Activity-Based Protein Profiling of Microbes

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Abstract

Activity-Based Protein Profiling (ABPP) in conjunction with multimodal characterization techniques has yielded impactful findings in microbiology, particularly in pathogen, bioenergy, drug discovery, and environmental research. Using small molecule chemical probes that react irreversibly with specific proteins or protein families in complex systems has provided insights in enzyme functions in central metabolic pathways, drug-protein interactions, and regulatory protein redox, for systems ranging from photoautotrophic cyanobacteria to mycobacteria, and combining live cell or cell extract ABPP with proteomics, molecular biology, modeling, and other techniques has greatly expanded our understanding of these systems. New opportunities for application of ABPP to microbial systems include: enhancing protein annotation, characterizing protein activities in myriad environments, and reveal signal transduction and regulatory mechanisms in microbial systems.

Introduction

Activity-based protein profiling (ABPP) is a powerful strategy for dissecting the function of microbial systems relevant to human health, bioenergy production, and the environment. Characterization of enzymes, transporters, protein localization, regulatory dynamics, and new drug discoveries has been accomplished using chemical probes with advanced proteomic techniques. ABPP encompasses the application of an increasingly broad collection of probe motifs that vary in both protein reactivity and mode of binding in diverse biological samples. Chemical probes have been intuitively applied to cell extracts and living systems with characterization by gel electrophoresis, fluorescence microscopy, and/or mass spectrometry. To date, ABPP in microbial research have only begun to be applied, but great promise remains in research such as uncovering the mechanisms by which energy-relevant microbes and communities and microbial pathogens function and respond to external stimuli. Here, we discuss the basis of ABPP and its application to microbial systems.

ABPP—Probe Design Strategies

We broadly apply the term ABPP to a diverse collection of probes with multiple modes of reactivity *in vitro* and in living organisms (Table 1): (1) Mechanism-based probes report on function by reaction dependent upon the catalytic mechanism of the target enzymes; (2) Probes that mimic small-molecules/metabolites for identifying specific protein interactions, e.g., drugs or small molecule probes with electrophilic or photoreactive moieties; and, (3) Probes that report on protein regulatory modifications. Each probe type consists of three moieties: a reactive group that forms an irreversible covalent bond with a target protein, a binding or spacer group (e.g., protein substrate or metabolite) that directs a probe toward a protein or protein family and may also impart cell permeability, and a reporter tag for rapid and sensitive measurement of labeled enzymes by fluorescence or mass spectrometry-based proteomics (Figure 1). Probes are often designed “tag-free” with an alkyne or azide compatible with the bioorthogonal Huisgen-Sharpless [3+2] cycloaddition reaction (“click chemistry”) for multimodal reporter tag attachment [1]. This also decreases probe size, thereby minimizing undesirable impacts on reactivity with target proteins, and increasing cell permeability [2-5].

Activity-Based Protein Profiling with Antibiotic Derived Probes

Activity-based strategies are well suited for characterizing the mode of action by which drugs inhibit or activate an enzyme, enzyme family, or metabolic pathway, and for unraveling non-canonical drug metabolism pathways. Several ABPP studies have focused on antibiotics and antibiotic resistance. In 2008, Staub et al. synthesized natural and synthetic beta-lactam probes for application in antibiotic resistant *Bacillus licheniformis*, *Listeria welshimeri*, and *Pseudomonas putida* [6]. The beta-lactam probes labeled and inhibited a subset of the penicillin binding proteins including the virulence-associated enzyme ClpP and a resistance associated beta-lactamase. These results indicate potential highly selective targets for fine-tuned antibiotic

discovery. A similar ABPP approach was utilized to characterize the effects of the nucleoside antibiotic showdomycin. A tag-free showdomycin probe was applied to *Staphylococcus aureus* and inhibited enzymes involved in cell wall biosynthesis, notably the essential MurA1 and MurA2 [7].

In 2011, Eirich et al. sought to profile the targets of the antibiotic vancomycin. Despite an established mode of action, questions remain about the drug. These questions prompted researchers to develop a series of vancomycin probes enabled with a photoreactive moiety for binding target proteins and were then employed to identify non-canonical vancomycin targets in live *Staphylococcus aureus* and *Enterococcus faecalis*. Two uncharacterized targets were identified with likely roles in vancomycin's efficacy: autolysin in *S.aureus* and an ABC transporter in *E. faecalis* [8]. In a similar approach, derivatives of the β -lactam-containing antibiotic cephalosporin were used to identify active penicillin-binding proteins (PBPs) in both *Bacillus subtilis* PY79 and an unencapsulated derivative of D39 *Streptococcus pneumoniae*. The probes were applied *in vivo* to explore cell wall PBPs during cell division. Fluorescence microscopy indicated cephalosporin C-based probes as more selective than a penicillin V analogue, which labels all PBPs [9]. Similar approaches may lead to rational design of drug variants with improved selectivity and potency, and for identifying potential routes of microbial drug resistance.

