Mapping Enzyme Active Sites in Complex Proteomes

Gregory C. Adam,[†] Jonathan Burbaum,[‡] John W. Kozarich,[‡]
Matthew P. Patricelli,*,[‡] and Benjamin F. Cravatt*,[†]

*Contribution from The Skaggs Institute for Chemical Biology and
The Departments of Chemistry and Cell Biology, The Scripps Research Institute,
10550 North Torrey Pines Road, La Jolla, California 92037, and
ActivX Biosciences, Inc., 11025 North Torrey Pines Road,
La Jolla, California 92037*

Received September 10, 2003; E-mail: cravatt@scripps.edu; mattp@activx.com

Abstract: Genome sequencing projects have uncovered many novel enzymes and enzyme classes for which knowledge of active site structure and mechanism is limited. To facilitate mechanistic investigations of the numerous enzymes encoded by prokaryotic and eukaryotic genomes, new methods are needed to analyze enzyme function in samples of high biocomplexity. Here, we describe a general strategy for profiling enzyme active sites in whole proteomes that utilizes activity-based chemical probes coupled with a gel-free analysis platform. We apply this gel-free strategy to identify the sites of labeling on enzymes targeted by sulfonate ester probes. For each enzyme examined, probe labeling was found to occur on a conserved active site residue, including catalytic nucleophiles (e.g., C32 in glutathione S-transferase omega) and bases/acids (e.g., E269 in aldehyde dehydrogenase-1; D204 in enoyl CoA hydratase-1), as well as residues of unknown function (e.g., D127 in 3β-hydroxysteroid dehydrogenase/isomerase-1). These results reveal that sulfonate ester probes are remarkably versatile activity-based profiling reagents capable of labeling a diversity of catalytic residues in a range of mechanistically distinct enzymes. More generally, the gel-free strategy described herein, by consolidating into a single step the identification of both protein targets of activity-based probes and the specific residues labeled by these reagents, provides a novel platform in which the proteomic comparison of enzymes can be accomplished in unison with a mechanistic analysis of their active sites.

Introduction

In the postgenome era, chemical and biological researchers are confronted with an unprecedented challenge of assigning molecular and cellular functions to the numerous protein products encoded by prokaryotic and eukaryotic genomes. The field of proteomics aims to accelerate this process by developing and applying methods for the global analysis of protein expression and function in samples of high biocomplexity.[1] Proteomic methods for the quantitative analysis of protein expression, including two-dimensional (2D) gel electrophoresis[2] and isotope coded affinity tagging (ICAT),[3] have provided important information on the relative expression levels of proteins in cell, tissue, and fluid samples. Still, these approaches, by measuring protein abundance, provide only an indirect assessment of protein activity and, therefore, may fail to detect important posttranslational forms of protein regulation such as those mediated by protein–protein and/or protein–small molecule interactions.[4] To address this challenge, a chemical strategy

for functional proteomics has been introduced called activity-based protein profiling (ABPP) that utilizes active site-directed probes to determine the functional state of enzymes in complex proteomes.[5,6] Chemical probes for ABPP consist of at least two elements: (1) a reactive group (RG) for binding and covalently modifying the active sites of many members of a given enzyme class or classes, and (2) one or more reporter groups, like biotin and/or a fluorophore, for the rapid detection and isolation of probe-labeled enzymes. Because ABPP probes, in general, possess moderately electrophilic RGs, they are poised to selectively modify enzyme active sites, which are enriched in nucleophilic residues important for catalysis.

To date, two general strategies for the design of ABPP probes have been introduced: (1) a directed approach, in which individual probes are designed to target specific enzyme classes,[5,7,8] and (2) a nondirected approach that utilizes libraries of probes to profile enzymes from several different classes.[9] Directed approaches for ABPP have capitalized on a rich history

† The Scripps Research Institute.
‡ ActivX Biosciences, Inc.
(1) Patterson, S. D.; Aebersold, R. H. *Nat. Genet.* **2003**, *33*, 311−323.
(2) Patton, W. F.; Schulenberg, B.; Steinberg, T. H. *Curr. Opin. Biotechnol.* **2002**, *13*, 321−328.
(3) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994−999.
(4) Kobe, B.; Kemp, B. E. *Nature* **1999**, *402*, 373−376.

