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Research Paper

Profiling the specific reactivity of the proteome with non-directed activity-based probes

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Abstract

Background: The field of proteomics aims to characterize dynamics in protein function on a global level. However, several classes of proteins, in particular low abundance proteins, remain difficult to characterize using standard proteomics technologies. Recently, chemical strategies have emerged that profile classes of proteins based on activity rather than quantity, thereby greatly facilitating the analysis of low abundance constituents of the proteome.

Results: In order to expand the classes of proteins susceptible to analysis by activity-based methods, we have synthesized a library of biotinylated sulfonate esters and applied its members to complex proteomes under conditions that distinguish patterns of specific protein reactivity. Individual sulfonates exhibited unique profiles of proteome reactivity that in extreme cases appeared nearly orthogonal to one another. A robustly labeled protein was identified as a class I aldehyde dehydrogenase and shown to be irreversibly inhibited by members of the sulfonate library.

Conclusions: Through screening the proteome with a non-directed library of chemical probes, diverse patterns of protein reactivity were uncovered. These probes labeled protein targets based on properties other than abundance, circumventing one of the major challenges facing contemporary proteomics research. Considering further that the probes were found to inhibit a target enzyme's catalytic activity, the methods described herein should facilitate the identification of compounds possessing both selective proteome reactivities and novel bioactivities.

Keywords: Proteomics; Sulfonate; Activity-based; Probe; Aldehyde dehydrogenase

1. Introduction

With the complete sequence of the human genome nearly in hand, biological research is entering a new era in which experimental focus will shift from identifying novel genes to determining the function of gene products. In efforts to accelerate the functional analysis of products expressed by the genome, researchers have developed several technologies that permit the study of biomolecules collectively, rather than individually. These global experimental strategies include: (1) genomics, or the analysis of a cell's complete transcript repertoire (transcriptome) [1,2], and (2) proteomics, or the analysis of a cell's complete protein repertoire (proteome) [3,4]. In the field of genomics, advances in gene array technologies have permitted researchers to compare in a single experiment the levels of several thousand transcripts across two or more test samples [1,2,5]. Recent studies have demonstrated how gene chips can be used to discover and predict new subclasses of cancer based on their distinctive patterns of gene expression [6,7]. Still, by measuring dynamics in mRNA abundance, genomics approaches offer only an indirect assessment of protein quantity and function. Considering that mRNA levels are often poor predictors of protein abundance [8,9], the degree to which genomics data are reflective of changes in protein function remains unclear.

Recently, proteomics initiatives have emerged with the goal of characterizing dynamics in the abundances of proteins themselves [3,4,10]. Considerable experimental challenges face the analysis of the proteome, especially when compared to the technically more feasible profiling of the

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transcriptome. Unlike mRNA molecules, proteins (1) display tremendous chemical diversity (e.g. various co- and post-translational modifications, hydrophobic domains, etc.), (2) are not amplifiable, and (3) do not typically possess defined high affinity and high selectivity binding partners. Thus, perhaps not surprisingly, a general experimental method for total proteome analysis remains an elusive goal. To date, most proteomics studies have relied on two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS) as their separation and detection technologies, respectively [11]. Advances in 2DE-MS methods have increased their reproducibility and throughput for proteome analysis [12]. However, a significant fraction of the proteome still remains difficult to analyze by 2DE, including very large and small proteins, very basic and acidic proteins, membrane proteins, and low abundance proteins [11–13]. In order to permit the general and systematic profiling of such 2DE-resistant proteins, proteomics researchers have proposed that alternative protein separation technologies must be developed [11,13]. Regardless, even if the requisite advances in separation strategies are achieved, it is critical to recognize that as long as proteomics focuses on measuring exclusively changes in protein abundance, it will provide, like genomics, only an indirect assessment of dynamics in protein function. Numerous post-translational events that regulate protein activity, especially those mediated by protein–protein and/or protein–small molecule interactions, will remain undetected by standard protein abundance-based techniques.

We have initiated a research program aimed at generating chemical probes that profile components of the proteome in an activity-dependent manner. In their broadest application, such activity-based probes (ABPs) would detect: (1) a significant, but manageable fraction of the proteome, (2) low abundance proteins and proteins with problematic biochemical properties (e.g. membrane proteins), and (3) dynamics in the activity of proteins independent of changes in their quantity. A prototype agent, a biotinylated fluorophosphonate (FP-biotin), was recently synthesized and shown to serve as an ABP for the serine hydrolase superfamily of enzymes [14]. Similarly, biotinylated epoxides and electrophilic ketones have been used to profile subclasses of the cysteine protease family [15–17]. In each of these examples, researchers exploited a wealth of previous knowledge on class-selective reactive groups and/or molecular scaffolds to create ABPs with predictable proteome reactivities (e.g. FPs for profiling serine hydrolases). However, for a number of protein families, selective active site-directed affinity reagents have not yet been identified, and whether such classes of proteins can be profiled in the manner illustrated above remains uncertain. Herein, we describe a non-directed approach for discovering new ABPs that bear both reactivities and selectivities compatible with whole proteome analysis. Additionally, we show that these chemical probes directly affect the activity of their target proteins, highlighting their potential utility in function-based screens as well as proteomics investigations.

2. Results

2.1. Selection and synthesis of biotinylated sulfonate esters as candidate ABPs

In order to facilitate the discovery of new ABPs, we adopted a non-directed strategy for profiling the chemical reactivity of the proteome. A library of candidate ABPs was synthesized based on the general scaffold outlined in Fig. 1. The structure of an ABP was conceptually divided into four pieces: a binding group (BG), a reactive group (RG), a linker (L), and a tag (T). The library's reactive group was selected as a sulfonate ester, based on the following criteria. Sulfonates are: (1) moderately reactive electrophiles, (2) relatively unexplored as protein labeling reagents in biological systems, and (3) facile to modify with a variety of chemical structures. Additionally, we hoped that by choosing a carbon electrophile as the library's reactive group, its bias towards particular enzyme

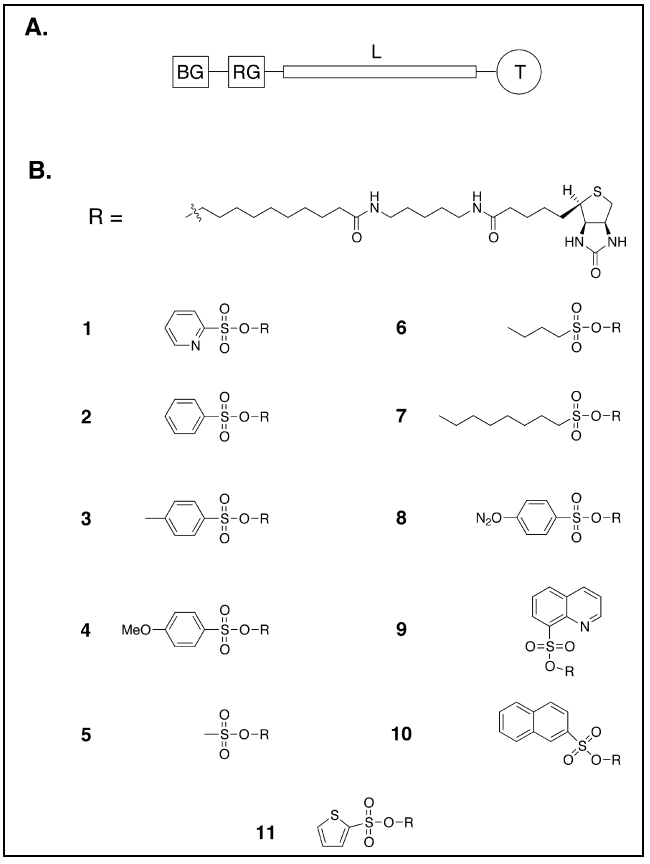


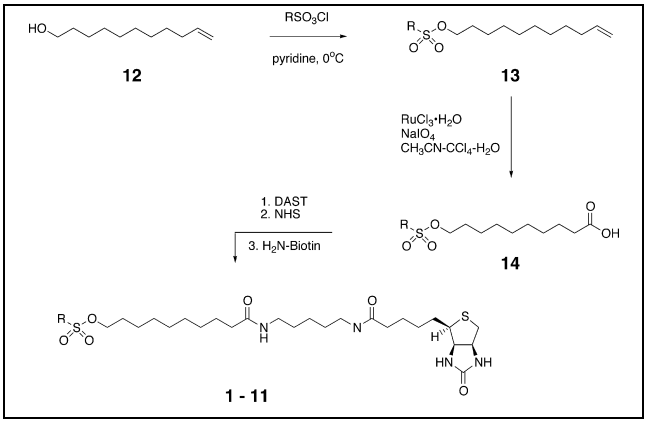
Fig. 1. A: General structure of an ABP, highlighting the probe's four main components: a binding group (BG), a reactive group (RG), a linker (L), and a tag (T). B: Structures of members of an ABP library, where the BG is varied, the RG is a sulfonate ester, the L is an extended alkyl chain, and the T is biotin.

classes would be minimized. Indeed, other carbon electrophiles like epoxides have been found to engage a large variety of enzymes through the alkylation of one of several different types of active site amino acids, including cysteine hydrolases (cysteines) [17], metallohydrolases (histidines) [18–20], glycosidases (glutamates/aspartates) [21], and proteosomal subunits (N-terminal amines) [22]. The library's binding group was combinatorially varied, while its linker moiety was kept constant and served as a bridge to the chemical tag (biotin). Through varying the binding group, we hoped to direct the sulfonate's specific proteome reactivity to different proteins and/or protein classes.

A series of biotinylated sulfonates (**1**–**11**; Fig. 1) were synthesized according to the three step sequence outlined in Scheme 1. The corresponding aryl or alkylsulfonyl chloride was added slowly to a solution of undecene-1-ol (**12**) dissolved in pyridine at 0°C to form the sulfonate (**13**). The procedure of Sharpless and colleagues was utilized to oxidatively cleave the terminal olefin, resulting in formation of the corresponding carboxylic acid (**14**) [23]. Treatment of **14** with diethylaminosulfur trifluoride followed by the addition of *N*-hydroxysuccinimide afforded the *N*-hydroxysuccinimidyl ester intermediate. The latter compound was reacted with commercially available 5-(biotinamido)pentylamine (NH_2-biotin, Pierce) in methanol to form the desired biotinylated sulfonate ester.