Recently, an ABPP approach was employed with 4-hydroxyderricin, an isolate from the plant *Angelica keiskei* which features antibiotic potency against several strains of bacteria including *Staphylococcus aureus*. The 4-hydroxyderricin probe bound and inhibited seryl-tRNA synthetases (STS), enzymes crucial for the biosynthesis of proteins. Sample alkylating pretreatments resulted in reduced probe labeling suggesting that the probe was binding to one or more Cys residues in the protein. Mutation studies were then performed to selectively replace each of the five cysteines with alanine; interestingly all five mutant enzymes displayed no

catalytic activity emphasizing that a perturbation of just one Cys is sufficient to interfere with the amino acylation of tRNAs by STS. The results suggest that 4-hydroxyderricin inhibits STS activity by the alkylation of essential Cys residues within the enzyme and may induce apoptosis by halting protein biosynthesis. These results point towards a previously unrecognized role in regulation by this enzyme and illuminate it as a novel drug target [10]*.

Activity-Based Protein Profiling of Enzyme Function in Microbial Pathogens

Serine Proteases

Serine proteases are a widespread group of proteolytic enzymes critical to numerous physiological processes and implicated in microbial virulence mechanisms. Several probes to broadly characterize the serine hydrolase family of enzymes via a fluorophosphonate (FP) reactive group have been developed following initial work by Cravatt and coworkers [11]. While these probes are widely employed in mammalian systems, microbial research has lagged. Using a FP-probe multiplexed with a probe that targets enzymes with reactive cysteines in the fungus *Aspergillus fumigatus*, a human pathogen that is difficult to detect and for which no known virulence proteins are known, our group found that in the presence of host serum the organism alters physiological functions throughout numerous metabolic processes [12]. A follow-up study comparing *A. fumigatus* to two highly related but non-pathogenic fungi further confirmed the metabolic attenuation of *A. fumigatus* in the presence of the host, and the disparate metabolism in the presence of host was not identified in the other fungi [13]. Thereby, ABPP facilitated an improved understanding of the fungi's pathogenesis mechanism, and an array of proteins was identified as potential disease candidate biomarkers.

In a bacterial study, FP-probes detected the catalytic integrity of active site residues of the Rhomboid protein GlpG, a highly conserved intramembrane serine protease, in *E. coli*.

Residues were identified located on the cytoplasmic side of the lipid bilayer required for maximal proteolytic activity, highlighting the utility of ABPP in characterizing enzyme function [14].

Kinases/ATPases

Protein kinases and ATPases phosphorylate a wide range of proteins by hydrolysis of ATP and transfer of a single phosphate moiety, a critical mechanism of intracellular signaling. Commencing with work by Patricelli, researchers have created probes as ATP mimics that selectively label active kinases and other ATPases in native proteomes [15]. The limited functional annotation for *Mycobacterium tuberculosis* is a common problem in microbial research that can be addressed using ABPP. To better characterize the biology of *M. tuberculosis*, and in pursuit of novel drug targets, Ansong et. al. created a tag-free ATP probe resulting in the characterization of ~325 ATPases, including 72 previously identified as hypothetical or unknown [16]. Many of the identified proteins are both unique to Mycobacteria and essential for infection, suggesting specialized functions in mycobacterial physiology and pathogenicity. Two subsequent studies evaluated ATPase function during latency of the pathogen. Oxygen is an environmental cue that triggers *M. tuberculosis* to be either in a latent or active state, but the mechanism used to regulate this oxygen-dependent replication switch is undetermined. Wolf et. al. characterized ATPases in *M. tuberculosis* in both regular and hypoxic growth conditions, finding differential function in several proteins involved in energy metabolism and lipid biosynthesis in hypoxic versus normal growth [17]. The second study also evaluated aerated versus hypoxic growth and determined that kinase inhibition led to a bacterial survival defect and that specifically, the PknB kinase is a critical regulator of the oxygen-dependent replication switch and is a promising drug target [18]**.

