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Activity-based proteomics: enzymatic activity profiling in complex proteomes

Review Article

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Summary. In the postgenomic era new technologies are emerging for global analysis of protein function. The introduction of active site-directed chemical probes for enzymatic activity profiling in complex mixtures, known as activity-based proteomics has greatly accelerated functional annotation of proteins. Here we review probe design for different enzyme classes including serine hydrolases, cysteine proteases, tyrosine phosphatases, glycosidases, and others. These probes are usually detected by their fluorescent, radioactive or affinity tags and their protein targets are analyzed using established proteomics techniques. Recent developments, such as the design of probes for in vivo analysis of proteomes, as well as microarray technologies for higher throughput screenings of protein specificity and the application of activity-based probes for drug screening are highlighted. We focus on biological applications of activity-based probes for target and inhibitor discovery and discuss challenges for future development of this field.

Keywords: Activity-based proteomics – Functional proteomics – Enzymatic activity profiling – Active site-directed chemical probes

1. Introduction

In the postgenomic era researchers are now confronted with the task of assigning functions to tens of thousands of proteins. However, global analysis of changes in gene transcription and translation by abundance-based genomic and proteomic approaches provides only indirect information about protein function. Differences in protein expression, modification and activity cannot directly be deduced from those data, since RNA levels do not necessarily correlate with protein abundance and, even more importantly, with protein or enzyme activity. Moreover, the amount of proteins present at a certain state of a cell might not correlate with enzyme activities responsible for the metabolic fluxes, cell management and signal transduction. Since protein activities are highly regulated post-translationally, proteins can be abundant yet possess little activity. Therefore, elucidation of changes in protein activity is the ultimate goal of proteomics. Furthermore, proteins interact functionally in vivo. Thus protein–protein and protein-small molecule interactions also need to be evaluated in processes of interest.

A quickly evolving technology ideally suited for the global analysis of protein function is activity-based proteomics. Different aspects of this field have recently been reviewed (Speers and Cravatt, 2004a; Hemelaar et al., 2004b; Drahl et al., 2005; Pohl, 2005). However, here we would like to provide a more comprehensive overview. The detection of protein activity can be facilitated using activity labels, so called activity-based probes. Basically, an activity label is a molecule consisting of a properly positioned reactive site which forms a covalent bond with the target and a tag for visualization and/or purification of the covalently bound target (Fig. 1). Moreover, a binding group, which acts as recognition site and enhances specificity for a certain enzyme class, may be included in the probe. The reactive group and the tag are separated by a linker so that neither reaction of the reactive group with enzymatic active sites nor affinity recognition/detection of the tag is sterically hindered by the other part of the probe. Mechanistic activity-based probes react with a catalytic amino acid residue in the active site of the enzyme following a 1:1 stoichiometry and in an

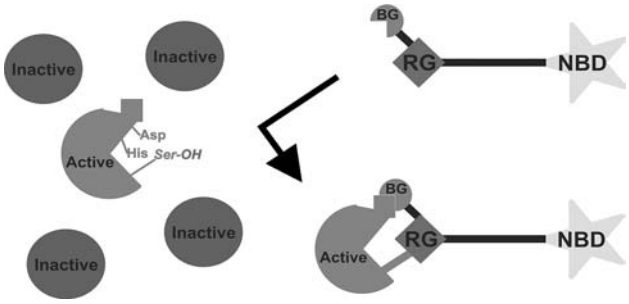


Fig. 1. The principle of activity-based proteomics: An activity-based probe, which contains a reactive group (RG) for covalent binding of a nucleophile (Nu) in the enzyme active site, a binding group (BG) as recognition element and a reporter-tag for detection/isolation, captures an active target protein in a complex proteome

activity-dependent manner, leading to irreversible inhibition of the target enzyme. To date mechanistic probes have been designed for different enzyme classes, namely serine hydrolases (Liu et al., 1999; Patricelli et al., 2001; Adam et al., 2002c; Kidd et al., 2003) including serine proteases (Williams et al., 1989; Bock, 1992a, b; Grabarek et al., 2002), lipases (Deussen et al., 2000a, b; Schmidinger et al., 2005) and PAF-acetylhydrolases (Deigner et al., 1999), cysteine proteases including caspases (Thornberry et al., 1994; Nicholson et al., 1995), papains (Greenbaum et al., 2000, 2002a, b; Bogyo et al., 2000; Nazif and Bogyo, 2001; Verhelst and Bogyo, 2005) and ubiquitin- and ubiquitin-like specific proteases (Borodovsky et al., 2002, 2005), threonine proteases (Bogyo et al., 1997; Kessler et al., 2001; Wang et al., 2003a), tyrosine phosphatases (Kumar et al., 2004) and glycosidases (Vocadlo and Bertozzi, 2004; Hekmat et al., 2005).

For completeness, it has to be noted that other chemical probes have been designed that target noncatalytic residues on proteins. These so-called affinity-based probes rely on highly selective tight binding to targets to be useful probes for distinct protein families. Thus the probe scaffold is of high importance and usually analogous to the respective substrates or cofactors. The affinity-based probes differ from activity-based probes in that they do not necessarily require an active enzyme for modification. Examples for such probes are affinity alkylating probes that modify a strong nucleophile or electrophile in the vicinity of the active-site pocket or probes that can be activated by subsequent addition of chemicals or UV light (Jeffery and Bogyo, 2003; Drahl et al., 2005). Probes mimicking ATP or GTP have been designed to target nucleotide-binding proteins (Zoller et al., 1981; Colman, 1983; Hohenegger et al., 1997). Other examples are chemical probes for carbohydrate-binding proteins, which contain a carbohydrate as a binding group and a photoreactive group for crosslinking to the target protein (Ballell et al., 2005), or photoactive affinity probes specific for metalloproteases based on zinc-chelating hydroxamate (Saghatelian et al., 2004; Chan et al., 2004).