(5) Liu, Y.; Patricelli, M. P.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14694−14699.
(6) (a) Cravatt, B. F.; Sorensen, E. J. *Curr. Opin. Chem. Biol.* **2000**, *4*, 663−668. (b) Adam, G. C.; Sorensen, E. J.; Cravatt, B. F. *Mol. Cell. Proteomics* **2002**, *1*, 781−790.
(7) (a) Kidd, D.; Liu, Y.; Cravatt, B. F. *Biochemistry* **2001**, *40*, 4005−4015. (b) Patricelli, M. P.; Giang, D. K.; Stamp, L. M.; Burbaum, J. J. *Proteomics* **2001**, *1*, 1067−171. (c) Jessani, N.; Liu, Y.; Humphrey, M.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 10335−10340.

of mechanistic studies of particular enzyme classes to create chemical probes with predictable proteome reactivities. By incorporating as the RG well-known affinity labels, ABPP probes have been developed that target, for example, serine hydrolases[5,7] and cysteine proteases.[8] In each of these cases, the designed probes have been shown to label their target enzymes in an activity-based manner, distinguishing, for example, active enzymes from their inactive zymogens and/or inhibitor-bound forms.[5,7,8]

Although directed ABPP has proven effective for addressing enzymes such as hydrolases, which possess known covalent inhibitors, this strategy is less straightforward to apply to many other enzyme classes that lack cognate affinity labels. To expand the number of enzyme classes addressable by ABPP, a nondirected, or combinatorial strategy has been introduced in which libraries of candidate probes are screened against complex proteomes for specific protein labeling events, which were defined as those that occurred in native, but not heat-denatured proteomes.[9] Heat-sensitive probe−protein reactions were predicted to occur in structured small molecule-binding sites that would often determine the biological activity of proteins, such as the active sites of enzymes. Using this nondirected method for ABPP, a library of probes bearing a sulfonate ester (SE) RG was screened against cell and tissue proteomes and found to label members of at least nine mechanistically distinct enzyme classes.[9] These probe−enzyme reactions were heat-sensitive and competed by substrates/cofactors, suggesting that they occurred in enzyme active sites. Still, direct evidence that SE probes label the active sites of their enzyme targets required a general strategy to identify probe-modified residues in these proteins. Such a method would also clarify whether SE probes label catalytically important residues, conceivably a prerequisite for these agents to serve as effective tools for ABPP. Indeed, unlike hydrolase-directed ABPP probes, which could be expected to label the serine or cysteine nucleophile of their target enzymes, the residues modified by SE probes are more difficult to predict, especially considering that several SE-labeled enzymes do not utilize covalent catalysis.[9]

Here, we describe the use of a novel gel-free platform for ABPP to map the sites of SE labeling on several enzymes isolated from complex proteomes. These studies reveal that SE probes are remarkably versatile activity-based profiling reagents capable of labeling a diversity of catalytic residues in a range of mechanistically distinct enzymes.

Results and Discussion

A Gel-Free Strategy for Activity-Based Protein Profiling (ABPP). To convert the standard ABPP method, which utilizes 1D or 2D gels to separate and detect probe-labeled proteins,[5,7−9] to a gel-free platform suitable for identifying probe-labeling sites, several methodological changes were introduced (Figure 1). Following treatment with a rhodamine-tagged phenyl-SE probe (PS-rhodamine), proteomes were denatured and their
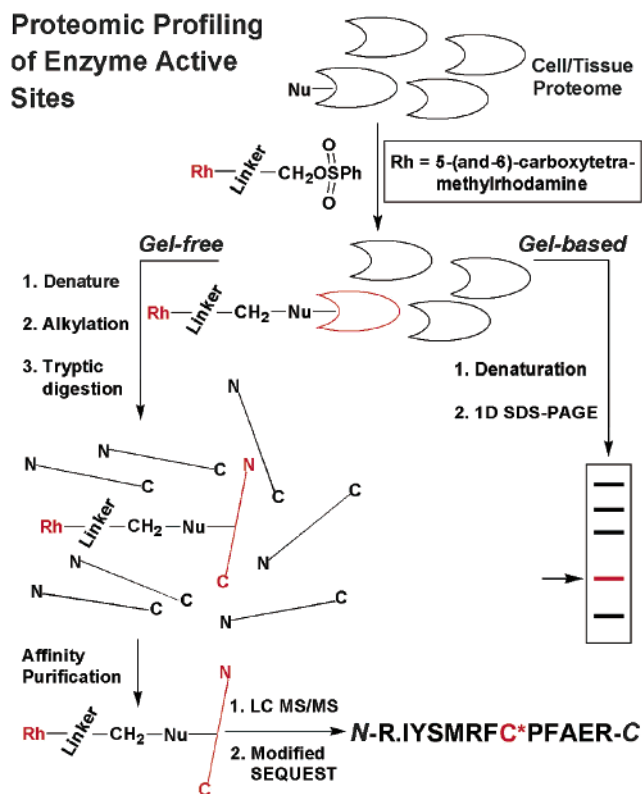
Figure 1. A comparison of gel-based and gel-free ABPP, highlighting how the latter method is used to identify sites of probe labeling on enzymes isolated from complex proteomes. PS-rhodamine-labeled active site peptide shown is for GST-ω (see Table 1).

