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Activity-Based Protein Profiling in Vivo Using a Copper(I)-Catalyzed Azide-Alkyne [3 + 2] Cycloaddition

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The field of proteomics is confronted with the awesome task of assigning structure and function to the tens of thousands of protein products encoded by prokaryotic and eukaryotic genomes. To realize this goal, new technologies are required that permit the analysis of many proteins in parallel and in samples of high complexity. Proteomic researchers also must face the challenge of characterizing proteins in a dynamic cellular environment in which these biomolecules are subject to myriad posttranslational modifications and the actions of various activators and inhibitors.¹ Here, we report an advanced strategy for activity-based protein profiling (ABPP) that addresses these important needs.

ABPP is a chemical proteomic method that employs active site-directed probes with broad target selectivity, either within or among enzyme classes, to simultaneously visualize changes in the activity of many proteins in cell, tissue, and fluid samples.² To date, ABPP probes have been developed for several enzyme classes, including serine³ and cysteine hydrolases⁴ and multiple oxidoreductases.^{5,6} However, current protocols for ABPP require that cells and tissues are first homogenized before treatment with probes. As a result, proteins are removed from their native cellular environment, and the effects of endogenous activators/inhibitors and subcellular localization on protein function may be obscured. Thus, there is a need to advance ABPP so that this strategy can be generally applied to viable cells and organisms.

A persistent technical challenge facing the utilization of ABPP probes in vivo is the adverse effect of their bulky reporter tags (fluorophore/biotin), which may inhibit cellular uptake and unduly influence probe distribution within the cell. In select cases, ABPP probes have been used to label enzymes in living cells,⁷ but these proteins reside in organelles, such as the lysosome, that can be accessed by chemical reagents without the need to cross cell membranes. Thus, we anticipated that a general strategy for carrying out ABPP in vivo would require a method for attaching the reporter tag to the probe after proteins have been covalently labeled. Toward this end, we sought to engineer a pair of biologically inert coupling partners into the tag and probe that would react at useful rates at submillimolar concentrations to produce a stable product in high yield. Examples of bio-orthogonal coupling reactions include the Staudinger ligation of azides with triaryl phosphines,⁸ the ketone/aldehyde-hydrazine reaction,⁹ and Huisgen's 1,3-dipolar azide-alkyne cycloaddition.¹⁰ However, these reactions can be quite slow, especially in aqueous solutions of high biomolecular complexity, and thus may not be directly applicable for detecting activity-based labeling events in whole proteomes.

In the course of their click chemistry endeavors,¹¹ the Sharpless group has reported a Cu(I)-catalyzed, stepwise analogue of Huisgen's concerted triazole synthesis¹² which they and the Finn group have recently used to modify purified virus particles with fluorescent dyes.¹³ To test whether this ligation reaction could be applied to profile enzyme activities in whole proteomes, we synthesized a