For separation and analysis of proteins tagged by activity-based probes standard proteomics techniques, such as one and two-dimensional gel electrophoresis, one or two-dimensional liquid chromatography (LC) and (tandem) mass spectrometry (MS, MS/MS) using either matrix assisted laser desorption (MALDI) or electrospray (ESI) as ionization methods, are utilized. Although these classic methods alone have highly facilitated global protein analysis they suffer from several shortcomings due to a number of reasons. Most importantly, individual protein levels vary widely, and there is no amplification method comparable to polymerase chain reaction (PCR) used in genomics to amplify RNA/DNA molecules. Thus low abundant proteins represent a major challenge in proteomics. Moreover, these methods are used for protein identification and protein quantification, but fail to provide functional information about protein activity and interaction.

The most mature method for comparative proteomics is two-dimensional gel electrophoresis, which involves separation according to the isoelectric point followed by separation by size in the second dimension (Goerg et al., 2004). Proteins are quantified according to the intensity by which they are stained by various chromogenic or fluorescent protein dyes. For protein identification, in general, spots are then excised from the gel, tryptically digested and subsequently analyzed by mass spectrometry. Although two-dimensional gel electrophoresis is a powerful method for imaging qualitative and quantitative changes in proteomes, it fails to detect proteins at the extremes of separation either by size or isoelectric point, is insufficiently sensitive for low-abundance proteins and rather ineffective for membrane proteins.

More complete coverage of the proteome is provided by separation of tryptically digested crude samples by charge and hydrophobicity during two-dimensional liquid chromatography coupled to tandem mass spectroscopy (Link et al., 1999; Washburn et al., 2001). This method is called multidimensional protein identification technology (MudPIT). However, it quantifies neither abundance nor activity. For quantification of protein mass by mass spectrometry a chemical modification strategy called isotope-coded-affinity tagging (ICAT) was developed (Gygi et al., 1999). The ICAT reagent contains a reactive group that covalently binds amino acids (e.g. cysteines), an isotopically light or heavy linker, and an affinity tag (e.g. biotin). The isotopic differences permit protein abundance comparisons between two samples after alkylation of the

samples with either the light or the heavy compound, affinity isolation of combined and tryptically digested samples and subsequent quantitation by LC-MS based on the ratio of light and heavy signals. Improved approaches analogous to ICAT, such as iTRAQ^{TM} (Ross et al., 2004), have been developed. The latter technique is based upon chemically tagging the N-terminus of peptides generated from protein digests that have been isolated from cells in, for example, two different states. The two labeled samples are then combined and analyzed by LC-MS/MS. Fragmentation of the tag attached to the peptides generates a low molecular mass reporter ion that is unique to the tag used to label each of the digests. Measurement of the intensity of these reporter ions enables relative quantification of the peptides in each digest.

Because of the extensive dynamic range of the proteomic space it is extremely beneficial to produce fractions of the proteome for in depth analysis independent of the protein separation method subsequently used. Activity-based proteomics, next to permitting the detection of protein activity rather than just abundance, also simplifies the proteome by concentrating on only one enzyme class. Therefore, active proteins are detected with high sensitivity and even at low abundance.

2. Building blocks of activity-based probes

a. Reactive groups of activity-based probes

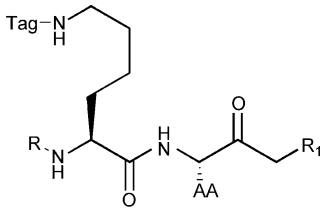
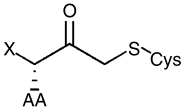
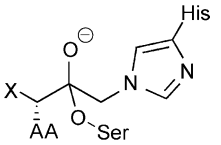
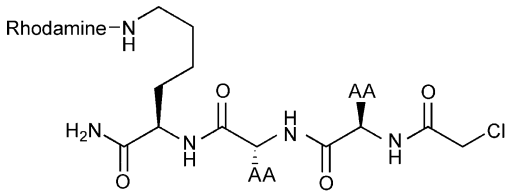
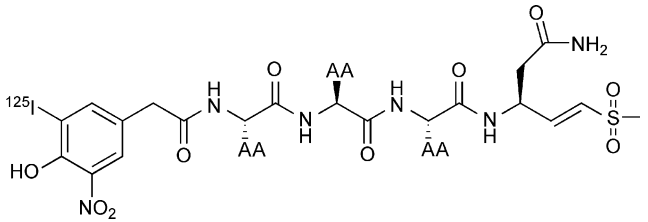
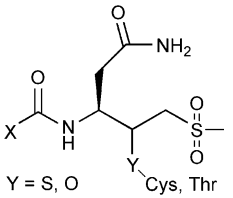
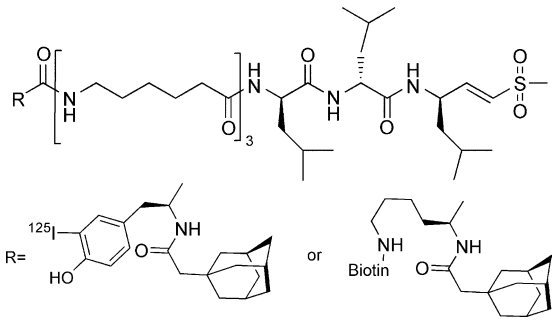
The challenge of activity-based proteome profiling lies in the selection of the appropriate chemical molecules used as probes to determine enzyme activities of interest in a given sample. Unlike in conventional proteomics where, at least hypothetically, all proteins regardless of their acivity are labeled and identified to a comparable extent, the results of activity based proteome profiling experiments always depend on the chemical probes used. Many researchers have tailored the structure of mechanism-based probes to specifically address enzymes or certain enzyme classes of their interest. However, in the Cravatt lab emphasis has been undertaken to extend the set of mechanism-based activity recognition probes for enzyme classes which are not accessible for activity-based proteomics yet (Adam et al., 2001, 2002a, b, c, 2004; Speers et al., 2003; Barglow and Cravatt, 2004; Speers and Cravatt, 2004b). Therefore, proteomes were incubated with non-directed probes bearing an electrophilic sulfonate or chloroacetamide moiety, respectively. Captured enzymes were analyzed and the site of the covalent probe-enzyme interaction elucidated. However, the proteins captured by this non-directed approach belong to very different enzyme classes ranging from oxido-reductases to serin-hydrolases and others.