2.2. *Evaluating the specific proteome reactivity of biotinylated sulfonates*

To determine whether members of the sulfonate library specifically targeted proteins in the proteome, a method was developed to rapidly distinguish a probe's specific and non-specific proteome reactivities. Each sulfonate (5 μM) was reacted with two versions of a rat testis proteome: a native proteome and a denatured proteome (generated by preheating the protein sample for 5 min at 80°C). After 30 min at 25°C, the sulfonate–proteome reactions were quenched by adding one reaction volume of standard sodium dodecyl sulfate (SDS)–PAGE loading buffer and analyzed by SDS–PAGE and avidin blotting. A sulfonate's specific and non-specific proteome reactivity were defined as those protein targets that displayed heat-sensitive and heat-insensitive labeling, respectively. All sulfonates except the octylsulfonate **7** labeled at least one member of the testis proteome in a heat-sensitive manner (Fig. 2A,B). Interestingly, four general patterns of specific proteome reactivity were observed among the sulfonates. The *p*-toluenesulfonate **3**, butylsulfonate **6**, and naphthylsulfonate **10** each specifically labeled one 55 kDa protein (Fig. 2A,B; single arrowhead). The *p*-methoxybenzenesulfonate **4** and methylsulfonate **5** labeled four members of the proteome in a heat-sensitive manner (Fig. 2A; 55 kDa (single arrowhead), 42 kDa (double arrowhead), 40 kDa (triple arrowhead), and 32 kDa (single dot)), but also showed significant additional reactivity with the preheated proteome. The benzenesulfonate **2** reacted with three of the four proteins labeled by **4** and **5**, failing to specifically label only the 40 kDa protein. The *p*-nitrobenzenesulfo-



Scheme 1. Synthetic route for the generation of biotinylated sulfonate esters.

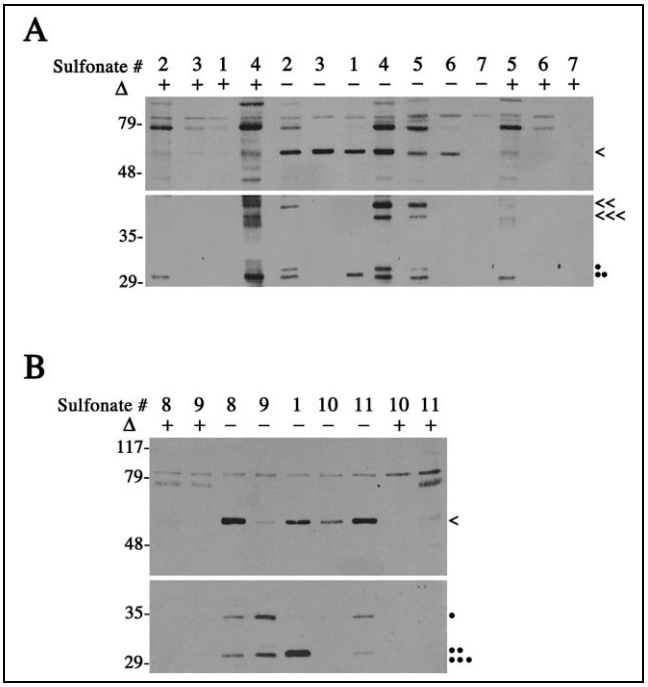


Fig. 2. Specific and non-specific proteome reactivities of sulfonates **1**–**7** (A) and **8**–**11** (B). For A and B, each sulfonate's reactivity with both a heated and unheated version of a rat testis proteome is shown (standard reaction conditions: 5 μM sulfonate, 0.5 μg/μl testis protein, 50 mM Tris–HCl, pH 8.0, 100 mM NaCl; 30 min reaction, 25°C). Sulfonate-labeled proteins were detected by SDS–PAGE (7.5 μg protein/lane) and avidin blotting. Highlighted with arrowheads and dots are proteins that reacted with sulfonates in a heat-sensitive manner (see text). The proteins labeled in the lanes containing preheated proteomes (Δ) were all considered 'non-specific' sulfonate reactivities, except an 80 kDa protein which represented an endogenous avidin-reactive protein (i.e. also observed in proteomes not treated with sulfonates; see Fig. 4B). Different film exposure times are presented for the high (45–100 kDa, 1× time exposure) and low (27–45 kDa, 4× time exposure) molecular mass proteins to permit the signals of labeled proteins to be shown prior to film saturation.

nate **8**, quinolinesulfonate **9**, and thiophenesulfonate **11** each reacted specifically with a 55 kDa protein, as well as two additional proteins poorly labeled by the other reagents (Fig. 2B; 36 kDa (single dot) and 30 kDa (triple dot)). Finally, the pyridylsulfonate **1** labeled a 55 kDa protein and a 31 kDa protein (Fig. 2A,B, single arrowhead and double dot, respectively), the latter protein appearing uniquely reactive with **1** among the sulfonates that were surveyed. Importantly, most of the sulfonate probes with the exception of **2**, **4**, and **5** showed low or negligible reactivity with the preheated proteome.

Although several sulfonate probes showed overlapping patterns of specific proteome reactivity, their relative reactivities with individual proteins differed considerably. For example, a 36 kDa protein reacted more strongly with quinolinesulfonate **9** than with pyridylsulfonate **1**, *p*-nitrobenzenesulfonate **8** or thiophenesulfonate **11**, while a 55 kDa protein displayed the opposite probe selectivity (Fig. 2B). To further examine the different proteome reactivities exhibited by individual sulfonates, the labeling pattern of pyridylsulfonate **1** was compared to that of methylsulfonate **5** and quinolinesulfonate **9** in side-by-side analyses (Fig. 3A,B, respectively). Sulfonates **1** and **5** exhibited quite distinctive patterns of specific proteome reactivity, with **1** most strongly labeling 55 and 31 kDa proteins (Fig. 3A, left panel, double arrowheads) and **5** most strongly labeling 42 and 32 kDa proteins (Fig. 3A, left panel, single arrowheads). A comparison of the proteome reactivities of **1** and **9** identified two proteins that showed preferred reactivity with **1** (Fig. 3B, left panel, 55 kDa and 31 kDa; double arrowheads) and two proteins that showed enhanced reactivity with **9** (Fig. 3B, left panel, 36 kDa and 30 kDa; single arrowheads).

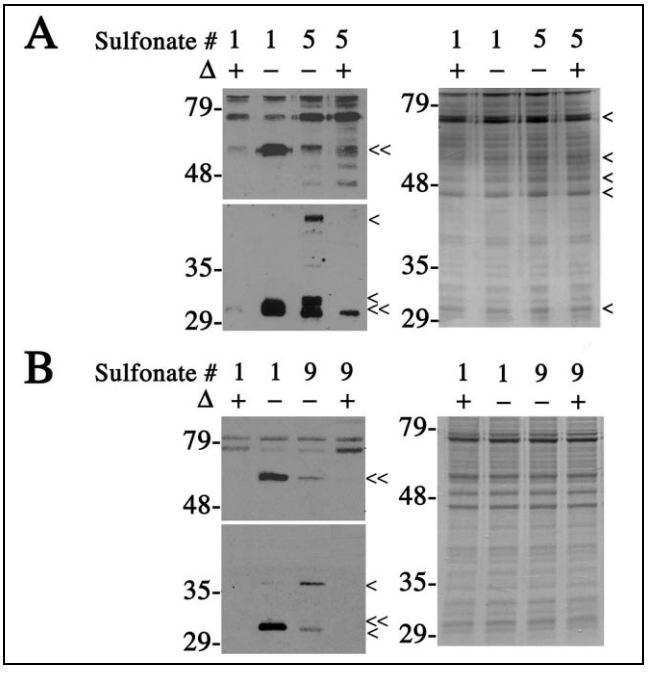


Fig. 3. Side-by-side comparisons of the proteome reactivities of sulfonates **1**, **5**, and **9**. A: Left panel, proteome reactivities of sulfonates **1** and **5**. Heat-sensitive protein reactivities selective for **1** and **5** are highlighted (double and single arrowheads, respectively). Right panel, Coomassie blue-stained protein gel of samples treated with **1** and **5**. Arrowheads highlight abundant proteins correlating in molecular mass with proteins labeled by **5** in the preheated proteome. B: Left panel, proteome reactivities of sulfonates **1** and **9**. Heat-sensitive protein reactivities selective for **1** and **9** are highlighted (double and single arrowheads, respectively). Right panel, Coomassie blue-stained protein gel of samples treated with **1** and **9**. For A and B, different film exposure times are presented for the high (45–100 kDa, 1× time exposure) and low (27–45 kDa, 4× time exposure) molecular mass proteins to permit the signals of labeled proteins to be shown prior to film saturation.

The greater non-specific reactivity of **5** was also evident in these side-by-side comparisons, as this agent labeled several proteins in a heat-insensitive manner that were unreactive towards **1** and **9**. Notably, a Coomassie blue-stained protein gel revealed that the proteins labeled by **5** in the preheated proteome represented very abundant proteins (Fig. 2C, right panel, single arrowheads), consistent with the notion that heat-insensitive labeling reflects a non-specific form of sulfonate reactivity. In contrast, the sulfonate library's heat-sensitive proteome reactivity showed no such bias towards abundant proteins. Finally, it is interesting to note that little correlation was observed between the magnitudes of a sulfonate's specific and non-specific forms of proteome reactivity, as several probes displaying low reactivity with the preheated proteome exhibited specific reactivities that equaled or exceeded in intensity those exhibited by probes with high non-specific reactivities.

2.3. Parameters that influence a sulfonate's specific proteome reactivity

The following features of the sulfonate–proteome reaction were varied in order to test their influence on the observed specific and non-specific protein labeling patterns: time, sulfonate concentration, pH, and the presence/absence of scavenging nucleophiles. For these studies, the reactivity of pyridylsulfonate **1** with the testis proteome was examined. The two testis proteins specifically targeted by **1** were labeled at similar rates, with their signal intensities increasing from 5 to 40 min and then plateauing from 40 to 120 min (Fig. 4A). The absence of additional reactivity from 40 to 120 min could signify that the proteins had labeled to completion by 40 min, or alternatively, that the concentration of **1** in the reaction was significantly depleted by these later time points (this latter explanation seems less likely when taking into account that pyridylsulfonates were quite stable to the assay conditions employed; see below).

Sulfonate **1**'s specific and non-specific proteome reactivities were evaluated over a range of probe concentrations (1–50 μM). From 1 to 10 μM, sulfonate **1** showed specific reactivity with the 31 and 55 kDa proteins that increased in intensity with increasing concentrations of reagent (Fig.

