to promote pro-survival membrane biogenesis pathways, and abrogation of these pathways results in an increased level of cell death. This study provides an example for another, noncanonical role for caspase-1 activity in response to bacterial pathogenesis.

FLICA as a Commercial Probe for Caspase-1. While the ABP FLICA provides a useful readout of caspase-1 activation in the study of host−pathogen interactions, caution must be taken when using these tools for imaging purposes. Several studies have shown that FMK-based probes are not selective for their caspase target and instead label additional active caspases and other classes of proteases such as cathepsins.[10,68−70] This has led to some confusion about how to interpret results generated using the FLICA probes.

For example, studies used FLICA with flow cytometry to show that caspase-1 activation by *Legionella* requires the cytosolic receptor NAIP5.[71] However, a 6-fold increase in the level of FLICA labeling was also observed in infected *Casp1*$^{-/-}$ macrophages compared to that in uninfected cells, which the authors attributed to cross-reactivity of the probe with other caspases. The use of these unselective probes to detect caspase-1 activity was subsequently questioned in a conflicting report.[72] This particular issue was finally resolved when biochemical reconstitution and mouse knockout experiments definitively proved a role for both NAIP5 and NLRC4 in the activation of caspase-1 upon detection of cytosolic flagellin from bacteria, including *Legionella*.[73,74]

FLICA labeling also produced confusing results in the previously highlighted work examining the role of caspase-1 autoproteolytic activation in cytokine processing and pyroptosis.[60] This study showed that when FLICA-labeled macrophages were examined by microscopy, labeling was found in areas where caspase-1 was not detected by a selective caspase-1 antibody. This led the authors to question the specificity of FLICA. Despite these reports and the known lack of selectivity for FMK-based probes, the scope of targets labeled by FLICA upon bacterial infection has not been analyzed biochemically.

Development and Use of AWP28 To Study Pyroptosis. Our laboratory has attempted to address the limitations of FLICA by creating an optimized activity-based probe for selective visualization of caspase activation in response to bacterial infection. This recently developed probe called AWP28 makes use of the AOMK electrophile that has greater overall selectivity for cysteine protease targets coupled with a more caspase-1-targeted peptide backbone.[10] Because of the overlapping substrate specificities of many proteases, including caspases, it remains difficult to target individual caspases.[75] While AWP28 labels multiple active caspases upon infection, the optimized properties of this probe allow the labeled caspases to be analyzed and identified by gel electrophoresis. Therefore, it was possible to use AWP28 to show that apoptosis is robustly triggered by *Salmonella* infection in macrophages lacking the ability to activate pyroptosis through the caspase-1 pathway. Furthermore, these studies confirmed that caspase-1 activity is required to bypass the apoptotic response, identifying a possible feedback mechanism between two critical cell death programs. This work may provide insight into a potential intracellular arms race between the host and pathogens capable of suppressing caspase-1 activation and also highlights the balance that exists between pro- and anti-inflammatory cellular responses.

CONCLUSIONS AND FUTURE USES OF CHEMICAL PROBES FOR STUDYING BACTERIAL PATHOGENESIS

Using small molecule tools to study biological processes can be complicated by difficulties accessing the intracellular environment. This is especially true in the case of bacteria, which possess many layers of resistance to chemical warfare. The outer membrane of Gram-negative bacteria is notoriously difficult to penetrate using small molecules, and many bacterial species possess arrays of efflux pumps.[76,77] It therefore can be difficult to use chemical probes to study the internal physiology of bacterial pathogens. Fortunately, there have been some successes developing cell permeable small molecule tools upon which to build. Activity-based probes (ABPs) tagged with a bioorthogonal moiety such as an azide or alkyne have been used to label intracellular enzymes in bacterial culture.[37]

Broadening the enzymatic targets of ABPs will also be critical to obtaining a holistic view of the molecular mechanisms involved in pathogenesis. The development of new ABP scaffolds requires an intimate understanding of the target enzyme family's mechanism of catalysis, because these probes harness this mechanism to label in an activity-dependent manner. A recently created probe targeting histidine kinases is promising, as these enzymes are involved in bacterial two-component signaling that can be critical for regulating bacterial virulence.[78,79]

The application of ABPs has diverged into two vastly different directions that both take advantage of their ability to report enzyme activity. One approach focuses on the use of general structures and electrophiles to globally profile the activity of a specific type of nucleophilic mechanism. For example, iodoacetamide has been used in reactive cysteine profiling to determine how the activity of nucleophilic cysteine residues varies within and between enzymes in complex proteomes.[80] These general techniques take advantage of powerful proteomics methods and can provide insight into how whole families of enzymes are regulated under distinct biological conditions. General ABPs can be used to determine the targets of compounds that create phenotypes of interest using competitive activity-based protein profiling.[13,29,81] Such probes are also capable of rapidly profiling differences between virulent and avirulent bacterial strains[36] and may therefore provide a means of functionally assessing different isolates. These techniques have a great deal of untapped potential for understanding pathogenic mechanisms in diverse strains.

A second application involves using ABPs for imaging purposes. In these cases, highly selective compounds are needed to ensure that the observed readout can be attributed to the activity of a specific target enzyme. This can be a difficult task because many families of related enzymes have overlapping substrate profiles.[75] This means the selectivity of an ABP can often not be made absolute by modifying the specificity region alone. One way to improve the selectivity of chemical probes is to make use of complementary genetic techniques. For example, "bump-hole" approaches can be used to modify an enzyme of interest so it is a unique target for a complementary chemical probe.[82] Recently, this approach has been applied to protease targets as well.[83,84] This technique is viable for studying bacterial virulence mechanisms in genetically tractable pathogens.

dx.doi.org/10.1021/bi400854d | *Biochemistry* 2013, 52, 5985−5996

Imaging enzyme activation is invaluable for dynamic host−pathogen interactions that are regulated post-translationally, and it will be exciting to see how this is accomplished in the future.

Many chemical biology tools languish following the proof of concept stage because they lack the simplicity and robustness necessary to answer biological questions. In contrast, activity-based probes are an example of a powerful technology that can be used to rapidly gain biological insight into a variety of enzymes and systems, including bacterial infection and host responses. The studies discussed here highlight the power of using these tools to dissect mechanisms of bacterial pathogenesis.

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Funding

A.W.P. was supported by a National Science Foundation Graduate Research Fellowship. This work was funded by National Institutes of Health Grant R01 EB005011 (to M.B.).

Notes

The authors declare no competing financial interest.

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