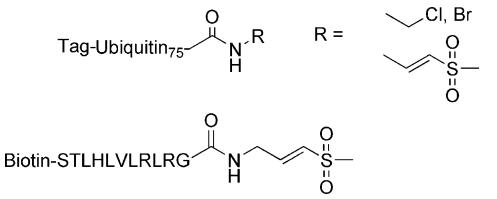
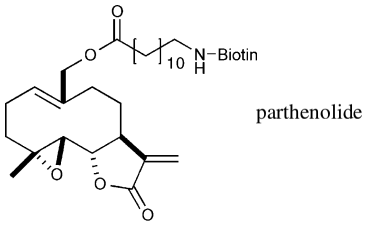
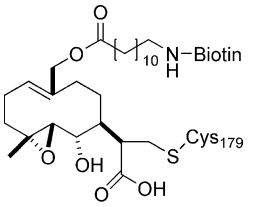
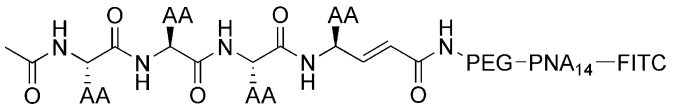
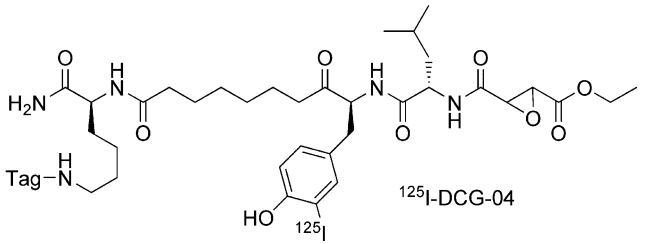
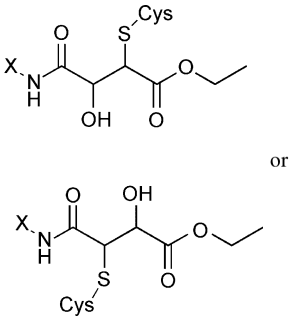
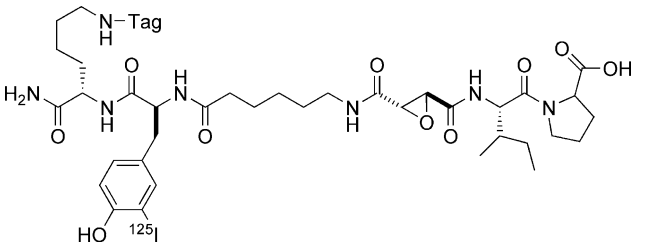
The advantage of activity-based proteomics is that only enzymes of interest are labeled and that the generated protein (enzyme) patterns are less complex. Several enzyme classes were subject to investigation using activity-based probes, prominent examples of the chemical structure of such probes, the target enzyme classes and the resulting probe-enzyme complexes are depicted in Table 1.

α-Halomethylketone, acyl- and aryl-oxymethylketone and chloroacetamide containing polypeptides are intensively used for the investigation and characterization of cysteine and serine proteases. The methylene carbon of those inhibitors is directly attacked by the soft thiolate nucleophile of cysteine proteases whereas the hard alcoholate nucleophile of serine proteases reacts with the carboxylic carbon resulting in a tetrahedral intermediate state. The intermediate state is resolved by the negatively charged oxygen which attacks the methylene carbon yielding an epoxide. This epoxide is then opened by a histidine residue of the enzyme's active site restoring the tetrahedral configuration at the carboxylic carbon. Fluoromethyl and acyl- and aryl-oxymethylketones are specific for cysteine proteases, whereas chloromethylketones are inhibitors of serine and cysteine proteases. Due to the high reactivity of the chloromethylketone-based inhibitors leading to unspecific background staining, these probes are not common for activity-based proteomics.

Polypeptidevinylsulfones are irreversible cysteine protease inhibitors, which are rather stable, unreactive towards nucleophiles and need the catalytic machinery of cysteine proteases for activation. Depending on their polypeptide chain, they are inhibitors for cruzain, rhinovirus 3C proteases, cathepsins and other cysteine proteases. Polypeptidevinylsulfones also inhibit the enzymatic activity of proteasomes (Bogyo et al., 1997; Wang et al., 2003a). These multifunctional proteolytic complexes belong to the family of threonine proteases, using their N-terminal threonine as active site nucleophile. Tuning of the polypeptide residues yields inhibitors capable of distinguishing between the subunits of the proteasome exerting the trypsin-, the caspase-, and the chymotrypsin-like enzymatic activities, respectively (Nazif and Bogyo, 2001). The anti-inflammatory parthenolide was linked to a biotin reporter tag via allylic oxidation of this natural compound. The biotinylated as well as the natural compound were shown to interact with IKKα and IKKβ subunits of the IκB kinase complex. Parthenolide contains an epoxide as well as an α,β-unsaturated lactone moiety. Experiments using reduced parthenolide abolished covalent tagging of overexpressed IKKβ subunits. This findings indicate that