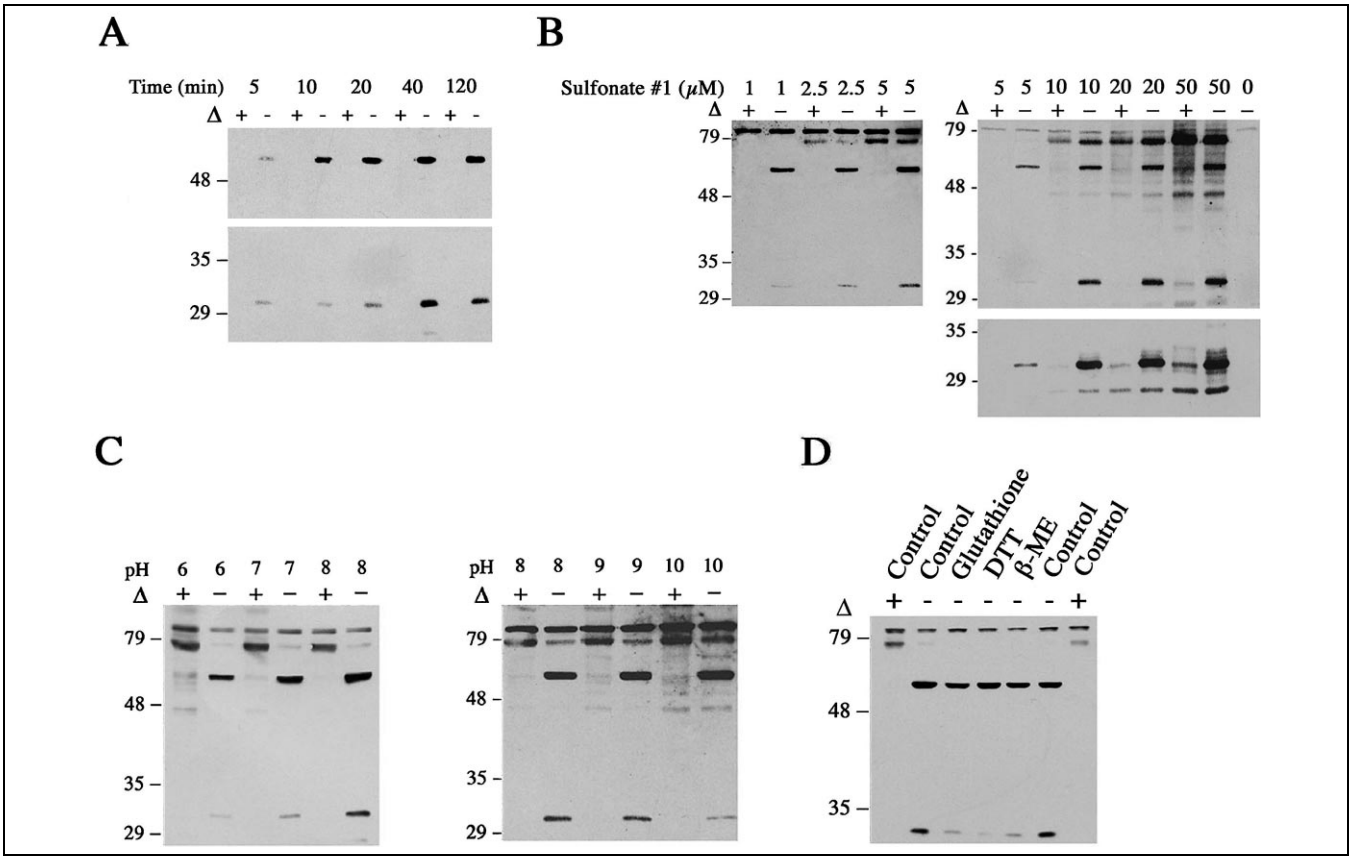


Fig. 4. Parameters that affect the proteome reactivity of pyridylsulfonate **1**. A: Time-dependence of **1**–proteome reaction (5 μM of **1**, 0.5 μg/μl protein, 50 mM Tris–HCl, pH 8.0). B: Concentration-dependence of **1**–proteome reaction. Left panel, 1, 2.5, and 5 μM concentrations of **1** were reacted with the testis proteome (0.5 μg/μl protein, 50 mM Tris–HCl, pH 8.0, 30 min reaction). Right panel, 5, 10, 20, and 50 μM concentrations of **1** were reacted with the testis proteome (0.5 μg/μl protein, 50 mM Tris–HCl, pH 8.0, 30 min reaction). Short (upper right panel) and long (lower right panel) film exposures of these reactions are shown. Note the presence of an endogenous 80 kDa avidin-reactive protein in the lane containing an untreated testis proteome. C: pH-dependence of **1**–proteome reaction (5 μM of **1**, 0.5 μg/μl protein, 30 min reaction). Left panel, reactions conducted from pH 6.0 to 8.0. Right panel, reactions conducted from pH 8.0 to 10.0. D: Thiol-dependence of **1**–proteome reaction. Control reactions were conducted under standard conditions. Each thiol (2 mM) was added to the proteome prior to the addition of **1**.

4B). Over this concentration range, sulfonate **1** displayed very low levels of heat-insensitive reactivity with the proteome. From 10 to 50 μM of **1**, the signal intensity of the 31 kDa protein continued to increase, while the intensity of the 55 kDa protein remained relatively constant. Over this concentration range, sulfonate **1**'s non-specific labeling increased dramatically, especially in the higher molecular mass range where most of the abundant testis proteins reside. Importantly, however, no new specifically labeled protein targets were identified over this concentration range. Thus, a concentration range of 5–10 μM appeared optimal for maximizing sulfonate **1**'s specific versus non-specific proteome reactivity.

The non-specific and specific proteome reactivities of sulfonate **1** showed different pH-dependencies, with the former appearing as an inverted bell-shape curve (higher background labeling at pH 6 and 9 than at pH 7 and 8) and the latter increasing in intensity from pH 6 to 8 and plateauing from pH 8 to 10 (Fig. 4C,D). Thus, reactions conducted at pH 7 and 8 produced the highest level of specific reactivity, while at the same time resulting in the lowest degree of non-specific reactivity.

Sulfonate **1**'s intrinsic reactivity with nucleophiles was examined by conducting proteome reactions in the presence of millimolar concentrations of free thiols (glutathione, β-mercaptoethanol, or dithiothreitol (DTT)). If this sulfonate displayed a high reactivity with generic nucleophiles, then the probe's effective concentration in thiol-treated proteome reactions should be greatly reduced, resulting in a significant decrease in the signal intensity of specifically labeled proteins. However, none of the tested thiols affected the labeling intensity of the 55 kDa protein, indicating that sulfonate **1**'s intrinsic reactivity with nucleophiles is low (Fig. 4D; see below for more evidence in support of this notion). In contrast, a moderate decrease in the labeling intensity of the 31 kDa protein was detected in the presence of free thiols, possibly indicating that this protein's structure/activity is sensitive to these reagents (e.g. if this protein's tertiary structure is stabilized by disulfide bonds).

2.4. Molecular identification of a protein labeled by biotinylated sulfonates

The screening method described above was enacted to rapidly identify protein targets specifically labeled by members of the sulfonate library. By defining these 'specific protein targets' as ones that displayed heat-sensitive reactivity with sulfonates, we hoped to restrict our focus to proteins whose activities would be affected by sulfonate labeling. The assumption inherent to this strategy was that heat-sensitive labeling was reflective of an event taking place within a structured portion of a protein suitable for small molecule binding. Such structures were anticipated to often represent either ligand binding pockets of receptors or active sites of enzymes. As such, if a sulfonate reacted specifically with one of these sites on a receptor or an enzyme, its reaction might in turn be expected to affect the activity of this protein. In order to test this premise, we pursued the molecular identification of the 55 kDa protein specifically labeled by several members of the sulfonate library.

A tissue blot with sulfonate **1** revealed that the labeled 55 kDa protein was most abundant in soluble fractions of rat liver (data not shown), and therefore the protein was purified from this source. The 55 kDa protein was partially purified by Q-Sepharose anion exchange chromatography. Aliquots of the flow-through and elution fractions of this column were labeled with **1**, and the 55 kDa protein was identified in the flow-through fractions. These fractions were combined, labeled with 5 μM **1** for 30 min, and the protein separated from excess sulfonate by size exclusion chromatography. The protein sample was then treated with avidin agarose beads to isolate the **1**-labeled 55 kDa protein. Elution of the avidin-bound proteins was achieved by adding one volume of standard SDS–PAGE loading buffer and heating (90°C, 5 min). This avidin-based affinity purification procedure provided a highly concentrated sample of the 55 kDa protein that was separated by SDS–PAGE and either blotted with avidin (Fig. 5A) or stained with Coomassie blue. The 55 kDa protein was excised from the stained gel, treated with trypsin, and the resulting peptide mixture analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS. MS-Fit and ProFound searches of protein databases identified the protein as cytosolic 2 class I aldehyde dehydrogenase (cALDH-I; nine tryptic peptides ranging from 1189 to 2055 Da matched this enzyme, 50% total sequence coverage; Fig. 5A), a member of a superfamily of NAD^+-dependent enzymes responsible for the oxidation of endogenous and exogenous aldehydes to carboxylic acids [24,25].

2.5. Recombinant expression of cALDH-I

In order to confirm the specific reactivity of cALDH-I with sulfonate **1**, this protein was recombinantly expressed in both eukaryotic and prokaryotic systems. The cALDH-I cDNA was subcloned into the pcDNA3 mammalian expression vector and then transfected into COS-7 and MCF-7 cells. cALDH-I-transfected COS-7 and MCF-7 cells both expressed a 55 kDa protein that labeled strongly with sulfonate **1** (Fig. 5B). In contrast, this sulfonate-reactive protein was not detected in mock-transfected versions of each cell type. cALDH-I was also recombinantly expressed in *Escherichia coli* using the pTrcHis system. Lysates from cALDH-I-transformed *E. coli* were treated with sulfonate **1** and found to express a single reactive protein of the predicted size for the cALDH-I enzyme bearing an N-terminal histidine tag (60 kDa; Fig. 5C). The His-tagged cALDH-I was purified from *E. coli* lysates by sequential metal affinity and gel filtration chromatography. This prokaryotic expression system routinely provided 15 mg/l culture volume of purified cALDH-I enzyme.

Members of the sulfonate library displayed a relatively broad range of apparent reactivities with cALDH-I in the soluble testis proteome, with some probes labeling the 55 kDa enzyme quite strongly and others displaying little or no reactivity towards this protein (see Fig. 2). In order to determine whether the sulfonate labeling events observed for the 55 kDa protein in the testis proteome reflected reactivity primarily with cALDH-I, several sulfonate probes were incubated with cALDH-I-transfected COS-7 extracts. Interestingly, the relative reactivities of sulfonates with the COS-expressed cALDH-I enzyme matched closely their relative labeling efficiencies with the 55 kDa testis protein (Fig. 5D), indicating that the sulfonate labeling pattern observed at this molecular mass in the testis proteome was predominantly due to cALDH-I.