thiols were reduced and alkylated with dithiothreitol (DTT) and iodoacetamide, respectively. Proteomes were then digested with trypsin, and the resulting tryptic peptide mixtures were incubated with anti-rhodamine antibodies to affinity capture PS-rhodamine-labeled peptides, which were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and the SE-QUEST[10] search algorithm to concurrently identify the protein targets of PS-rhodamine and the specific residues labeled by this activity-based probe. Confidence values in the search results were determined using variants of previously described scoring methods[11] (Experimental Procedures).

Identification of Probe Labeling Sites on Enzyme Targets of PS-Rhodamine by Gel-Free ABPP. Five known enzyme targets of PS-rhodamine[9] [glutathione S-transferase omega (GST-ω), aldehyde dehydrogenase-1 (ALDH1), enoyl CoA hydratase-1 (ECH1), dimeric dihydrodiol dehydrogenase (DDH), and 3β-hydroxysteroid dehydrogenase/isomerase-1 (3HSD1)] were transfected into COS-7 cells, and cell proteomes were analyzed by gel-free ABPP. A single probe-modified peptide was identified for each enzyme, and tandem MS analysis predicted a specific site of probe labeling on each peptide, with the exception of the DDH peptide, which was predicted to be modified on one of two residues by PS-rhodamine (Table 1, asterisks).[12] Interestingly, literature searches revealed that, for most of the enzymes, probe labeling occurred on a conserved

(8) (a) Greenbaum, D.; Medzihradszky, K. F.; Burlingame, A.; Bogyo, M. *Chem. Biol.* **2000**, *7*, 569−581. (b) Faleiro, L.; Kobayashi, R.; Fearnhead, H.; Lazebnik, Y. *EMBO J.* **1997**, *16*, 2271−2281. (c) Borodovsky, A.; Ovaa, H.; Kolli, N.; Gan-Erdene, T.; Wilkinson, K. D.; Pleogh, H. L.; Kessler, B. M. *Chem. Biol.* **2002**, *9*, 1149−1159.

(9) (a) Adam, G. C.; Sorensen, E. J.; Cravatt, B. F. *Nat. Biotechnol.* **2002**, *20*, 805−809. (b) Adam, G. C.; Sorensen, E. J.; Cravatt, B. F. *Mol. Cell. Proteomics* **2002**, *1*, 828−835. (c) Adam, G. C.; Cravatt, B. F.; Sorensen, E. J. *Chem. Biol.* **2001**, *8*, 81−95.

(10) Eng, J. K.; McCormack, A. L.; Yates, J. R. *J. Am. Soc. Mass. Spectrom.* **1994**, *5*, 976−989.

(11) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. *Anal. Chem.* **2002**, *74*, 5383−5392.

(12) Equivalent results were obtained when mouse tissue proteomes were analyzed, indicating that gel-free ABPP can identify sites of probe labeling on both native and recombinantly expressed enzymes.

Table 1. Sites of Labeling Predicted for the PS-Rhodamine ABPP Probe

enzyme	labeled peptide	site of labeling	catalytic residue
GST-ω	R.IYSMRFC*PFAER	C32	yes
ALDH1	K.RVTLE*LGGK	E269	yes
ECH1	K.EVDMGLAAD*VGTLQR	D204	yes
3HSD1	K.GTQNLLEACVQASV-PAFIFCSSVD*VAGPNSYK	D127	unknown
DDH	R.ATDWNQAGGGLLD*L-GIY*CVQFLSMIFGAQKPEK	D176	unknown
		Y180	yes

catalytic residue. For example, GST-ω was labeled on its cysteine nucleophile C32,[13] while ALDH1 and ECH1 were modified on carboxylate residues that function as catalytic bases/acids in these enzymes (E269[14] and D204,[15] respectively). The labeling sites in DDH, D176 and Y180, are both conserved, and the latter has been shown to be important for catalysis.[16] In contrast to these examples, 3HSD1, an integral membrane enzyme for which structural information is lacking, was labeled on D127, a residue of unknown function.