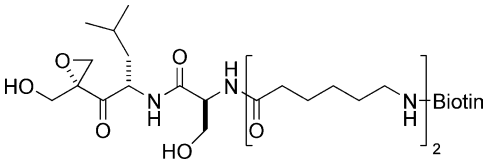
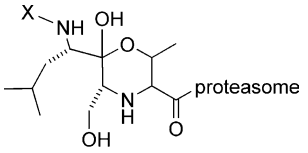
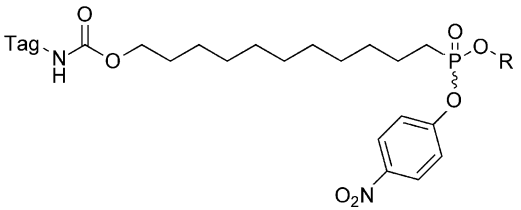
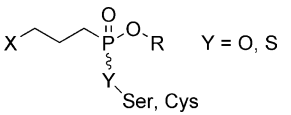
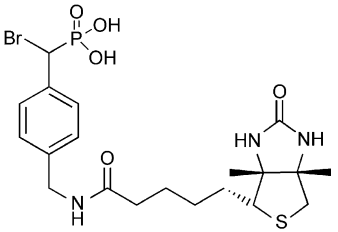
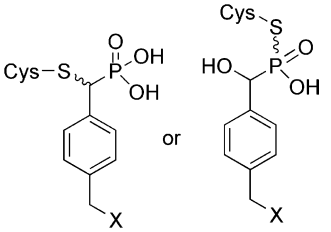
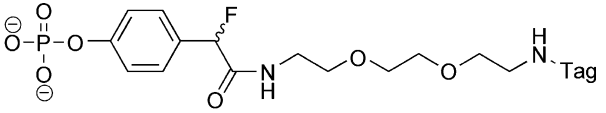
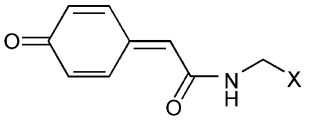
Table 1. Probes for activity-based proteome profiling

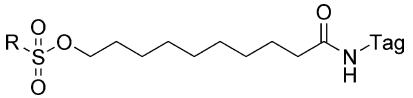
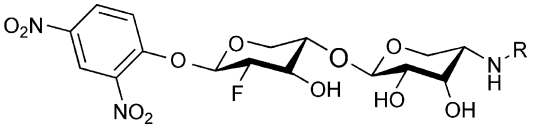
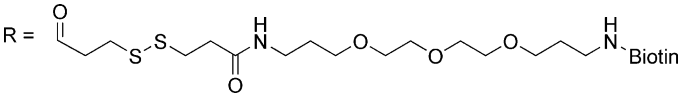
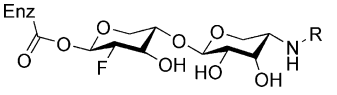
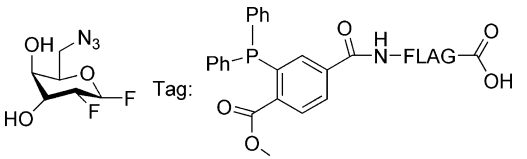
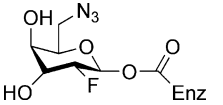
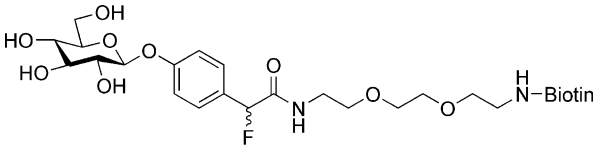
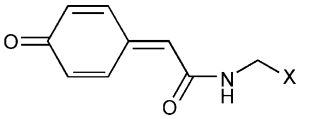
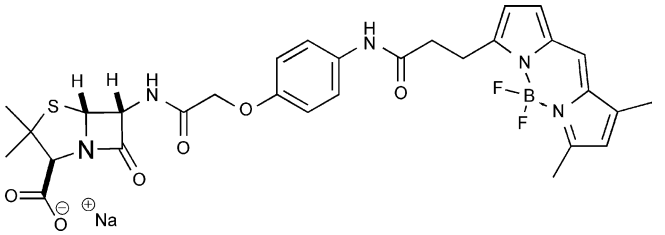
Structure	Target enzymes	Probe-enzyme complex	Ref.
α-halomethyl-, acyl-, aryloxymethylketones, vinylsulfones			
 R = Amino acyl, R_1 = F, Cl, acyl-, aryloxy	Interleukin-1β converting enzyme, Thrombin, falcipain, papain, calpains, cathepsins, serine and cystein proteases	Cystein-protease:  Serine-protease: 	Williams et al. (1989), Bock (1992a, b), Thornberry et al. (1994), Smolewski et al. (2001), Powers et al. (2002), Grabarek et al. (2002), Grabarek and Darzynkiewicz (2002)
	GST-z, hydroxypyruvate reductase, ATP-citrat lyase, malic enzyme etc.	non-directed approach	Barglow and Cravatt (2004)
	20S proteasome subunits, cysteine proteases		Bogyo et al. (1997), Nazif and Bogyo (2001), Kisselev and Goldberg (2001), Wang et al. (2003a)
	26S proteasome	see above	Kessler et al. (2001)