2.6. Pyridylsulfonates are time-dependent inhibitors of cALDH-I catalytic activity

cALDH-I-catalyzed oxidation of propionaldehyde to propionic acid was measured by observing the reduction of NAD^+ to NADH at 340 nm. The observed Michaelis constant for propionaldehyde ($K_m = 4.2$ μM) displayed by the His-tagged recombinant cALDH-I was comparable to the reported literature value for this enzyme ($K_m = 6.5$ μM) [26]. To examine the effect of sulfonates on cALDH-I's catalytic activity, the enzyme was treated with varying concentrations of 2-pyridylsulfonyl octanoate (**15**), a variant of **1** lacking the probe's biotin tag. Sulfonate **15** inhibited cALDH-I's catalytic activity in a time-dependent manner that increased in rate from 2.5 to 15 μM inhibitor (Fig. 6A). Concentrations of **15** greater than 15 μM inactivated cALDH-I at a rate that was too fast to measure under the assay conditions employed. The average $K_{obs}/[I]$ value calculated from reactions conducted at 2.5, 5.0, 7.5, 10, and 15 μM **15** was $(9.7 \pm 1.8) \times 10^3$ M^{-1} min^{-1}. Although the inactivation of cALDH-I at each concentration of **15** could be fit to pseudo-first order kinetics, extrapolation of these reactions back to time zero did not

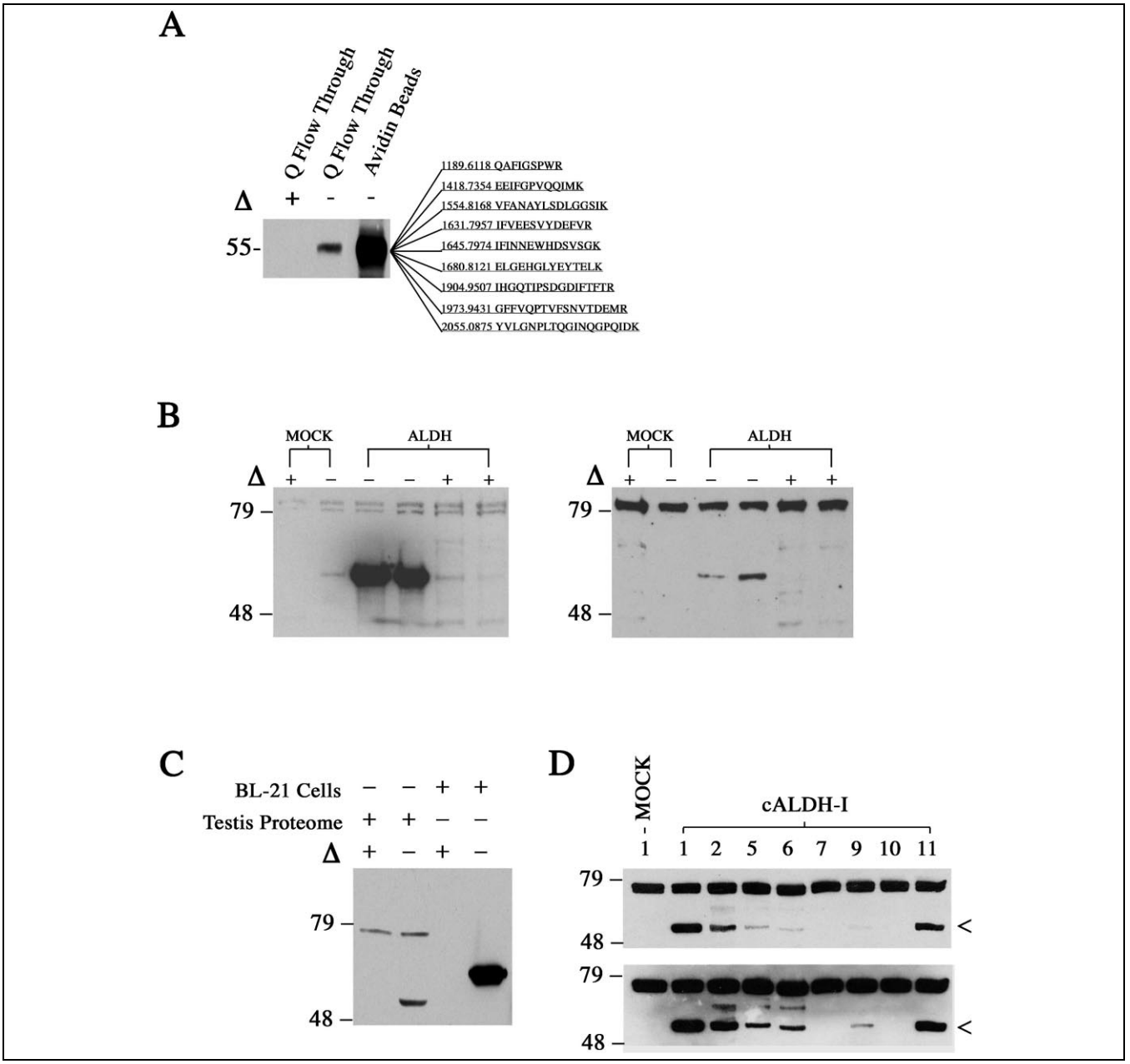


Fig. 5. Identification of a 55 kDa specifically labeled sulfonate target as cALDH-I. A: Avidin-based affinity isolation of the 55 kDa **1**-labeled protein. Shown is an avidin blot of samples containing the partially purified 55 kDa protein (Q Flow Through) and the affinity-isolated 55 kDa protein (Avidin Beads). Also shown are the tryptic peptides from this protein that identified it as cALDH-I. B: Sulfonate **1** labels recombinant cALDH-I in eukaryotic expression systems. Protein samples from COS-7 (left panel) and MCF-7 (right panel) cells transfected with the cALDH-I cDNA or empty vector (mock) were reacted with **1** and resolved by SDS–PAGE and avidin blotting. A strongly labeled 55 kDa protein was identified only in the cALDH-I-transfected cells. C: Sulfonate **1** labels recombinant cALDH-I in prokaryotic expression systems. A protein sample of *E. coli* BL-21 cells transformed with a His-tagged version of cALDH-I was reacted with **1** and resolved by SDS–PAGE and avidin blotting. A strongly labeled 60 kDa protein was identified, corresponding to the predicted molecular mass of cALDH-I with an appended N-terminal histidine tag. D: Reactivity of recombinantly expressed cALDH-I with members of the sulfonate library. Sulfonate probes (numbers shown above lanes) were reacted with cALDH-I-transfected COS-7 extracts and the reactions comparatively analyzed by SDS–PAGE and avidin blotting. A mock-transfected COS extract treated with sulfonate **1** was also run as a control. Note that the relative reactivity of sulfonates with the COS-expressed cALDH-I enzyme (arrowhead) matched closely their relative labeling efficiencies with the 55 kDa protein of the testis proteome (Fig. 2). Top and bottom panels represent 1 and 5 min film exposures, respectively.

predict 100% enzyme activity. Considering that cALDH-I is a homotetrameric protein [27], one possible explanation for these data was that individual cALDH-I subunits exhibited different rates of reactivity with **15**. In general support of this notion, the kinetics of reactions conducted at lower concentrations of **15** appeared biphasic in nature, with time points preceding 50% enzyme inhibition predicting a slightly faster rate of cALDH-I inactivation than time points at which greater than 50% cALDH-I activity was inhibited. Finally, incubating **15** in the reaction buffer

(20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM DTT) for 60 min prior to the addition of cALDH-I did not affect the inhibitor's potency, indicating that this sulfonate was stable to the assay conditions employed (including the presence of excess free thiols).

To probe the nature of **15**'s interaction with cALDH-I, competition studies were performed with both propionaldehyde and NAD^+. Recombinant cALDH-I was treated with 10 μM **15** for 10 min either in the presence or absence of 25 μM propionaldehyde or 50 μM NAD^+ and the percentage of enzyme activity remaining was determined (Table 1). Propionaldehyde had no detectable effect on **15**'s inactivation kinetics. In contrast, 50 μM NAD^+ significantly reduced **15**'s inhibition of cALDH-I, and higher concentrations of NAD^+ completely protected the enzyme from inactivation (data not shown).

2.7. Features of pyridylsulfonates responsible for cALDH-I inactivation

Analogs of **15** were synthesized in which the agent's octyl and pyridyl substituents were replaced with ethyl (**16**) and methyl (**17**) groups, respectively. cALDH-I was incubated for 60 min with 50 μM of either **15**, **16**, or **17**, and the percentage of cALDH-I activity remaining was determined. While **15** completely inactivated cALDH-I under these conditions, **16** and **17** produced weak and no inhibition, respectively. A K_{obs}/[I] value of 0.25 M^{-1} min^{-1} was calculated for **16**, representing a second order inhibition rate constant 40 000 times lower than that determined for **15**. **15**–**17** were also tested for their ability to block sulfonate **1**'s reactivity with cALDH-I in the soluble testis proteome. The testis proteome was preincubated for 30 min with each non-biotinylated sulfonate at concentrations of 5 or 50 μM. The proteome samples were then treated with 5 μM **1** and the reaction mixtures incubated for 30 min prior to analysis by SDS–PAGE and avidin blotting. Consistent with the inhibition kinetics described above, only **15** blocked the labeling of cALDH-I by **1** in the testis proteome (Fig. 6B). Collectively, these data highlight that the interaction of sulfonate **1** with cALDH-I depends on the chemical nature of both the linker and binding groups of the inhibitor.