In more targeted quantitative proteomic studies, a hypothemycin derived probe was developed to interrogate trypanosome Cys-Asp-Xaa-Gly motif (CDXG) kinases when coupled

with RNA interference strategies. The two kinases eliciting the greatest reduction in probe labeling after exposure to hypothemycin, TbGSK3short and TbCLK1, are also the only CDXG kinases whose knockdown by RNAi significantly reduced cell growth [19]. Combined, these results provide strong evidence that these kinases are integrally involved in *T. brucei* infection mechanisms, and demonstrate the value of ABPP for drug/inhibitor target selectivity and potency.

High-throughput methods with ABPP have also been used in bacterial kinase studies to identify new antibiotics that reduce streptokinase transcription without inhibiting bacterial growth. Streptokinase is a bacterial activator of human plasmin and enhances Group A Streptococcus (GAS) virulence. From the screening seven compounds were identified as inhibitors, and three were developed into tag-free photoreactive probes for subsequent determination of their cellular targets [20]. This research is still in the preliminary stages of biological testing, but the overall strategy from inhibitor identification, to probe development, and chemoproteomic target identification is highly promising for developing new antibiotics in the future.

Fatty Acid Synthases

Intriguing research ventured into the realm of *in vitro* and *in vivo* labeling of native acyl carrier proteins (ACPs) in both type I and II fatty acid synthases. Pantetheinyl-probes were applied to bacteria and found to cross the cell membrane before transformation intracellularly into CoA analogues *in vivo* by the endogenous CoA biosynthetic pathway. These studies successfully used the probes to enrich carrier proteins and allowed for the visualization and proteomic identification of fatty acid ACPs in *E. coli*, *B. subtilis*, *S. oneidensis*, and *B. brevis* lysates [21-23].

Glycoside Hydrolases

Renewable energy research has focused on the degradation and fermentation of lignocellulosic biomass and non-food crop fibers to produce biofuels. Historically, identifying functional enzymes responsible for biomass degradation is very challenging. A suite of probes based upon aglycone, N-halogenated glycosylamine, and 2-deoxy-2-fluoroglycoside scaffolds were developed for labeling cellulases, hemicellulases, xylanases, and other glycoside hydrolases [24]*. The suite was applied to the highly efficient lignocellolytic anaerobic bacterium *Clostridium thermocellum*, and proteomics provided a picture of the functional assembly of the cellulosome, a cell-adherent cellulolytic assemblage of proteins. A subsequent study designed to facilitate biorefinery and biotechnology applications of glycoside hydrolases used the same probes to characterize the functional response of enzymes to mild and significant perturbations in temperature, pH, and culture duration in the fungus *Trichoderma reesei* [25]. The suite of probes for glycoside hydrolases provides opportunities for optimizing biofuel processing conditions, and identifying enzymes for incorporation into enzyme “cocktails” for potentially improving biofuel production.

ABPP to Characterize Protein Redox Dynamics

Reduction and oxidation (redox) is a common post-translational protein modification playing myriad rolls in biological systems, including significant and often elusive roles in signaling and regulation. Cysteine thiol alkylating reagents such as iodoacetamide and N-ethylmaleimide have been used to quantitatively characterize redox reactive cysteines in biological systems, and can be applied in conjunction with isotopic labeling for highly precise quantitation [26,27]. To decipher proteins sensitive to H₂O₂ stress in *Pseudomonas aeruginosa* and *Staphylococcus aureus*, differing oxidation conditions and subsequent cysteine redox status were profiled using isotopic iodoacetamide probes with tandem proteolytic methods to identify probe labeled Cys residues [28]. Biochemical experiments and chemoproteomics elucidated highly sensitive Cys thiols, including the master quorum sensing regulator LasR, and redox-

regulated activities for acetaldehyde dehydrogenase ExaC, arginine deiminase ArcA, and glyceraldehyde 3-phosphate dehydrogenase GAPDH [29]**.