	Deubiquitinating enzymes, ubiquitin and ubiquitin-like proteases	see above	Borodovsky et al. (2001, 2002, 2005), Hemelaar et al. (2004a, b), Ovaa et al. (2004)
	IKKβ subunit	proposed adduct 	Kwok et al. (2001)
	Cathepsins, calpains, caspases, cysteine proteases	see above	Winssinger et al. (2001, 2002), Harris et al. (2004), Winssinger and Harris (2005)
epoxyketones			
	Cathepsins, falcipain-2, cruzain, papain, calpain		Bogyo et al. (2000), Greenbaum et al. (2000, 2002a, b)
	Selective for papain family cysteine proteases	see above	Verhelst and Bogyo (2005)

(*continued*)

Table 1 (*continued*)

Structure	Target enzymes	Probe-enzyme complex	Ref.
 dihydroeponemycin-biotin	20S proteasome, targets of angiogenesis		Sin et al. (1998)
	***p*-nitrophenyl- and fluorophosphonates, phosphates**		
 R = alcoxy moieties (ethoxy, cholesteryl, dialkylglyceryl, etc.)	Serine hydrolases, lipases, esterases, fatty acid synthase, cystein proteases, serine proteases		Liu et al. (1999), Barglow and Cravatt (2004), Cravatt and Sorensen (2000), Jessani et al. (2002, 2004, 2005a, b, c), Kidd et al. (2003), Leung et al. (2003, 2005), Birner-Gruenberger et al. (2004), Jessani and Cravatt (2004), Sieber et al. (2004), Speers and Cravatt (2004a), Zimmermann et al. (2004), Alexander and Cravatt (2005), Birner-Gruenberger et al. (2005), Evans et al. (2005), Schmidinger et al. (2005)
	Protein tyrosine phosphatases		Kumar et al. (2004)
 LCL	Protein tyrosine phosphatase	 active intermediate	Lo et al. (2002)

miscellaneous activity-based probes			
 R = octyl, quinolinyl, naphthyl, thiophenyl, etc.	Epoxide hydrolase, enoyl CoA hydratase, acetyl CoAacetyltransferase, aldehyde dehydrogenase 1 and 7 GSTO 1-1, etc.	non-directed approach	Adam et al. (2001, 2002c), Speers et al. (2003), Speers and Cravatt (2004a, b, 2005)
 	Xylanases, Glucanases		Hekmat et al. (2005)
	β-retaining glycosidases	 Tag attachment after inhibition via Staudinger ligation	Vocadlo and Bertozzi (2004)
	β-glucosidases	 active intermediate	Tsai et al. (2002)
	penicillin-binding proteins		Zhao et al. (1999)

AA Amino acid residue; *Tag* biotin or a fluorophore or both, unless indicated otherwise; *X* activity-based probe residue; *PNA* peptidonucleic acid; *FITC* fluorescein; *PEG* polyethyleneglycol

the active site cysteine of the IKKβ subunit is bound to biotinylated parthenolide via Michael addition (Kwok et al., 2001), a common mechanism also found for the vinylsulfone-based inhibitors described above.

Polypeptide-epoxyketones have been used extensively for the mapping of cathepsins and papain-like cysteine proteases. The nucleophilic cysteine is thereby irreversibly attached to the epoxyketone through nucleophilic ring opening of the epoxide. Epoxomicin and the semisynthetic activity-recognition probe dihydroeponemycin-biotin (Table 1) are natural epoxyketones which specifically inhibit the *N*-terminal threonine of proteasomes. In contrast to a simple ring opening of the epoxide upon attack of a cysteine, the latter inhibitors form a six-membered morpholine ring structure with the *N*-terminal threonine residue of the proteasome. Dihydroeponemycin-biotin also targets enzymes in angiogenesis in bovine aortic endothelial cells (Sin et al., 1998).

p-Nitrophenyl-, and fluorophosphonates have been used to characterize and inhibit serine and cysteine hydrolases for more than three decades. The labeling of these compounds with a reporter tag (biotin and/or a fluorophore) make them excellent probes for activity-based proteomics to identify lipases, esterases and proteases. The irreversible inhibition of these enzymes is caused by the nucleophilic exchange of the leaving group (*p*-nitrophenol or fluoride) with the active site nucleophilic serine or cysteine. A similar mechanism is proposed for the inhibition of protein tyrosine phosphatases by α-bromobenzylphosphonates. In contrast to the latter, the LCL probe (Table 1) unspecifically alkylates the target protein tyrosine phosphatases in the region of the active site via a quinone methide intermediate, which is generated by 1,6 elimination of the benzylic fluoride. Replacement of the phosphate by glucose following the same reaction mechanism of inhibition and/or tagging yields β-glucosidase sensing probes. This kind of activity-based probes could serve as general starting point for inhibitors addressing completely different enzyme activities. However, these probes do not necessarily interact with the catalytic machinery of the target enzyme resulting in possible multiple labeling of the target enzyme and elevated background signal. These effects mainly depend on the structure of the target biocatalyst making predictions difficult. The LCL probe was successfully used within complex proteome samples, whereas the β-glucosidase probe produced high unspecific background staining in such samples (Lo et al., 2002; Tsai et al., 2002). 2,4-Dinitrophenyl-, and fluoro-2-deoxy-2-fluoro sugars are irreversible suicide inhibitors for a variety of sugar cleaving enzymes trapping the active site catalytic nucleophile. They have been applied for proteomic analysis of plant secreted proteomes.

b. Tags of activity-based probes

In general, a radioactive group, a fluorophore or affinity groups, predominantly biotin, have been used as reporter tags for activity-based probes. The introduction of a reporter tag to activity-based probes facilitated the directed identification of enzymes classes on the proteome level. However, the reporter tag of such probes has to be carefully selected with respect to its chemical properties. Factors like polarity, size, charge, structure and chemical reactivity of the reporter tag have a large impact on the reactivity of the inhibitors towards the target enzymes. Especially large reporter tags can have a detrimental effect on the inhibition/recognition profile of the compound (Kessler et al., 2001).