Each predicted site of probe labeling was mutated to alanine or a structurally conserved nonnucleophilic residue (e.g., Y180 to phenylalanine), and mutant enzymes were expressed in COS-7 cells as epitope-tagged proteins by transient transfection. For the mutants of GST-ω, ALDH1, ECH1, and 3HSD1, gel-based ABPP revealed that PS-rhodamine labeling was reduced by >90% as compared to their wild-type (WT) counterparts (Figure 2A−D). In each case, Western blotting confirmed equivalent expression levels for the WT and mutant proteins. In contrast, both the D176A and the Y180F mutants of DDH showed strong probe labeling (Figure 2E). However, the D176A/Y180F double mutant exhibited significantly reduced reactivity with PS-rhodamine, suggesting that this probe could label either residue in DDH.[17] Collectively, these data indicate that gel-free ABPP accurately predicted the sites of probe labeling for the five enzymes examined.

Confirmation that PS-Rhodamine Labeling of ALDH1 Occurs on Its Catalytic Base E269, but not Its Catalytic Nucleophile C302. The apparently exclusive labeling of ALDH1 on E269 was initially surprising given that this enzyme uses a cysteine nucleophile for catalysis (C302, which is activated by E269 to form a covalent intermediate with aldehyde substrates[14]) and possesses a second cysteine residue (C303) in its active site. To test whether these cysteine residues may represent sites of probe labeling that eluded detection by gel-free ABPP, their alanine mutants were tested for reactivity with PS-rhodamine. The C302A, C303A, and C302A/C303A mutants all showed WT levels of PS-rhodamine labeling (Figure 2B), supporting that E269 is the exclusive site of probe modification in ALDH1.

Evidence that D127, the Site of PS-Rhodamine Labeling in 3HSD1, Is an Active Site Residue. The site of probe labeling

in 3HSD1 was intriguing, as initial database searches suggested that D127 is not a conserved residue among members of the 3HSD superfamily. Notably, however, these enzymes, despite all sharing strong sequence homology, actually segregate into two functional subtypes − those, like 3HSD1, with dehydrogenase/isomerase activity and those with ketosteroid reductase activity.[18] Further sequence analysis revealed that D127 was conserved in members with the former, but not the latter activity (Figure 3). Additionally, a homology model of 3HSD1 recently constructed based on the UDP galactose-4-epimerase structure[19] places D127 in the predicted substrate-binding pocket. Together, these findings suggest that D127 may represent an active site residue that contributes to the enzymatic activity of 3HSD1. Consistent with this idea, PS-rhodamine labeling of 3HSD1 was competed by the substrate dehydroepiandrosterone (DHEA, IC_{50} = 15.6 μM; Figure 4A). Additionally, preincubation of 3HSD1 with 10 μM PS-rhodamine for 1 h blocked >90% of the enzyme's catalytic activity (WT-3HSD1, 80 ± 1.5 pmol/min·mg; PS-rhodamine-treated WT-3HSD1, 7.5 ± 0.8 pmol/min·mg; n = 2/group). Finally, when assayed for conversion of ³H-DHEA to 4-androstene-3, 17-dione, the D127A mutant exhibited an ∼100-fold reduction in activity as compared to WT-3HSD1 (Figure 4B and C). Collectively, these data provide evidence that D127 is an active site residue and suggest that it may play a role in 3HSD1 catalysis and/or substrate recognition. More detailed kinetic studies with purified WT- and D127A-3HSD1 proteins will be required to distinguish among these possibilities.

Conclusions

In summary, we report the use of a gel-free version of ABPP to map the sites of probe labeling on enzymes isolated from whole proteomes. In comparing this approach to gel-based ABPP, several advantages of the former method are apparent. First and foremost, gel-free ABPP consolidates into a single step the identification of both the protein targets of activity-based probes and the specific residues labeled by these reagents. This attribute should prove particularly valuable for screening new probes, as direct evidence for active site labeling can be achieved in parallel with target determination. Such was the case for PS-rhodamine, which was found to label conserved active site residues in each of the enzymes investigated. Given that most of these residues play important roles in catalysis, these results suggest that PS-rhodamine should act as a genuine activity-based proteomic probe for the majority of its enzyme targets. More generally, the diversity of amino acids labeled by PS-rhodamine (aspartate, cysteine, glutatmate, tyrosine) highlights the versatility of the SE reactive group and suggests that it may serve as the basis for proteomic profiling agents for many enzyme classes. Although further studies will be required to understand how PS-rhodamine is capable of targeting such a diverse collection of enzymes, it is interesting to speculate that the general hydrophobicity of this probe coupled with its tempered reactivity may enable it to selectively bind and label a variety of enzyme active sites, while at the same time showing limited nonspecific interactions with the remainder of the proteome.

Gel-free ABPP also offers a complementary method for resolving probe-labeled enzymes that comigrate by SDS-PAGE.