Scheme 1

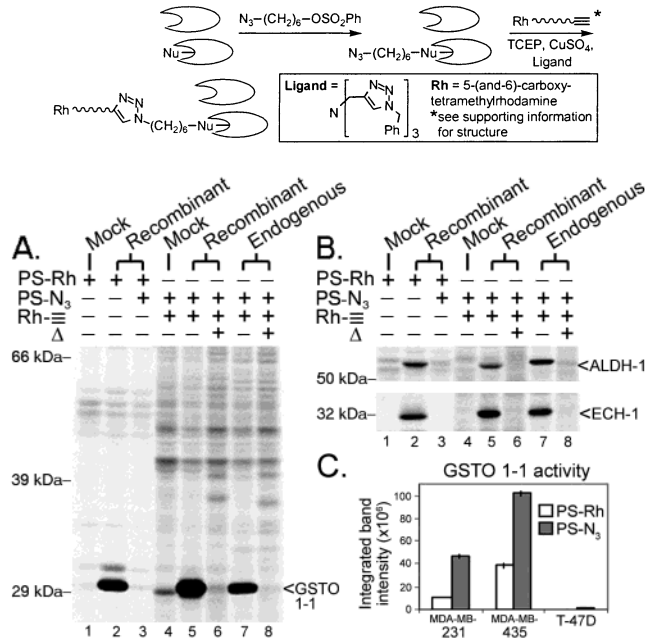


Figure 1. In vitro labeling of enzymes in complex proteomes by PS-Rh and PS-N₃. Labeling of GSTO 1-1 (A), ALDH-1 (B, upper panel), and ECH-1 (B, lower panel) in recombinant (transfected COS cells) and endogenously expressed (MDA-MB-435 cells, mouse liver, and mouse heart, respectively) forms. Mock, transfected with empty vector. (C) GSTO 1-1 activity in human breast cancer lines measured by in-gel fluorescence scanning of PS-Rh- and PS-N₃-labeled proteomes (*n* = 3 per group; arbitrary units). Fluorescent gel images shown in gray scale.

rhodamine-alkyne tag (Rh≡) and an azide-derivatized phenyl sulfonate ester reactive group (PS-N₃) (Scheme 1).

Previous studies have identified several enzymes labeled in an active site-directed manner by a rhodamine-tagged phenyl sulfonate probe (PS-Rh) including glutathione *S*-transferases (GSTO 1-1), aldehyde dehydrogenases (ALDH-1), and enoyl CoA hydratases (ECH-1).^{5,6} To initially test the azide-alkyne cycloaddition reaction in whole proteomes, homogenates of GSTO 1-1-transfected COS cells (2 mg/mL protein in phosphate buffer, pH 8.0) were treated with PS-N₃ (2.5 μM) for 30 min, followed by Rh≡ (20 μM), CuSO₄ (1 mM), tris(carboxyethyl)phosphine (TCEP; 1 mM), ligand (2 mM), and 5% *tert*-butyl alcohol. Upon addition of ligand, proteins precipitated out of solution; however, this phenomenon did not significantly impede the cycloaddition reaction. After 1 h, proteins were separated from excess reagents by centrifugation and analyzed by SDS-PAGE and in-gel fluorescence scanning.

Robust labeling of GSTO 1-1 was observed following the cycloaddition reaction (Figure 1A, lane 5). This labeling was heat sensitive (lane 6) and depended on CuSO₄ (Supporting Information).

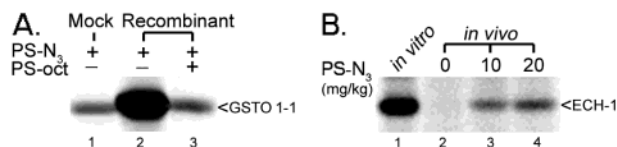


Figure 2. In vivo labeling of proteins by PS-N₃. (A) Labeling of GSTO 1-1 in viable COS cells. Pretreatment of cells with PS-oct (100 μ M) blocked labeling. (B) Labeling of ECH-1 in mice. PS-N₃ was administered to mice (0, 10, 20 mg/kg; i.p.) and after 1 h, animals were sacrificed and heart tissue removed and analyzed by click chemistry-based ABPP. In vitro lane: PS-N₃ labeling of ECH-1 in mouse heart homogenate.