Radioactive activity-based probes, mostly labeled by ^{125}I (Bogyo et al., 1997, 2000; Greenbaum et al., 2000, 2002b; Nazif and Bogyo, 2001; Kessler et al., 2001; Wang et al., 2003a; Verhelst and Bogyo, 2005), are detected by phosphoimaging after one- or two-dimensional gel electrophoresis of labeled proteins. Higher throughput but less resolution is achieved by using a dot-blot based system (Greenbaum et al., 2002b).

Fluorescent activity-based probes can be detected by their fluorescence after one (Grabarek et al., 2002; Schmidinger et al., 2005; Verhelst and Bogyo, 2005) or two-dimensional gel electrophoresis (Greenbaum et al., 2002a; Birner-Gruenberger et al., 2005) using an appropriate gel imager/laser scanner. However, proteins carrying fluorescent prosthetic groups, may generate some background emission and have to be taken into account by analyzing unlabeled samples as negative controls. Moreover, especially fluorophores with long excitation wavelengths, which are, in general, preferred over fluorophores with short excitation wavelengths because of lower background fluorescence, are rather bulky and polar and may affect probe recognition by some enzymes. Activity-based probes differing solely in the fluorophore can be used to multiplex activity profiles of proteomes. Fluorophosphonates with the same reactive group and linker but different fluorophores, fluorescein and tetramethylrhodamine were used to compare the serine hydrolase profiles of rat liver and testis in the same lane of a SDS-PAGE gel (Patricelli et al., 2001). Very little spectral overlap was observed with the two fluorescent probes allowing for the signals of each sample to be readily distinguished. However, the authors did not show whether the two proteomes produced a similar profile with each of the two probes so that pos-

sible probe discrimination in dependence of the fluorophore could be neglected. In contrast, Greenbaum et al. (2002a) demonstrated that derivatives of the epoxyketone DCG-04, containing different Bodipy fluorophores, labeled, when incubated with purified cathepsins, the same bands although with different relative intensities. Thus huge differences in protease activity could be monitored by separating Red-DCG-04 and Blue-DCG-04 labeled samples in one 2D-gel.

Detection of biotinylated probes involves more time consuming Western blotting procedures using avidin instead of antibodies (Williams et al., 1989; Thornberry et al., 1994; Bogyo et al., 1997; Liu et al., 1999; Deussen et al., 2000a, b; Adam et al., 2001, 2002b; Kessler et al., 2001; Kidd et al., 2003; Kumar et al., 2004; Borodovsky et al., 2005; Hekmat et al., 2005; Verhelst and Bogyo, 2005). However, fluorescent and radioactive tags are detected with much higher sensitivity compared to biotinylated probes (Patricelli et al., 2001). Moreover, biotin-containing proteins, such as carboxylases, are rather abundant in proteomes and produce a high intrinsic background. Proteins bound to the biotinylated probe are distinguished from intrinsic biotin-carrying proteins by sample denaturation and identification of activity-probe labeled proteins which are only tagged in native but not in heat-denatured samples (Liu et al., 1999). But since the biotin tag allows affinity isolation of the labeled proteins on avidin columns as well as affinity detection by Western blotting procedures it has been used more often than the radioactive or fluorescent tags. However, if available, a suitable antibody against the fluorophore can also be used for affinity isolation of the labeled proteins (Adam et al., 2004).

A combination of fluorophore and biotin tags in trifunctional probes allows facilitated detection of labeled enzymes via the fluorescent tag and affinity purification via the biotin (Adam et al., 2002c). However, the resultant probes are rather large and bulky molecules and the tags may therefore sterically discriminate recognition of the reactive group by some enzymes. As an alternative to biotin, other affinity labels such as an influenza hemagglutinin (HA)-tag (Borodovsky et al., 2002) or a FLAG (DYKDDDDK)-tag (Vocadlo and Bertozzi, 2004) have also been used. Both tags allow immunoprecipitation of probe-labeled proteins with monoclonal antibodies. Thus, in contrast to biotin, the intrinsic background is negligible.

c. Linkers of activity-based probes

The linker can be used to introduce a third functional group, e.g. a cleavage site to produce cleavable activity-based probes. Since the non-covalent bonds between biotin and avidin are among the strongest known elution conditions for affinity isolation are rather harsh and involve high concentrations of detergent and high temperatures. Thus, a disulfide linker, which is easily cleaved under mild reductive conditions, was introduced between the biotin and the reactive group of biotinylated activity-based probes (Vanwetswinkel et al., 1995; Deussen et al., 2000a, b; Ichikawa and Ichikawa, 2001; Hekmat et al., 2005). Although the disulfide linker appeared to be useful in most studies with enzyme preparations or small proteomes, problems might arise with samples containing proteins with free cysteines or exhibiting mild reductive conditions since the disulfide bond is rather labile and prone to sulfide exchange reactions leading to unspecific labeling of proteins (Ichikawa and Ichikawa, 2001). As an alternative to the disulfide linker, a tobacco etch virus protease (TEV) cleavage site was used for elution of probe-labeled peptides from streptavidin beads by incubation with TEV (Speers and Cravatt, 2005).

Isotopically light and heavy spacers can also be used as linkers to produce an ICAT activity-based probe. An ICAT probe selective for cysteine proteases was developed by van Swieten et al. (2004). The authors presented the synthesis of two pairs of isotopically coded spacers and incorporated them into a known cysteine protease inhibitor, the biotinylated epoxysuccinate DCG-04. The inhibitory profile of the label was not altered by derivatization of DCG-04. Thus the novel probes can be used for quantitative functional proteomics studies on the cathepsin family of cysteine proteases. This concept may be extended toward other activity-based probes and other isotopic coded spacers.