(13) Board, P. G.; et al. *J. Biol. Chem.* **2000**, *275*, 24798−24806.
(14) Hurley, T. D.; Weiner, H. *Adv. Exp. Med. Biol.* **1999**, *463*, 45−52.
(15) Modis, Y.; et al. *Structure* **1998**, *6*, 957−970.
(16) Arimitsu, E.; Aoki, S.; Ishikura, K.; Matsuura, K.; Hara, A. *Biochem. J.* **1999**, *342*, 721−728. For analysis of the catalytic function of Y180, see: Asada, Y.; Aoki, S.; Ishikura, S.; Usami, N.; Hara, A. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 333−337. The role of D176 in catalysis has not yet been tested.
(17) Consistent with this premise, the D176A and Y180F mutants were found by gel-free ABPP to be labeled by PS-rhodamine on Y180 and D176, respectively.
(18) Clarke, T. R.; Bain, P. A.; Greco, T. L.; Payne A. H. *Mol. Endocrinol.* **1993**, *7*, 1569−1578.
(19) Thomas, J. L.; Duax, W. L.; Addlagatta, A.; Brandt, S.; Fuller, R. R.; Norris, W. *J. Biol. Chem.* **2003**, *278*, 35483−35490.
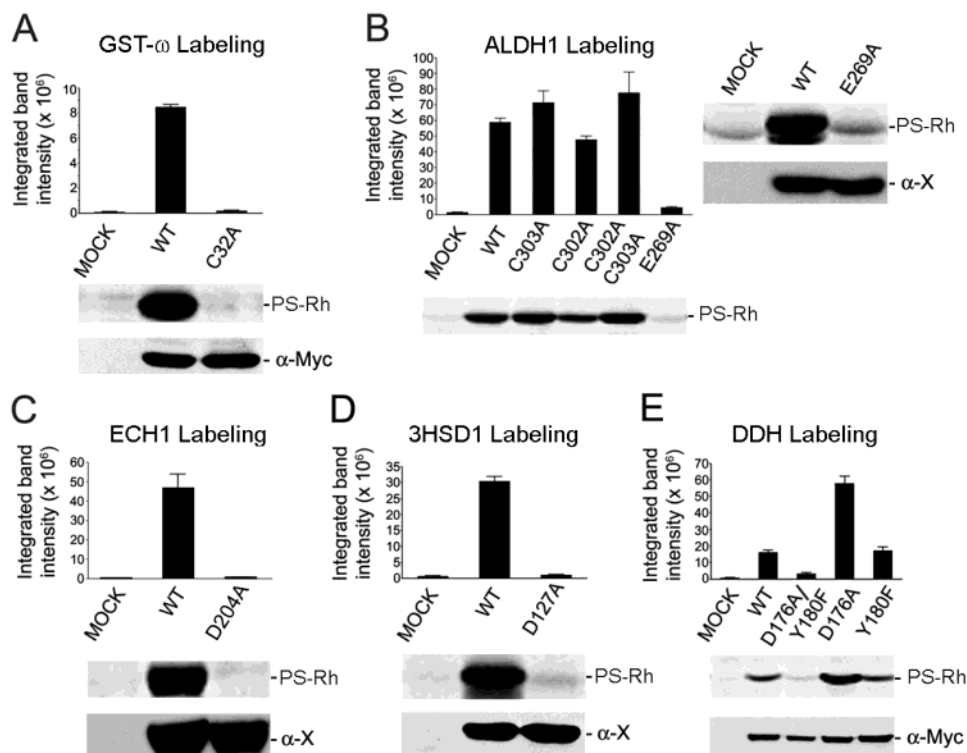
Figure 2. Mutagenesis of predicted sites of probe labeling. Enzymes were expressed in COS-7 cells by transient transfection as described previously,[9] and cellular proteomes were treated with 5 μM PS-rhodamine (PS-Rh) for 1 h. Labeling was quantified by in-gel fluorescence scanning ($n = 4$/group; arbitrary units; PS-Rh signals shown in gray scale). α-Myc, anti-Myc antibodies; α-X, anti-X-press antibodies. Mock, transfected with empty vector.

```
N-IFCSSVDVAGPNS-C    mouse HSD/isomerase-1
N-IYCSTVDVAGPNS-C    rat HSD/isomerase-1
N-IYCSTVDVAGPNS-C    rat HSD/isomerase-4
N-IYCSTVDVAGPNS-C    rat HSD/isomerase-2
N-IFSSSVDVAGPNS-C    mouse HSD/isomerase-6
N-IYTSSIDVAGPNS-C    hamster HSD/isomerase-1
N-IYTSSIDVAGPNS-C    hamster HSD/isomerase-2
N-IYTSSIEVAGPNS-C    human HSD/isomerase-1
N-IYTSTLEVAGPNS-C    monkey HSD/isomerase-1
N-IYSSSTGVAGPNS-C    hamster 3-ketosteroid reductase
N-IYSSSTGVAGPNS-C    rat progesterone reductase
N-IYSSSVLVAGPNS-C    mouse 3-ketosteroid reductase-1
N-IYSSSVLVAGPNS-C    mouse 3-ketosteroid reductase-2
```