2.8. Multiplexing ABPs increases proteome coverage

Although individually, biotinylated sulfonates only profiled a modest fraction of the proteome, their application together, or in combination with other ABPs ('multiplexing') may significantly augment the number of proteins visualized in a single assay. To test this notion, the rat testis proteome was treated with either 2.5 μM sulfonate **1**, 5 μM FP-biotin, or a mixture of 2.5 μM **1** and 5 μM FP-biotin [14], and the resulting heat-sensitive labeling profiles were visualized by SDS–PAGE and avidin blotting. As can be seen in Fig. 7, applying a mixture of sulfonate **1** and FP-biotin to the testis proteome effectively detected in a single sample the proteins labeled by both probes individually. Notably, a preheated sample treated with the same probe mixture displayed a very low level of labeling that was comparable to the non-specific reactivity observed when each probe was tested alone. These data suggest that by multiplexing ABPs, one can significantly

Table 1
Competition studies for **15** with propionaldehyde and NAD^+ in cALDH

Substrate	Sulfonate **15** (μM)	Activity remaining (%)
–	–	100
–	5	15
50 μM NAD^+	5	61
25 μM Propionaldehyde	5	14

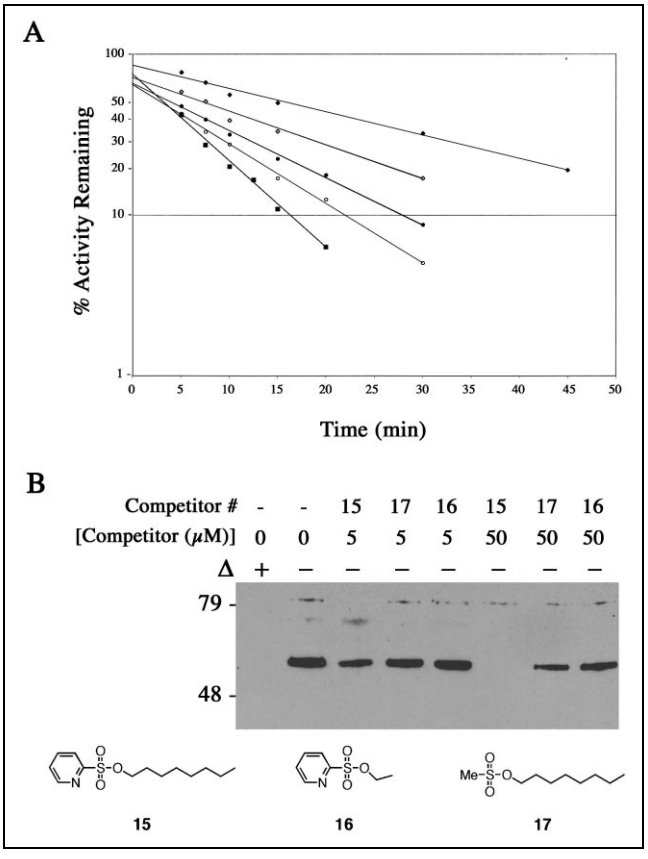


Fig. 6. Sulfonates are time-dependent inhibitors of cALDH-I. A: Time-dependent inactivation of cALDH-I as a function of sulfonate **15** concentration. Recombinant, purified cALDH-I was incubated with different concentrations of **15** and at the time points shown, aliquots of the reaction were removed and assayed for enzyme activity using 1 mM propionaldehyde and 0.5 mM NAD^+ as substrate and cofactor, respectively. Concentrations of **15** were: solid diamonds, 2.5 μM; hollow diamonds, 5 μM; solid circles, 7.5 μM; hollow circles, 10 μM; solid squares, 15 μM. B: Competition reactions between sulfonate **1** and structural analogs **15**–**17**. Sulfonate **15** effectively blocked the labeling of cALDH-I by **1** in the testis proteome. Analogs of **15** in which this sulfonate's pyridyl and octyl groups were replaced with methyl and ethyl groups, respectively (**16** and **17**, respectively), did not block the labeling of cALDH-I by **1**.

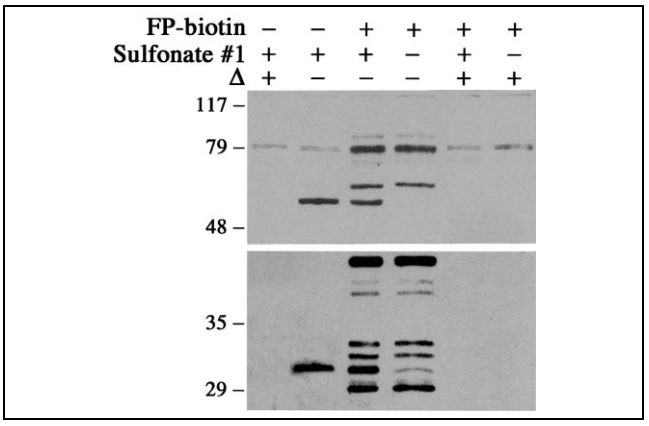


Fig. 7. Multiplexing ABPs increases the number of protein activities detected in a single proteome. Shown is a comparison of the heat-sensitive labeling patterns of a testis proteome treated with pyridylsulfonate **1** (2.5 μM), FP-biotin (4 μM), or a mixture of pyridylsulfonate **1** (2.5 μM) and FP-biotin (4 μM). The mixture-treated proteome exhibited a labeling profile similar to that predicted from merging the profiles of the proteome treated with each ABP alone.

increase the coverage of specific protein reactivities detectable in a single proteome assay.

3. Discussion

Genomics and proteomics aim to characterize on a global level changes in the expression of genes and proteins, respectively. Both methods generate data that are anticipated to reflect dynamics in molecular and cellular function. Considering that proteins are the main components of the cell responsible for executing its various activities, proteomics data could be viewed as bringing one conceptually closer to the goal of understanding complex cellular function. However, proteomics initiatives face serious methodological challenges that have limited their impact, especially when compared to the technically more feasible field of genomics. To date, the field of proteomics has relied primarily on 2DE and MS as its separation and detection methods, respectively [11–13]. Although a significant fraction of the proteome can be analyzed with 2DE-MS methods, numerous classes of proteins remain undetected, including very large/small, highly basic/acidic, membrane, and low abundance proteins [11–13]. Moreover, by measuring changes in protein abundance, standard proteomics experiments provide only an indirect assessment of dynamics in protein activity.

We have initiated a research program aimed at generating chemical probes that profile components of the proteome in an activity-dependent manner. For example, a biotinylated fluorophosphonate (FP-biotin) was recently described that acts as an ABP for the serine hydrolase superfamily of enzymes [14]. Similarly, other researchers have generated biotinylated epoxides and electrophilic ketones as profiling agents for subclasses of cysteine proteases [15–17]. Each of these ABPs measures changes in enzyme activity directly in complex proteomes, thereby offering a global visualization of dynamics in protein function independent of changes in protein abundance. Additionally, an ABP's biotin tag serves not only as a sensitive detection device for classes of low abundance and biochemically problematic proteins (e.g. membrane proteins; our unpublished data), but also as a means for rapidly isolating and identifying labeled proteins using avidin-based solid supports.

In the design of ABPs that targeted serine and cysteine hydrolases, researchers exploited a set of thoroughly characterized active site-directed reactive groups and/or chemical structures. However, for many enzyme and protein families, well-defined affinity labeling reagents have not yet been identified. In order to expedite the discovery of new ABPs that show protein selectivities and reactivities compatible with complex proteome analyses, we have synthesized a library of biotinylated sulfonate esters and applied these agents to complex proteomes using an assay that distinguished their specific and non-specific protein reactivities. This assay involved comparing a sulfonate's reactivity with native and heat-denatured versions of the proteome. Proteins that showed heat-sensitive sulfonate reactivity were considered 'specific protein targets', while those that were labeled in both native and heat-denatured proteomes were defined as 'non-specific protein targets'. We hypothesized that proteins reacting with members of the sulfonate library in a heat-sensitive manner would likely possess structured sites for small molecule interaction, and that these sites would often determine the biological activity of the protein (e.g. ligand binding pockets of receptors or active sites of enzymes). In such cases, sulfonate labeling was hoped to affect the function of the protein target.

Members of the sulfonate library displayed strikingly different specific and non-specific proteome reactivities. At low micromolar concentrations, most sulfonates exhibited heat-sensitive reactivity with a discrete number of proteins, while at the same time showing very low or negligible reactivity with the heat-denatured proteome. Individual sulfonates displayed unique patterns of specific reactivity, and even subtle variations in the sulfonate's binding group resulted in significant changes in proteome labeling. For example, the pyridyl (**1**), benzene (**2**), and *p*-tosyl (**3**) sulfonates, despite their structural similarity, all displayed distinct specific proteome reactivities, with **1** labeling 31 and 55 kDa proteins, **2** labeling 32, 42, and 55 kDa proteins, and **3** labeling exclusively a 55 kDa protein. A few sulfonates, like the *p*-methoxybenzenesulfonate **4**, exhibited a high degree of non-specific reactivity, labeling numerous proteins in both heat-denatured and native proteomes. All of these non-specific targets represented proteins of high abundance (as judged by Coomassie blue staining), suggesting that a sulfonate's heat-insensitive labeling was more reflective of its intrinsic reactivity than a

selective association with particular constituents of the proteome. In contrast, the sulfonate library's heat-sensitive labeling showed no such bias towards abundant proteins, indicating that these reactivities resulted from specific small molecule–protein interactions. We speculate that an expanded library of ABPs in which not only the binding group, but also the linker and reactive group are varied will uncover additional probes that display distinct patterns of specific proteome reactivity.

Perhaps not surprisingly, the ratio of a sulfonate's specific versus non-specific reactivity depended strongly on the concentration of the agent applied to the proteome. For example, sulfonate **1** showed negligible heat-insensitive labeling at 1–5 μM concentrations, but as its concentration approached 50 μM, the probe began to label numerous proteins in both the native and heat-denatured proteomes. Interestingly, no additional heat-sensitive reactivities were detected when the concentration of **1** was raised from 5 to 50 μM. Collectively, these data highlight the importance of determining for a given electrophilic probe the concentration range over which the agent's ratio of specific versus non-specific reactivity is maximized.

To examine the sulfonate library's specific proteome reactivities in more detail, a heat-sensitive 55 kDa protein target was isolated by avidin-based affinity chromatography and identified as cALDH-I. ALDHs constitute a superfamily of enzymes that utilize the cofactor NAD^+ ($NADP^+$) to oxidize exogenous and endogenous aldehydes to carboxylic acids through formation of a thioester intermediate with an active site cysteine nucleophile [28–30]. The ALDH family is divided into three subclasses: the homotetrameric cytosolic class I and the mitochondrial class II isoforms and the homodimeric cytosolic class III isoforms [25,27]. Genome sequencing projects have identified numerous ALDHs in *Drosophila melanogaster* (28 ALDHs), *Caenorhabditis elegans* (13 ALDHs), and *Saccharomyces cerevisiae* (13 ALDHs) [31]. In humans, at least 16 ALDH genes have already been characterized [32]. Mammalian ALDHs are noted for their roles in retinoic acid biosynthesis [33], the detoxification of nitrogen-mustard based chemotherapeutic agents [34–36], and the catabolism of ethanol to acetic acid [37]. cALDH-I, a quantitatively minor, though widely distributed cytosolic protein, appears to function in the retinoid signaling pathway by catalyzing the irreversible oxidation of retinal to retinoic acid [38,39].