The structure and polarity of the linker may influence probe specificity. Thus, a recognition site enhancing specificity for a certain enzyme class may be included in the linker. Similar ''maximal coverage'' serine hydrolase activity profiles were obtained when either a hydrophobic alkyl chain or a hydrophilic polyethyleneglycol moiety were used as a linker in biotinylated fluorophosphonates (Liu et al., 1999; Kidd et al., 2003). Kinetic analysis, however, revealed that several serine hydrolases reacted at different rates with each probe. For activity-based probes that target proteases specificity elements in the form of peptides or peptide-like structures have been incorporated into the linker. Usually two to four amino acids in this part of the probe confer specific binding to protease active sites (Nazif and Bogyo, 2001). The use of very large peptides or proteins providing a very high degree of target specificity may be advantageous in some cases. For ex-

ample, a recombinantly expressed HA-tagged ubiquitin was modified with an electrophilic reactive group by an intein-based chemical ligation method to covalently bind ubiquitin specific proteases (Borodovsky et al., 2001, 2002). Recombinant HA-ubiquitin-intein-chitin binding domain fusion protein was bound to a chitin affinity column. On-column cleavage of the ubiquitin-intein junction was induced by the addition of β-mercaptoethane sulfonic acid. The resulting thioester was then subjected to chemical ligation with the reactive group generating the desired activity-based probe. This method of making activity-based probes from recombinant proteins may be very useful in the design of probes for other enzymes or protein-binding domains that require large protein recognition elements for specificity.

However, the main specificity elements may also be contained within the structure of the reactive group. Using a non-directed approach members of a probe library bearing a sulfonate ester chemotype were screened against complex proteomes for activity-dependent protein reactivity, which resulted in the labeling of at least six mechanistically distinct enzyme classes (Adam et al., 2002b). Recently, a set of fluorescently labeled *p*-nitrophenol esters of alkylphosphonates with two selector domains differing in polarity and stereochemistry on both sides of the phosphonate was synthesized (Schmidinger et al., 2005). One of the selector domains thus served as a linker between the reactive group and the fluorophore. Probe selectivity was analyzed using different (phospho-) lipase, esterase and cholesterol esterase preparations indicating that the chemical structure of the selector domains highly influenced enzyme recognition. Probes with very low specificity but also derivatives with high specificity for certain subgroups of lipolytic enzymes, such as lipases and cholesterol esterases, were identified. A combination of these probes thus allowed rapid identification and classification of serine hydrolases.

3. Applications of activity-based probes

a. Target identification

A major application is the discovery and identification of novel protein targets for a certain activity-based probe. Thus, on the one hand, the activity and/or function of so far uncharacterized proteins can be elucidated. On the other hand, targets of known irreversible inhibitors can be identified and may help to explain the bioactivity of these agents. Moreover, activity-based probes can also be used to identify novel disease-associated enzymes by comparative profiling of activities in healthy and/or diseased cells/tissues. In addition, activities can be identified that are more prominent in one of the examined states and thus may act as protein activity markers for a disease and/or provide some insight into the underlying (patho-) physiological processes.

Malaria

In an elegant study, DCG-04, an epoxysuccinate probe specific for the papain class of cysteine proteases, was used to identify the cysteine protease falcipain 1 as being involved in host cell invasion by the human malaria parasite *Plasmodium falciparum* (Greenbaum et al., 2002c). Falcipain 1 was the only protease upregulated during the invasive merozoite stage of the parasite life cycle, and thus may represent an attractive target for antimalarial drugs. The authors supported this hypothesis by identifying falcipain 1-specific inhibitors by screening of chemical libraries that blocked parasite invasion of cultured human erythrocytes. Notably, these inhibitors were discovered by activity-based profiling methods described below.

Obesity/energy homeostasis

A Rhodamine tagged α-chloroacetamide, which is a non-directed probe, was applied to discover several enzyme activities differentially expressed in lean and obese (ob/ob) mice (Barglow and Cravatt, 2004). One of these enzymes, hydroxypyruvate reductase, which was 6-fold upregulated in ob/ob livers, participates in the conversion of serine to glucose, suggesting that this unusual metabolic pathway may contribute to gluconeogenesis selectively in states of obesity.

Hydrolysis of triacylglycerols and cholesteryl esters is a key event in energy homeostasis of animals. However, many lipolytic activities still await their molecular identification. A novel protocol for concomitant analysis of lipases in complex proteomes was developed by Birner-Gruenberger et al. (2005). Fluorescent activity-based probes mimicking lipid substrates, including dialkylglycero and cholesteryl phosphonates (Schmidinger et al., 2005), were used to label the proteome of mouse adipose tissue. Analysis by 2D-gel electrophoresis and LC-MS/MS led to the identification of all known intracellular lipases, as well as a number of novel candidates. One of them was recently shown to be involved in triacylglycerol mobilization in adipocytes and therefore named adipose triglyceride lipase (ATGL) (Zimmermann et al., 2004). Functional characterization of transiently expressed enzymes demonstrated that lipolytic and esterolytic activi-

ties could be well discriminated using a small set of structurally differing fluorescent probes. Thus the authors presented the first map of the lipolytic proteome of mouse adipose tissue and demonstrated the general applicability of their method for rapid profiling and identification of lipolytic activities in complex biological samples.