Interestingly, TCEP or ligand was also required, but not both (Supporting Information), suggesting that endogenous sources of reductant or stabilizers of Cu(I) may exist in the proteome. Time course and concentration dependence experiments indicated that complete labeling could be achieved in 1 h with 50 μ M Rh \equiv (Supporting Information). Click chemistry-based ABPP also detected GSTO 1-1 at endogenously expressed levels in homogenates of the human breast cancer cell line MDA-MB-435 (lane 7), as well as two additional PS-Rh targets, ALDH-1 and ECH-1, in COS cells and mouse tissue proteomes (Figure 1B, lanes 5 and 7). For all three enzymes (GSTO 1-1, ALDH-1, ECH-1), excess PS-N₃ blocked labeling by PS-Rh (Figure 1A and B lane 3), indicating that this azide reagent modifies the active sites of these enzymes. In contrast, some targets of the PS-Rh probe were not sensitive to competition by PS-N₃ (e.g., phosphofructokinase¹⁴) and, correspondingly, were not visualized with click chemistry-based ABPP (data not shown). These enzymes may interact with both the sulfonate and rhodamine groups of PS-Rh. Finally, the levels of GSTO 1-1 in three breast cancer lines were quantified by both click chemistry-based and standard ABPP methods (Figure 1C). Consistent with previous findings,⁶ both approaches determined that the estrogen receptor (ER)-negative lines MDA-MB-435 and MDA-MB-231 expressed more GSTO 1-1 than the ER-positive line T-47D. Notably, stronger fluorescent signals were observed in cell proteomes treated with PS-N₃ than with PS-Rh, suggesting that removal of the rhodamine tag accelerated the rate of sulfonate labeling of GSTO 1-1. For other enzyme targets (ALDH-1 and ECH-1), similar signals were observed with standard and click chemistry-based ABPP (Figure 1B, lanes 2 and 5, respectively). Collectively, these results reveal that azide-alkyne cycloaddition chemistry can be used to profile enzyme activities in complex proteomes with a sensitivity that rivals standard ABPP methods.

We next tested whether click chemistry-based ABPP could detect protein activities in vivo. Viable COS cells expressing GSTO 1-1 were treated with 10 μ M PS-N₃ for 1 h, thoroughly washed, homogenized, and reacted with Rh \equiv . A strong fluorescent signal was seen for GSTO 1-1 (Figure 2A), indicating that PS-N₃ labeled this protein in living cells. Consistent with this labeling event being active site-directed, fluorescence was abolished by pretreatment of cells with 100 μ M octyl phenyl sulfonate (PS-oct) (Figure 2A). We then tested whether PS-N₃ could label enzyme targets in living animals. Mice were injected with 0–20 mg/kg of PS-N₃, sacrificed after 1 h, and heart tissue removed, homogenized, and reacted with Rh \equiv . A clear fluorescent signal coinciding with the molecular mass of ECH-1 was observed in heart tissue from mice treated with 10 or 20 mg/kg PS-N₃, but not in sham-injected mice (Figure 2B). Collectively, these data indicate that azide-alkyne cycloaddition chemistry can be used to profile enzyme activities in living cells and organisms.

In summary, we have demonstrated the ability to label enzymes in vitro and in vivo with an azido ABPP probe and detect these labeled proteins in whole proteomes by copper(I)-catalyzed ligation with a rhodamine-alkyne reagent. This methodology offers several

potential advantages relative to standard methods for ABPP. First, replacement of the bulky fluorescent tag with a sterically inconspicuous azide group should furnish probes that are more able to distribute in an unbiased manner within a living cell, tissue, or organism. Likewise, the variable and often antagonistic effect of the fluorescent tag on probe binding affinity for specific proteins is also eliminated. Finally, the use of azide-alkyne cycloaddition chemistry should streamline probe synthesis by removing the need to generate and purify large quantities of structurally diverse fluorophore-tagged reagents. However, a current limitation of click chemistry-based ABPP is the higher background signals that were observed with this method as compared to standard ABPP (Figure 1). These background signals were dependent on the concentration of PS-N₃ (Supporting Information), which appears to exhibit a greater inherent reactivity than PS-Rh. Accordingly, we anticipate that designing N₃-probes with higher affinity for their cognate enzymes will enable their application to proteomes at much lower concentrations, achieving a corresponding increase in signal-to-noise. With these refinements, cycloaddition-based ABPP should emerge as a unique and versatile method at the forefront of strategies for functional proteome analysis.

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Supporting Information Available: Synthesis and experimental protocol; characterization of cycloaddition reaction (PDF). This material is available free of charge via the Internet at: <http://pubs.acs.org>

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