The heat-sensitive reactivity of cALDH-I with sulfonate **1** was confirmed by expressing this enzyme in both eukaryotic and prokaryotic systems. Additionally, sulfonate **15** (a non-biotinylated version of **1**) was found to act as a time-dependent irreversible inhibitor of cALDH-I, exhibiting a $k_{obs}/[I]$ value of 9.7×10^3 M^{-1} min^{-1} (similar inhibitory properties were observed for sulfonate **1**; data not shown). Excess NAD^+ protected cALDH-I from inhibition by **15** (as well as from labeling by **1** in complex proteomes; data not shown), consistent with the notion that this sulfonate labeled cALDH-I at or near its active site. Analogs of **15** in which the agent's pyridyl and octyl groups were replaced with methyl and ethyl groups, respectively, were inactive as cALDH-I inhibitors, indicating that both the binding group and linker components of **15** contributed significant binding interactions. Nonetheless, saturation kinetics could not be achieved with **15** even at concentrations as high as 20 μM, indicating that its binding affinity for cALDH-I is relatively weak. These data, in conjunction with the recognition that sulfonate **1** reacted with only two proteins in the testis proteome, suggest that an ABP displaying a combination of tempered reactivity and modest binding affinity can engage in protein labeling events of high selectivity.

The relatively potent and selective inhibition of cALDH-I by **15** raises several intriguing issues. First, what is the mechanism by which **15** reacts with cALDH-I? Although it is attractive to speculate that the enzyme's cysteine nucleophile represents the site of sulfonate labeling, the cALDH-I–**15** adduct has not yet been isolated and characterized. If cALDH-I's nucleophile does prove to be the site of reactivity, then sulfonates appended with the appropriate binding group(s) could act as active site-directed profiling agents for the ALDH family as a whole. Indeed, considering that many ALDHs are 55 kDa in size, at present we cannot exclude the possibility that our sulfonate library is reacting with more than one ALDH in the proteome. Finally, sulfonates like **15** could serve as lead compounds for the generation of highly selective and potent inhibitors of ALDHs, an enzyme family of significant therapeutic importance. On this note, disulfiram, a relatively non-selective ALDH inhibitor, has been used clinically for many years in alcohol aversion therapy [40]. However, disulfiram's therapeutic utility is limited by its high reactivity in vivo, resulting in the non-specific carbamoylation of several serum and cellular proteins [41,42] and glutathione [43]. Thus, more selective and potent ALDH inhibitors may find application in the treatment of alcoholism, as well as various forms of cancer [34–36].

4. Significance

The chemical and biochemical approaches described herein represent an alternative strategy for analyzing the proteome that is both complementary and in some aspects superior to the standard 2DE-MS methods currently in practice. By treating complex proteomes with a library of chemical probes bearing a moderately reactive sulfonate ester, diverse patterns of specific protein reactivity were identified. Probes were characterized in terms of their heat-sensitive protein labeling patterns, permitting the discovery of reagents that profiled proteins based on properties other than abundance. Although such ABPs when applied individually to the proteome detected only a limited set of proteins, their utility in combination should

greatly enhance the characterization of a large number of low abundance proteins. Finally, the discovery that sulfonate probes not only labeled cALDH-I in complex proteomes, but also inhibited this enzyme's catalytic activity suggests that, at least in this one example, a screen for heat-sensitive labeling events accurately identified a small molecule–protein reaction that impacted the protein's biological function. If this correlation proves generalizable, non-directed approaches for profiling the specific reactivity of the proteome may generate chemical reagents applicable for both proteomics investigations and cell-based functional screens. In this latter case, one could envision screening a library of ABPs for compounds that possess a particular cellular bioactivity, and then comparing the proteome labeling patterns of biologically active and inactive library members to identify the affected protein target(s).

5. Materials and methods

5.1. Synthesis alkyl and aryl sulfonate esters

All reactions were carried out under an atmosphere of argon unless specified. Methylene chloride (CH_2Cl_2) was dried by passing through activated alumina columns. Commercial reagents of high purity were purchased and used without further purification unless otherwise noted. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AMX-400 instrument and calibrated to the residual solvent peak. The multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet).

The synthesis of 10-((2-pyridylsulfonyl)oxo)-*N*-biotinamidopentyldecanamide (**1**) is provided as a representative synthesis of the 11 biotinylated alkyl and aryl sulfonates (**1**–**11**).

*5.1.1. ((2-Pyridylsulfonyl)oxo)-10-undecene (**13**)*

A solution of ω-undecylenyl alcohol (**12**) (0.50 g, 2.91 mmol, 1.0 equivalents (equiv.)) in pyridine (4 ml) was cooled to 0°C and treated with 2-pyridylsulfonyl chloride (1.04 g, 5.87 mmol, 2.0 equiv.), prepared according to the procedure of Corey and colleagues [44]. The reaction mixture was kept at 0°C for 6 h, then partitioned between ethyl acetate (50 ml) and water (25 ml). The organic layer was washed with 10% aqueous HCl (2 × 50 ml) and saturated aqueous NaCl (50 ml), dried ($MgSO_4$), and concentrated under reduced pressure. Column chromatography (2% EtOAc/Hex) afforded **13** as a colorless oil (98%): ^{1}H NMR ($CDCl_3$, 400 MHz), δ 8.61 (m, 1H, Ar*H*), 7.89 (m, 2H, Ar*H*), 7.47 (m, 1H, Ar*H*), 5.67–5.60 (m, 1H, RC*H*=CH_2), 4.84–4.74 (m, 2H, RCH=C*H*$_2$), 4.21 (t, J = 6.4 Hz, 2H, C*H*$_2$OSO$_2$Pyr), 1.88 (m, 2H, C*H*$_2$CH=CH_2), 1.55 (p, 2H, J = 6.8 Hz, 2H, C*H*$_2$CH$_2$OSO$_2$Pyr), 1.20–1.08 (m, 12H); MALDI-FTMS 334.1433 ($C_{16}H_{25}NO_3S$+Na^+ requires 334.1447).

*5.1.2. 10-((2-Pyridylsulfonyl)oxo)-decanoic acid (**14**)*

Compound **13** (0.90 g, 2.88 mmol, 1 equiv.) in a biphasic solution composed of CCl_4–CH_3CN–H_2O (10 ml–10 ml–15 ml) with a total volume of 35 ml was treated sequentially with sodium periodate (2.53 g, 11.80 mmol, 4.1 equiv.) and ruthenium trichloride hydrate (0.005 g, 0.02 mmol, 0.03 equiv.). The reaction was stirred at 25°C overnight then partitioned between CH_2Cl_2 (100 ml) and 1 N aqueous HCl (2 × 100 ml). The organic layer was washed with saturated aqueous NaCl (100 ml), dried ($MgSO_4$) and concentrated under reduced pressure. Column chromatography (40% EtOAc/Hex) afforded **14** (80%): ^{1}H NMR ($CDCl_3$, 400 MHz), δ 8.84 (d, J = 4.0 Hz, 1H, Ar*H*), 8.11 (d, J = 5.9 Hz, 1H, Ar*H*), 8.05 (t, J = 6.0 Hz, 1H, Ar*H*), 7.65 (t, J = 3.3 Hz, 1H, Ar*H*), 4.37 (t, J = 6.6 Hz, 2H, C*H*$_2$OSO$_2$Pyr), 2.34 (t, J = 7.4 Hz, 2H, C*H*$_2$COOH), 1.70 (p, J = 8.0 Hz, 2H, *CH*$_2$CH$_2$COOH), 1.61 (p, J = 7.3 Hz, 2H, C*H*$_2$CH$_2$OSO$_2$Pyr), 1.25 (m, 10H): MALDI-FTMS (DHB) *m*/*z* 352.1202 ($C_{15}H_{23}NO_5S$+Na^+ requires 352.1189).

*5.1.3. 10-((2-Pyridylsulfonyl)oxo)-N-biotinamidopentyldecanamide (**1**)*

A solution of **14** (0.030 g, 0.09 mmol, 10 equiv.) in CH_2Cl_2 (1.5 ml) at −78°C was treated dropwise with (diethylamino)sulfur trifluoride (0.027 ml, 0.21 mmol, 22 equiv.), brought to 25°C, and stirred for 10 min. The reaction was then treated with one-half reaction volume of dimethylformamide containing *N*-hydroxysuccinimide (0.05 g, 0.04 mmol, 40 equiv.) and stirred for an additional 15 min at 25°C. The reaction mixture was partitioned between ethyl acetate (50 ml) and water (50 ml). The organic layer was washed with saturated aqueous NaCl (200 ml), dried (Na_2SO_4), and concentrated under reduced pressure to afford 10-((2-pyridylsulfonyl)oxo)-*N*-(hydroxysuccinyl)decanamide (as judged by crude ^{1}H NMR; data not shown). Without further purification, the intermediate was treated with 5-(biotinamido)-pentylamine (Pierce, 0.003 g, 0.009 mmol, 1.0 equiv.) in MeOH (0.04 ml) and stirred for 30 min. The solvent was evaporated under a stream of nitrogen, and the remaining residue was washed with ethyl acetate (2 × 2.5 ml), solubilized in a minimal volume of chloroform, transferred to a clean glass vial, and the solvent evaporated. This process was repeated to rid the desired biotinylated product of excess reagents and byproducts, affording **4** as a white film (0.004 g, 46%): ^{1}H NMR ($CDCl_3$, 400 MHz), δ 8.79 (d, J = 7.0 Hz, 1H, Ar*H*), 8.06 (m, 2H, Ar*H*), 7.61 (t, J = 7.3 Hz, 1H, Ar*H*), 6.05 (b s, 1H, N*H*), 5.91 (b s, 1H, N*H*), 5.60 (b s, 1H, N*H*), 4.77 (b s, 1H, N*H*), 4.54 (m, 1H), 4.39 (m, 1H+2H, C*H*$_2$OSO$_2$R), 3.22 (m, 4H, C*H*$_2$NHCOR), 3.11 (m, 1H), 2.92 (dd, J = 4.0 and 12.9 Hz, 1H), 2.76 (d, J = 13.3 Hz, 1H), 2.18 (m, 4H, C*H*$_2$CONHR), 1.67–1.28 (m, 26H); MALDI-FTMS (DHB) *m*/*z* 640.3209 ($C_{30}H_{49}N_5O_6S_2$+H^+ requires 640.3202).

*5.1.4. 10-((Benzenesulfonyl)oxo)-N-biotinamidopentyldecanamide (**2**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 7.93 (d, J = 7.0 Hz, 2H, Ar*H*), 7.67 (t, J = 7.4 Hz, 1H, Ar*H*), 7.59 (t, J = 7.3 Hz, 2H, Ar*H*), 6.06 (b s, 1H, N*H*), 5.87 (b s, 1H, N*H*), 5.63 (b s, 1H, N*H*), 4.79 (b s, 1H, N*H*), 4.53 (m, 1H), 4.37 (m, 1H), 4.07 (t, J = 6.4 Hz, 2H, C*H*$_2$OSO$_2$R), 3.26 (m, 4H, C*H*$_2$NHCOR), 3.09 (m, 1H), 2.93 (dd, J = 5.0 and 7.9 Hz, 1H), 2.76 (d, J = 13.0 Hz, 1H), 2.19 (m, 4H,

CH_2CONHR), 1.62–1.26 (m, 26H); MALDI-FTMS (DHB) *m/z* 639.3244 ($C_{31}H_{50}N_4O_6S_2+H^+$ requires 639.3245).