A challenge in characterizing protein redox dynamics is that cell lysis results in rapid oxidation, largely destroying the native intracellular redox state. Previously, multiple artificial reduction and oxidation steps have been used to induce protein redox while alkylating free thiols between each step in cell lysates, or by performing acid quenching concomitant with cell lysis [30,31]. To capture live cell biological redox phenomena in the biofuel relevant cyanobacteria *Synechococcus* sp. PCC 7002, tag-free electrophilic N-ethylmaleimide and iodoacetamide derived probes were redesigned to include a polyethylene glycol region to enable cell permeability. Two studies were designed to induce a change in cellular redox, and provide insight into post-translational redox modifications of proteins in metabolic pathways pertinent to biofuel or high value added small molecules. The first study evaluated a transition in carbon availability, from C-limited to C-starved then to C-replenished, with *in vivo* probe labeling throughout a 60-minute evaluation of redox dynamics [32]. Dramatic changes in the redox state were characterized for 176 proteins within 30 seconds of carbon reintroduction by label-free quantitative proteomics, with subsequent identification of probe-labeled Cys residues using the tandem protease cleavage method previously described [28,29]**. This study employed high-resolution confocal fluorescence microscopy and protein identification and LC-MS/MS-based proteomics approaches [32]. A second study profiled Cys thiol redox in cells experiencing nutrient replete, carbon limited, and nitrogen limited cells when transitioning from light to dark irradiance. Nutrient deficient cultures had increased levels of Cys thiol reduction than nutrient replete samples, a phenomenon indicative of unbalanced electron transport and ATP synthesis [33]. Both studies yielded novel proteins undergoing redox dynamics while also confirming well known redox regulated proteins, and provide new clues for redox 'switches' that may be coupled directly to metabolic output.

Conclusion

Human health, energy production, environmental remediation, and climate change all are tightly coupled to microbial physiology. As human microbiome research has already demonstrated, humans and the planet as a whole have a deep connection to microbes for nearly all aspects of life. However, the vast diversity of microbes, the plurality of microbial interactions, and the breadth of environments they reside places researchers at a point in which we have hardly scratched the surface of microbial discovery. Harnessing the utility of ABPP for continued microbial research will facilitate our understanding of protein functions, interactions, and regulation. Moving forward, the multimodal nature of ABPP enables the use of mass spectrometry-based proteomics, and also powerful microscopy and imaging techniques, such as fluorescence and electron microscopy. Taken together, microbial metabolism, community functions and assemblages, interactions, spatiotemporal dynamics, and response to perturbation can be untangled with increasing effectiveness.

Acknowledgements

We thank Richard D. Smith for his careful edits and insightful suggestions. Portions of this research were supported by the Department of Energy Office of Biological and Environmental Research Genome Sciences Program under the Pan-omics and Foundational Scientific Focus Area projects, and the DOE-BER Bioimaging Pilot Project program; additional resources supporting research described herein was provided via the Environmental Molecular Sciences Laboratory, a DOE-BER national scientific user facility at Pacific Northwest National Laboratory (PNNL) in Richland, Washington. PNNL is operated by Battelle for the DOE under contract DE-AC05-76RLO-1830.

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Table

Representative probes and their application		
Probe target	Structure	Applications
Antibiotic reactive proteins		Showdomycin derived probe was applied to <i>S. aureus</i> to identify the antibiotic's targets. Cell wall biosynthesis enzymes MurA1 and MurA2 were identified [7].
Serine proteases		Studies to identify virulence mechanisms in <i>A. fumigatus</i> [12,13]. Characterized Glpg in <i>E. coli</i> [14].
ATPases		Studies performed to determine pathogenicity and features of latency in <i>M.tuberculosis</i> [16-18]. PknB was identified as a critical regulator and replication switch [18].
Fatty acid synthases		Pantetheinyl probes crossed the cell membrane and were transformed into CoA analogues in <i>E. coli</i> , <i>S. oneidensis</i> , and <i>B. brevis</i> [21-23]
Glycoside hydrolases		Used to profile active cellulose degrading enzymes in <i>C. thermocellum</i> [24] and <i>T.reesei</i> [25] for biofuel application.
Redox reactive proteins		H ₂ O ₂ sensitive proteins were profiled in <i>P. aeruginosa</i> and <i>S. aureus</i> ; LasR, ExaC, ArcA, and GAPDH were among the highlighted targets [29]. Redox reactive proteins were profiled in vivo during various nutrient levels in <i>Synechococcus</i> [32,33].

Figure 1. “Tagged” versus “Tag-Free” activity-based protein profiling. “Tagged” probes directly incorporate the reporter or enrichment moiety directly into the probe structure. “Tag-free” probes are enabled for use in [3+2] Huisgen-Sharpless cycloaddition reactions, delivering the reporter moiety post-protein labeling. Both variants can be used for subsequent SDS-PAGE, fluorescence imaging, or LC-MS proteomic analysis of probe targets.

Figure

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Probe design and classification

Probe labeling of reactive proteins

Tagged ABPP

Tag-free ABPP