Figure 3. A comparison of sequences of 3HSD family members in the local region surrounding D127 in mouse 3HSD1. Note that all members of this enzyme class with documented dehydrogenase/isomerase activity possess an Asp or Glu residue at this position (in bold), while members showing ketosteroid reductase activity possess other residues at this position (e.g., G or L, italics). *N*, N-terminus, *C*, C-terminus.

Indeed, as long as such enzymes contain distinct active site sequences, their probe-labeled tryptic peptides should be resolvable by LC. The augmented resolution power afforded by gel-free ABPP should prove especially useful for visualizing the targets of probes that profile exceptionally large enzyme classes (e.g., fluorophosphonate probes[5,7] that target the serine hydrolase superfamily[20]). Finally, as appears to be the case for the PS-rhodamine target 3HSD1, gel-free ABPP may expose previously unrecognized active site residues in enzymes targeted by activity-based probes. Future efforts to combine gel-free ABPP with isotope labeling[3] and in vivo profiling[21] methods may provide a general proteomic platform in which the quantitative comparison of active enzymes can be accomplished in unison with a mechanistic analysis of their active sites.

Experimental Procedures

General Protocol for Gel-Free Activity-Based Protein Profiling (ABPP). Sample Preparation, Digestion Protocol, and Affinity Capture of Tryptic Peptides. Proteomes were labeled with PS-rhodamine as described previously.[9] Briefly, proteomes (1 mg/mL protein, total volume = 1 mL) were treated with 5 μM PS-rhodamine for 1 h at room temperature in 50 mM Tris, pH 8.0. The samples were then denatured by adding 1 volume of 12 M urea (prepared at 65 °C to allow for complete dissolution of the urea) and 1/50 volume of DTT from a fresh 1 M stock. The mixture was then heated to 65 °C for 15 min. Iodoacetamide was added to 40 mM, and the solution was incubated at 37 °C for 30 min. Next, the buffer was exchanged to 10 mM ammonium bicarbonate, 2 M urea by gel filtration (Bio-Rad 10DG columns). Following gel filtration, the samples were digested with 150 ng of trypsin (sequencing grade modified trypsin, Promega) per 10 μL of sample for 1 h at 37 °C. SDS was added to the resulting peptides to 1% final concentration, and the samples were heated to 65 °C for 5 min to inactivate the trypsin. One volume of antibody binding buffer (2X Phosphate buffered saline, 2% Triton X-100, 1% tergitol NP-40 type) was then added to the sample followed by 50 μL of agarose beads containing monoclonal anti-TAMRA antibodies (5 mg antibody/mL beads, ActivX Biosciences). Samples were incubated with the beads for 1 h, then washed three times with 1X antibody binding buffer containing 1% SDS, three times with 1X PBS, and three times with deionized water. The bound, probe-modified peptides were then eluted with 2 × 50 μL volumes of 50% CH$_3$CN, 0.1% TFA.

Identification of Probe Labeling Sites by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) and SEQUEST Search Algorithms. Eluted peptides were analyzed in data-dependent mode on a Finnigan LC DecaXP ion trap MS interfaced with an Agilent 1100 series capillary LC system. A 90 min gradient from 10% CH$_3$-CN/0.1% formic acid to 50% CH$_3$CN/0.1% formic acid was used. The

(20) Additional aspects of this platform, including its use with other activity-based probes, will be described elsewhere (Patricelli, M. P.; et al., manuscript in preparation).