*5.1.5. 10-((p-Toluenesulfonyl)oxo)-N-biotinamidopentyl-decanamide (**3**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 7.78 (d, J = 8.5 Hz, 2H, Ar*H*), 7.37 (d, J = 7.9 Hz, 2H, Ar*H*), 5.91 (b s, 1H, N*H*), 5.84 (b s, 1H, *NH*), 5.49 (b s, 1H, N*H*), 4.76 (b s, 1H, N*H*), 4.53 (m, 1H), 4.35 (m, 1H), 4.02 (t, J = 6.4 Hz, 2H, CH_2OSO_2R), 3.25 (m, 4H, CH_2NHCOR), 3.18 (m, 1H), 2.93 (dd, J = 5.0 and 7.9 Hz, 1H), 2.76 (d, J = 12.9 Hz, 1H), 2.46 (s, 3H, CH_3Ar), 2.19 (m, 4H, CH_2CONHR), 1.70–1.50 (m, 26H); MALDI-FTMS (DHB) *m/z* 653.3381 ($C_{32}H_{52}N_4O_6S_2+H^+$ requires 653.3401).

*5.1.6. 10-((4-Methoxybenzenesulfonyl)oxo)-N-biotinamidopentyl-decanamide (**4**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 7.86 (d, J = 8.8 Hz, 2H, Ar*H*), 7.03 (d, J = 8.8 Hz, 2H, Ar*H*), 5.96 (b s, 1H, N*H*), 5.85 (b s, 1H, N*H*), 5.57 (b s, 1H, N*H*), 4.84 (b s, 1H, N*H*), 4.53 (m, 1H), 4.37 (m, 1H), 4.01 (t, J = 6.5 Hz, 2H, CH_2OSO_2R), 3.90 (s, 3H, CH_3OAr), 3.25 (m, 4H, CH_2NHCOR), 3.17 (m, 1H), 2.95 (dd, J = 4.7 and 7.7 Hz, 1H), 2.73 (d, J = 12.9 Hz, 1H), 2.25 (m, 4H, CH_2CONHR), 1.63–1.26 (m, 26H); MALDI-FTMS (DHB) *m/z* 669.3381 ($C_{32}H_{52}N_4O_7S_2+H^+$ requires 669.335).

*5.1.7. 10-((Methylsulfonyl)oxo)-N-biotinamidopentyldecanamide (**5**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 6.00 (b s, 1H, N*H*), 5.85 (b s, 1H, N*H*), 5.60 (b s, 1H, N*H*), 4.81 (b s, 1H, N*H*), 4.53 (m, 1H), 4.37 (m, 1H), 4.35 (t, J = 6.2 Hz, 2H, CH_2OSO_2R), 3.26 (m, 4H, CH_2NHCOR), 3.18 (m, 1H), 2.93 (dd, J = 5.0 and 7.9 Hz, 1H), 2.76 (d, J = 12.9 Hz, 1H), 2.21 (m, 4H, CH_2CONHR), 2.05 (s, 3H, H_3CSO_3R), 1.75–1.27 (m, 26H); MALDI-FTMS *m/z* 577.3105 ($C_{26}H_{48}N_4O_6S_2+H^+$ requires 577.3088).

*5.1.8. 10-((Butylsulfonyl)oxo)-N-biotinamidopentyldecanamide (**6**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 5.93 (b s, 1H, N*H*), 5.84 (b s, 1H, N*H*), 5.53 (b s, 1H, N*H*), 4.82 (b s, 1H, N*H*), 4.54 (m, 1H), 4.37 (m, 1H), 4.21 (t, J = 6.2 Hz, 2H, CH_2OSO_2R), 3.26 (m, 4H, CH_2NHCOR), 3.19 (m, 1H), 3.09 (t, J = 4.1 Hz, 2H, CH_2SO_3R), 2.93 (dd, J = 4.6 and 7.4 Hz, 1H), 2.76 (d, J = 9.1 Hz, 1H), 2.21 (m, 4H, CH_2CONHR), 2.16–1.31 (m, 30H), 0.97 (t, J = 7.3 Hz, 3H); MALDI-FTMS (DHB) *m/z* 619.3530 ($C_{29}H_{54}N_4O_6S_2+H^+$ requires 619.3530).

*5.1.9. 10-((Octylsulfonyl)oxo)-N-biotinamidopentyldecanamide (**7**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 6.05 (b s, 1H, N*H*), 5.87 (b s, 1H, N*H*), 5.81 (b s, 1H, N*H*), 5.00 (b s, 1H, N*H*), 4.53 (m, 1H), 4.35 (m, 1H), 4.21 (t, J = 6.2 Hz, 2H, CH_2OSO_2R), 3.25 (m, 4H, CH_2NHCOR), 3.17 (m, 1H), 3.09 (t, J = 8.5 Hz, 2H, CH_2SO_3R), 2.93 (dd, J = 4.7 and 7.9 Hz, 1H), 2.76 (d, J = 12.9 Hz, 1H), 2.19 (m, 4H, CH_2CONHR), 1.86 (p, J = 7.9 Hz, 2H, $CH_2CH_2SO_3R$), 1.76–1.31 (m, 36H), 0.89 (t, J = 6.4 Hz, 3H); MALDI-FTMS (DHB) *m/z* 675.4173 ($C_{33}H_{62}N_4O_6S_2+H^+$ requires 675.4184).

*5.1.10. 10-((4-Nitrobenzenesulfonyl)oxo)-N-biotinamidopentyl-decanamide (**8**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 8.44 (d, J = 9.1 Hz, 2H, Ar*H*), 8.14 (d, J = 9.1 Hz, 2H, Ar*H*), 5.90 (b s, 1H, N*H*), 5.82 (b s, 1H, N*H*), 5.50 (b s, 1H, N*H*), 4.86 (b s, 1H, N*H*), 4.54 (m, 1H), 4.37 (m, 1H), 4.14 (t, J = 6.5 Hz, 2H, CH_2OSO_2R), 3.25 (m, 4H, CH_2NHCOR), 3.18 (m, 1H), 2.96 (dd, J = 5.0 and 7.9 Hz, 1H), 2.76 (d, J = 15.0 Hz, 1H), 2.21 (m, 4H, CH_2CONHR), 1.72–1.26 (m, 26H); MALDI-FTMS (DHB) *m/z* 684.3069 ($C_{31}H_{49}N_5O_8S_2+H^+$ requires 684.3095).

*5.1.11. 10-((8-Quinolinesulfonyl)oxo)-N-biotinamidopentyl-decanamide (**9**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 9.17 (m, 1H, Ar*H*), 8.53 (d, J = 5.8 Hz, 1H, Ar*H*), 8.50 (d, J = 6.4 Hz, 1H, Ar*H*), 8.16 (d, J = 6.7 Hz, 1H, Ar*H*), 7.70 (t, J = 7.6 Hz, 1H, Ar*H*), 7.61 (q, J = 3.9 Hz, 1H, Ar*H*), 6.06 (b s, 1H, N*H*), 5.93 (b s, 1H, N*H*), 5.73 (b s, 1H, N*H*), 5.08 (b s, 1H, N*H*), 4.54 (m, 1H), 4.35 (m, 1H), 4.30 (t, J = 6.5 Hz, 2H, CH_2OSO_2R), 3.27 (m, 4H, CH_2NHCOR), 3.18 (m, 1H), 2.96 (dd, J = 5.0 and 7.9 Hz, 1H), 2.77 (d, J = 15 Hz, 1H), 2.21 (m, 4H, CH_2CONHR), 1.68–1.18 (m, 26H); MALDI-FTMS 712.3189 ($C_{34}H_{51}N_5O_6S_2+Na^+$ requires 712.3173).

*5.1.12. 10-((2-Naphthalenesulfonyl)oxo)-N-biotinamidopentyl-decanamide (**10**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 8.49 (s, 1H, Ar*H*), 7.98 (t, J = 8.2 Hz, 2H, Ar*H*), 7.93 (d, J = 7.9 Hz, 1H, Ar*H*), 7.87 (d, J = 8.8 Hz, 1H, Ar*H*), 7.65 (p, J = 7.0 Hz, 2H, Ar*H*), 6.05 (b s, 1H, N*H*), 5.91 (b s, 1H, N*H*), 5.60 (b s, 1H, N*H*), 4.77 (b s, 1H, N*H*), 4.53 (m, 1H), 4.37 (m, 1H), 4.06 (t, J = 6.4 Hz, 2H, CH_2OSO_2R), 3.25 (m, 4H, CH_2CONHR), 3.17 (m, 1H), 2.95 (dd, J = 5.0 and 7.6 Hz, 1H), 2.77 (d, J = 14.0 Hz), 2.17 (m, 4H, CH_2CONHR), 1.71–1.20 (m, 26H); MALDI-FTMS (DHB) *m/z* 689.3379 ($C_{35}H_{52}N_4O_6S_2+H^+$ requires 689.3401).

*5.1.13. 10-((2-Thiophenesulfonyl)oxo)-N-biotinamidopentyl)-decanamide (**11**)*

^{1}H NMR ($CDCl_3$, 400 MHz), δ 7.74 (t, J = 5.0 Hz, 2H, Ar*H*), 7.16 (t, J = 3.8 Hz, 1H, Ar*H*), 5.90 (b s, 1H, N*H*), 5.84 (b s, 1H, N*H*), 5.47 (b s, 1H, N*H*), 4.75 (b s, 1H, N*H*), 4.53 (m, 1H), 4.35 (m, 1H), 4.12 (t, J = 6.4 Hz, 2H, CH_2OSO_2R), 3.26 (m, 4H, CH_2NHCOR), 3.19 (m, 1H), 2.95 (dd, J = 5.3 and 7.6 Hz, 1H), 2.76 (d, J = 12.9 Hz, 1H), 2.17 (m, 4H, CH_2CONHR), 1.67–1.26 (m, 26H); MALDI-FTMS (DHB) *m/z* 645.2817 ($C_{29}H_{48}N_4O_6S_3+H^+$ requires 645.2809).