(21) Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686−4687.
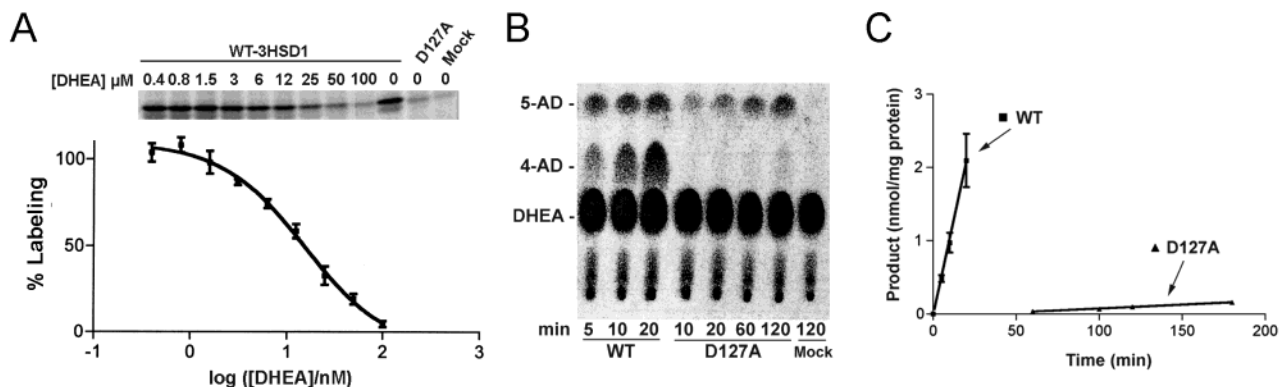
Figure 4. Evidence that D127, the site of PS-rhodamine labeling in 3HSD-1, is an active site residue. (A) Inhibition of PS-rhodamine labeling of 3HSD-1 by the substrate dehydroepiandrosterone (DHEA). (B) and (C) Analysis of the enzymatic activities of WT- and D127A-3HSD1. (B) Representative thin-layer chromatography plate showing the conversion of ^{3}H-dehydroepiandrosterone (DHEA) to the dehydrogenase product, intermediate 5-androstene-3, 17-dione (5-AD), and the final isomerase product 4-androstene-3, 17-dione (4-AD). For these assays, 0.25 mg/mL protein was used for both WT and D127A-3HSD1. Note that, at the time points shown, WT-3HSD1 reactions exhibited steady-state levels of the intermediate 5-androstene-3,17-dione, while the D127A-3HSD1 reactions show a lag phase in reaching steady-state levels of this product. Because of the slower activity of the D127A-3HSD1 enzyme, the steady-state kinetics shown for this enzyme in (C) were obtained at later time points (60−180 min) using 1 mg/mL protein.

resulting MS/MS data were searched using SEQUEST. The results of the SEQUEST searches were first filtered to remove any results that did not yield singly probe-modified peptides as their highest scoring match. Determining labeling sites for activity-based probes such as sulfonate esters (SEs), which may react with one of several potential amino acids in enzyme active sites, presented several technical challenges. First, the probes react on a single site for each enzyme, thus providing only a single probe-labeled peptide for identification. This aspect, which is general to ABPP, has been addressed through the use of improved scoring methods, which combine standard SEQUEST output scores (Xcorr and ΔCN) with general peptide properties such as charge and mass.[11,20] These composite scores provide an absolute measure of data quality that is independent of peptide charge state or mass and thereby make the distinction of correct and incorrect results more robust.

A quantitative assessment of confidence in each search result required a significantly different approach than those described previously.[11] Reported methods of statistical analysis attempt to fit two distributions to each MS/MS data set that represent correct and incorrect search results based on prior knowledge of the general shape of these distributions. These methods require that enough data are present to define two distinct populations of scores. In nearly all cases, SE probe-labeled samples possessed only a select number of positive search results that were insufficient in quantity to define a distribution of correct results. A simple method was developed to address these issues whereby each data set was searched once using the correct parameter for probe mass, and again using an incorrect mass (or multiple incorrect masses if additional data were needed) for the probe modification. The data from the search using the incorrect probe mass predict the shape and position of the (gamma) distribution that defines incorrect search results in that data set. The probability that each search result falls outside the population of incorrect search results in the data set can then be determined. Once the probability that each data point is correct has been determined, an overall score for each target protein/labeling site can be calculated. While most proteins label only at a single site, it is relatively common to observe either alternative trypsin cleavage patterns yielding overlapping but distinct peptides, multiple methionine oxidation states, and/or data collected on multiple charge states of the same peptide. All three of these situations can be viewed as independent data corroborating the same identification. When these multiple pieces of data are present, the probability of each independent data point can be summed to determine an overall confidence in the identified target/labeling site.

Gel-Based ABPP of Transfected COS-7 Cell Proteomes. cDNAs for enzyme targets were purchased as expressed sequence tags

(Invitrogen) and subcloned by the polymerase chain reaction into the eukaryotic expression vectors pcDNA3-max/His or pcDNA3-myc/His, which append an N- and C-terminal epitope tag to protein products, respectively. Point mutants were generated using the Quikchange procedure (Stratagene). All constructs were confirmed by DNA sequencing. Epitope-tagged wild-type (WT) and mutant enzymes were expressed in COS-7 cells by transient transfection, and cell proteomes were processed as described previously.[9] Mock-transfected cells (transfected with empty vector) were also processed for analysis. Proteomes (1 mg/mL protein) were treated with 10 μM PS-rhodamine (50X stock in DMSO) for 1 h at room temperature in 50 mM Tris, pH 8.0, after which reactions were quenched with 1 volume equivalent of SDS-PAGE loading buffer (reducing) and resolved by one-dimensional SDS-PAGE. The extent of probe labeling of enzyme targets was quantified by in-gel fluorescence scanning using a Hitachi FMBio IIe flatbed scanner. Data represent the mean ± standard deviation of four independent experiments. Equivalent protein expression for each WT and mutant enzyme pair was confirmed by Western blotting with anti-Myc or anti-X-press antibodies following manufacturer's guidelines (Invitrogen).

Inhibition of PS-Rhodamine Labeling of 3HSD1 by Substrate. WT 3HSD1 was expressed in COS-7 cells by transient transfection as described above, and the membrane fraction of transfected cells was isolated by centrifugation (100 000*g* for 1 h). Aliquots of the membrane proteome (1 mg/mL, 50 mM Tris, pH 8.0) were treated sequentially with the 3HSD1 substrate dehydroepiandrosterone (DHEA; concentrations ranging from 0.4 to 100 μM, 50X stocks in DMSO) and PS-rhodamine (5 μM, 100X stock in DMSO). Reaction mixtures were incubated at room temperature for 20 min, quenched, and analyzed as described above. Values for PS-rhodamine labeling of WT-3HSD1 at each concentration of competing substrate were divided by control values (PS-rhodamine labeling of WT-3HSD1 in the absence of competing substrate) and plotted as described previously[22] to provide an IC$_{50}$ value for inhibition of labeling of 15.6 μM (10.9−22.2 μM, 95% confidence limits). Data represent the mean ± standard deviation of three independent experiments.

Analysis of the Catalytic Activity of WT- and D127A-3HSD1 Enzymes. 3HSD1 activity was measured by following the conversion of ^{3}H-DHEA to ^{3}H-4-androstene-3,17-dione by thin-layer chromatography (TLC).[23] Membrane fractions of WT-3HSD1-, D127A-3HSD1-, and mock-transfected COS-7 cells were incubated with ^{3}H-DHEA (2.5 μM, 24.7 Ci/mmol, 25X in ethanol) in 50 mM Tris, pH 7.5, with 1

(22) Leung, D.; Hardouin, C.; Boger, D. L.; Cravatt, B. F. *Nat. Biotechnol.* **2003**, *21*, 687−691.
(23) Abbaszade, I. G.; Arensburg, J.; Park, C.-H.; Kasa-Vubu, J. Z.; Orly, J.; Payne, A. H. *Endocrinology* **1997**, *138*, 1392−1399.

mM NAD^+ at room temperature under the following conditions: WT-3HSD1 − 5, 10, and 20 min reactions with 0.25 mg/mL protein; D127A-3HSD1 − 60, 100, 120, and 180 min reactions with 1 mg/mL protein; mock − 180 min reactions with 1 mg/mL protein. At the indicated time points, reactions were extracted with 2.5 volumes of chloroform containing 15 mg/mL each of cold DHEA (substrate), 5-androstene-3,17-dione (5-AD, dehydrogenase product), and 4-androstene-3,17-dione (4-AD, isomerase product). The organic phase was removed, and the aqueous phase was extracted once more with 2.5 volumes of chloroform. The combined organic phases were dried under a stream of gaseous N_2, solubilized in 25 μL of chloroform, and steroid products were separated by TLC (10% ethyl acetate in chloroform; see Figure 4B for a representative TLC showing separation of steroid products). At the time points examined for both WT- and D127A-3HSD1, steady-state levels of the intermediate 5-AD were detected and linear kinetics were observed for formation of the final product 4-AD (105 ± 10 pmol/min·mg protein for WT-3HSD1; 1.0 ± 0.1 pmol/min·mg protein for D127A-3HSD1). Mock-transfected cells showed no detectable dehydrogenase activity. Although the dehydrogenase and isomerase steps could not be separately measured for WT- and D127A-3HSD1 proteins with this radiolabeled assay, the D127A mutant showed slower rates for formation of steady-state levels of the 5-AD intermediate, indicating that at least the dehydrogenase step was compromised

in this 3HSD-1 variant. Data represent the mean ± standard deviation of three independent experiments.

In separate experiments, WT-3HSD1-transfected cell proteomes were treated with 10 μM PS-rhodamine (50X stock in DMSO) or an equivalent volume of DMSO for 1 h at room temperature, followed by 2.5 μM ^{3}H-DHEA for 20 min. Reactions were processed and analyzed as described above. PS-rhodamine-treated samples were found to exhibit less than 10% of the 3HSD1 activity of DMSO-treated samples.

Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA038441G