*5.1.14. 1-(2-Pyridylsulfonyl)oxo-octane (**15**)*

To 3.0 ml of anhydrous triethylamine (23.04 mmol, 30 equiv.) at 0°C was added 1-octanol (0.10 g, 0.77 mmol, 1 equiv.) followed by the addition of 2-pyridylsulfonyl chloride in one portion. The mixture was kept at 0°C for 3 h followed by the addition of water (5 ml). The resulting mixture was extracted with diethyl ether (3 × 50 ml), then the organic extracts were combined and washed with aqueous $NaHCO_3$ solution (50 ml), dried ($MgSO_4$), and concentrated under reduced pressure. Column

chromatography (2% EtOAc/Hex) afforded **15** (99%): ^{1}H NMR ($CDCl_3$, 400 MHz), δ 8.68 (d, J = 7.0 Hz, 1H, Ar*H*), 7.94 (p, J = 8.8 Hz, 2H, Ar*H*), 7.54 (t, J = 6.4 Hz, 1H, Ar*H*), 4.26 (t, J = 6.7 Hz, 2H, C*H*$_2$OSO$_2$R), 1.61 (p, J = 7.9 Hz, 2H), 1.13 (m, 10H), 0.76 (t, J = 7.0 Hz, 3H); MALDI-FTMS 294.1130 ($C_{13}H_{21}NO_3S+Na^+$ requires 294.1134).

*5.1.15. 1-(2-Pyridylsulfonyl)oxo-ethane (**16**)*

To a solution of triethylamine (0.86 g, 8.51 mmol, 2.2 equiv.) in dichloromethane (3 ml) at 0°C was added ethanol (0.18 g, 3.87 mmol, 1 equiv.) followed by the addition of 2-pyridylsulfonyl chloride (0.83 g, 4.65 mmol, 1.2 equiv.). After stirring for 4 h at 0°C, the solution was concentrated under reduced pressure. The concentrate was dissolved in aqueous $NaHCO_3$ solution (50 ml) and extracted with diethyl ether (3×50 ml). The ether extracts were combined and washed with aqueous NaCl (50 ml), dried ($MgSO_4$), and concentrated under reduced pressure. Column chromatography (5% EtOAc/Hex) afforded **16** (95%): ^{1}H NMR ($CDCl_3$, 400 MHz), δ 8.70 (d, J = 4.7 Hz, 1H, Ar*H*), 7.95 (p, J = 6.4 Hz, 2H, Ar*H*), 7.56 (t, J = 5.3 Hz, 1H, Ar*H*), 4.37 (q, J = 7.0 Hz, 2H, C*H*$_2$OSO$_2$R), 1.29 (t, J = 7.0 Hz, 3H); MALDI-FTMS 188.0000 ($C_6H_9NOS+H^+$ requires 188.0376).

*5.1.16. 1-(Methanesulfonyl)oxo-octane (**17**)*

To a solution of triethylamine (0.12 g, 1.15 mmol, 1.5 equiv.) in dichloromethane (3 ml) was added octanol (0.10 g, 0.77 mmol, 1.0 equiv.) at 0°C followed by the addition of methanesulfonyl chloride (0.10 g, 0.85 mmol, 1.1 equiv.), over a period of 5 min. After 30 min at 0°C, the reaction mixture was diluted in dichloromethane (50 ml) and extracted with ice cold water (50 ml), ice cold 10% aqueous HCl (50 ml), saturated aqueous $NaHCO_3$ (50 ml), and with saturated aqueous NaCl (50 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to afford **17** (97%): ^{1}H NMR ($CDCl_3$, 400 MHz), δ 4.21 (t, J = 6.5 Hz, 2H, C*H*$_2$OSO$_2$Me), 2.99 (s, 3H, C*H*$_3$SO$_3$), 1.77 (p, J = 6.7 Hz, 2H), 1.35 (m, 10 H), 0.87 (t, J = 6.1 Hz, 3H).

5.2. Proteome sample preparation, labeling, and detection

Rat tissues were Dounce-homogenized in Tris buffer (50 mM Tris–HCl buffer, pH 8.0, 0.32 M sucrose). Tissue extracts were centrifuged sequentially at 1100×g (10 min), 22 000×g (30 min), and 105 000×g (60 min). The final supernatant (soluble fraction) was adjusted to 0.5 mg protein/ml with Tris buffer (without sucrose) and kept at 0°C until utilized. Unless otherwise indicated, reactions between protein samples and biotinylated reagents were conducted as follows: all biotinylated reagents were stored as stock solutions in DMSO at −20°C and then added directly to reactions with protein extracts, keeping the DMSO concentration constant at 1% of the total reaction volume. The reaction mixture was incubated at 25°C for 30 min (final concentration of the probe was 5 μM), then quenched by the addition of 1 volume equiv. of standard 2× SDS–PAGE loading buffer (reducing). Quenched reactions were run on SDS–PAGE (7.5 μg protein/gel lane) and transferred by electroblotting onto nitrocellulose membranes, which were blocked in Tris-buffered saline (TBS) with 1% Tween (TBS-Tween) and 3% (w/v) non-fat dry milk for either 1 h at 25°C or overnight at 4°C. Blots were then treated with an avidin–horseradish peroxidase conjugate (Bio-Rad, 1:1500 dilution) in TBS-Tween for 2 h at 25°C. The blot was washed with TBS-Tween three times (5 min/wash), treated with SuperSignal chemiluminescence reagents (Bio-Rad), and exposed to film for 0.1–20 min before development. For the pH-dependence studies, the following reaction buffers were used: pH 6–8: 50 mM Tris–HCl; pH 8–10: 50 mM Tris–HCl, 50 mM CAPS.

5.3. Enrichment and molecular characterization of a 55 kDa sulfonate-reactive protein

Rat liver soluble fractions were run over a Q-Sepharose column by using an AKTA FPLC (Amersham Pharmacia Biotech) and eluted with a linear gradient of 0–500 mM NaCl. Aliquots of the elution fractions (10×2.5 ml fractions) as well as the flow-through (3×2.5 ml fractions) were labeled with **1** as described above to identify the fractions containing the labeled proteins. The flow-through fractions, which contained the 55 kDa protein, were concentrated to 1 mg protein/ml followed by labeling 2.5 ml of the sample with **1** utilizing the standard conditions. After incubating the reaction for 30 min, it was applied to a PD-10 size exclusion column and eluted with 3.5 ml of pH 8, 50 mM Tris–HCl buffer. SDS (0.5% w/v) was added and the labeled samples heated to 90°C for 10 min in order to denature the proteins allowing for a more accessible biotin moiety. The sample was then diluted 2.5-fold (0.2% SDS) and incubated with 50–100 μl of avidin beads on a rotator for 1 h at 25°C. The eluant was then removed followed by washing with 5 ml of 0.2% SDS and three washes with pH 8, 50 mM Tris–HCl buffer. Standard 2× SDS–PAGE loading buffer was added followed by heating the sample to 90°C in order to elute the proteins labeled with **1** from the avidin beads. The eluant was run on an 8% Novex Tris–glycine gel and stained with Coomassie blue stain followed by destaining in a 30% methanol–water solution. The desired 55 kDa **1**-reactive protein was excised from the gel and digested with trypsin. The resulting peptides were analyzed by MALDI-TOF MS. The MALDI peptide data were utilized in both the MS-Fit search of the Protein Prospector databases (falcon.ludwig.ucl.ac.uk/mskome3.2.htm) and the ProFound search of the Proteometrics databases (www.proteometrics.com/prowl-cgi/ProFound.exe), which identified the protein as cALDH-I.

5.4. Recombinant expression and purification of cALDH-I

Primers were designed based on cALDH-I's cDNA sequence and used to amplify the enzyme's cDNA from a liver cDNA library (Clontech). The cALDH-I cDNA was subcloned into the prokaryotic expression vector, TrcHisA, sequenced, and transformed into *E. coli* BL-21 cells. Expression was induced with 1 mM isopropyl β-D-thiogalactoside when cultures grew to an OD_{600} of 0.6. After 4 h, the cells were pelleted and the supernatant removed. The cell pellet was resuspended in Tris buffer (20 mM Tris–HCl buffer, pH 8.0, 100 mM NaCl), lysed by treatment with lysozyme (1 mg/ml) for 30 min and then sonication. The soluble

fraction was isolated by centrifugation 39 800×*g* (25 min). The His-tagged cALDH-I was purified from the soluble fraction by rotating with Talon cobalt beads for 30 min at 4°C followed by centrifugation and removal of the lysate. After washing, the beads were eluted with 80 mM imidazole buffer and the eluted protein concentrated to 10 mg protein/ml. The concentrated protein solution was subjected to gel filtration chromatography (Superose 6 column, AKTA FPLC, Amersham Pharmacia Biotech). Gel filtration samples containing purified cALDH-I were combined, concentrated, and stored at −78°C in Tris buffer containing 1 mM DTT (final cALDH-I protein concentration, 1.5 mg/ml).

5.5. Expression of cALDH-I in eukaryotic cells

The cALDH-I cDNA was subcloned into the eukaryotic expression vector pcDNA3 and transiently transfected into COS-7 cells and MCF-7 cells by using methods described previously [14]. Transfected cells were harvested by scraping, resuspended in Tris buffer and their protein concentrations determined (D_c protein assay kit, Bio-Rad). Whole cell suspensions were labeled with the sulfonate **1** as described above.

5.6. Characterization of sulfonate reactivity of cALDH-I

cALDH-I-transfected COS-7 cells were harvested by scraping, resuspended in Tris buffer, Dounce-homogenized, and sonicated. The soluble fraction was separated by centrifugation and the protein concentration determined (D_c protein assay kit, Bio-Rad). The soluble lysate (0.5 μg/μl) was incubated with each biotinylated probe (2.5 μM, 1% DMSO) for 2 min at 25°C in Tris buffer (50 mM Tris–HCl, pH 8). A mock-transfected COS-7 sample was labeled with sulfonate **1** as a control. The reactions were quenched and analyzed by SDS–PAGE and avidin blotting as described above.

5.7. cALDH-I enzyme assay and inhibition studies

cALDH-I activity was determined at 25°C in Tris buffer (20 mM Tris–HCl, pH 8, 100 mM NaCl). Purified cALDH-I (0.2 μM) was preincubated with **15** (2.5–15 μM) in DMSO (30 μl, 3% total incubation volume) in a volume of 950 μl for 5–45 min. After preincubation of the enzyme with inhibitor, remaining catalytic activity was measured by adding NAD^+ (500 μM final concentration) and propionaldehyde (1 mM final concentration) in 50 μl of buffer. Production of NADH from the oxidation of propionaldehyde was monitored by measuring the change in absorbance at 340 nm for 2 min. In substrate competition assays, purified cALDH-I (0.2 μM) was preincubated with either NAD^+ (50 μM) or propionaldehyde (25 μM) and 10 μM of **15** for 10 min at 25°C in a volume of 950 μl. Remaining catalytic activity was monitored as described above.

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