Cancer

To identify active caspases in apoptotic cells, tumor cell lines bound to programmed cell death were treated with chloromethyl or acyloxymethyl ketones based on biotinylated peptides (Faleiro et al., 1997). The authors detected multiple species of CPP32 (caspase 3) and Mch2 (caspase 6) by affinity blotting after one and two-dimensional gel electrophoresis. They identified them by comparing two-dimensional Coomassie stained gels and affinity blots of affinity-isolated caspases, excision of the matched spots from the gels, digestion with Achromobacter protease I and sequencing of the peptides.

Ubiquitin specific proteases (USP), also known as deubiquitinating enzymes, which remove ubiquitin from ubiquitin conjugates, were profiled in normal, virus-infected and tumor-derived human cells using HA-tagged ubiquitin probes (Ovaa et al., 2004). Depending on tissue origin and stage of activation/differentiation different USP activity profiles were revealed. USPs and USP-associated proteins were identified by immunoprecipitation of tagged proteins and MS/MS analysis.

Serine hydrolase activities were profiled across a panel of human cancer cell lines using a Rhodamine tagged fluorophosphonate and one-dimensional gel electrophoresis (Jessani et al., 2002). A group of secreted and membrane-associated enzyme activities was discovered that were used for classification of cancer cells depending on the tumor, like breast carcinoma or melanoma. Interestingly, nearly all of these activities were down-regulated in the most invasive cancer lines examined by the authors, while a distinct set of activities, including urokinase, a secreted serine protease with a recognized role in tumor progression, and KIAA1363, a membrane-associated hydrolase, for which no previous link to cancer had been made, was upregulated. The latter protein was recently identified as a brain detoxifying enzyme for organophosphorous nerve poisons by comparing chlorpyrifos oxon (a bioactivated metabolite of the insecticide chlorpyrifos) and fluorophosphonate labeled mouse brain proteomes (Nomura et al., 2005).

In another cancer study, Jessani et al. (2004) investigated to which extent preparations of human cancer lines displayed similar properties in vitro (as culture) and in vivo (as xenograft), where important host factors may influence tumor biology. To address this question the authors established orthotopic xenograft tumors of the human breast cancer line MDA-MB-231 in the mammary fat pad of immunodeficient mice and compared their enzyme activity profiles obtained with Rhodamine tagged fluorophosphonate and sulfonate esters with those of the corresponding cell lines in culture. By this approach enzyme activities selectively expressed in culture and in xenografts, as well as host enzymes that either infiltrated or were excluded from xenograft tumors were identified. The elevated tumorigenic property of the cancer cells in xenografts as compared to the cells in culture was thus depicted by profound differences in their enzyme activity profiles.

Recently, a functional proteomics strategy combining activity-based protein profiling and MudPIT was introduced by Jessani et al. (2005b) for the streamlined analysis of primary human specimens, such as tumor biopsies. First homogenized tissue sections were treated with a Rhodamine tagged fluorophosphonate and analyzed by one-dimensional gel electrophoresis to provide serine hydrolase activity signatures visualized by in-gel fluorescence scanning. Hierarchical clustering of these profiles was used for functional classification of the samples. The MudPIT approach was then used for in-depth analysis of representative members of each class after treatment of proteomes with a biotinylated fluorophosphonate, enrichment of probe-labeled proteins using avidin-conjugated beads and on-bead trypsin digestion. Using this approach more than 50 serine hydrolase activities, nearly a third of which represented previously uncharacterized proteins, were identified in human breast tumors. Comparison with cDNA microarrays revealed enzymes whose activity but not mRNA abundance depicted the tumor class.

Target identification of bioactive natural products

Natural compounds that produce their biological effects through covalent modification of specific proteins and/or protein families can be used to design activity-based probes by introduction of a tag or a functional group to which a tag can be coupled (Drahl et al., 2005). The resultant probes can then be used for identification of the cellular target(s) of these compounds.

Simultaneous activities of many papain-like Cys proteases in extracts from various tissues and from different plant species were displayed and identified using a bio-

tinylated derivative of the Cys protease inhibitor E-64 (van der Hoorn et al., 2004). A covalent target of the natural compound FR182877, which promotes microtubule polymerization, induces cell-cycle arrest in human cancer cell lines and has anti-tumor effects in mouse models, was identified by activity-based proteomics to be carboxylesterase 1 (Adam et al., 2003). For this purpose, FR182877 was modified with an azide group and subsequently coupled to alkyne-derivatized Rhodamine or a Rhodamine-biotin bifunctional agent by biocompatible copper (I)-catalyzed azide-alkyne [3 + 2] cycloaddition. This coupling reaction was shown to be a very efficient and specific labeling procedure employing ring formation reaction between an alkyne and an azide moiety (Wang et al., 2003b). Notably, this reaction was also used for production of activity-based probes for in vivo applications (see below).

The natural product Wortmannin covalently binds to a nucleotide binding site in proximity to the substrate binding site of phosphatidylinositol-3-kinase thereby inhibiting the enzyme (Walker et al., 2000). This antifungal antibiotic is thus an example for a covalent inhibitor that targets the cofactor binding site and not the substrate binding site. A Rhodamine-tagged Wortmannin analogue was synthesized and used as affinity-based probe in proteomic studies to identify mammalian polo-like kinase as an additional target of this compound (Liu et al., 2005). This kinase is important for mitosis. It is overexpressed in various human cancers and may thus be a target for cancer therapy.

Another natural product, microcystin, an inhibitor of serine/threonine phosphatases, labels a noncatalytic cysteine residue in the intact active site of the target enzyme (Mackintosh et al., 1995). A Rhodamine-LR-microcystin conjugate was used to isolate and identify serine/threonine protein phosphatases and to record changes in phosphatase activity levels in Jurkat cells (Shreder et al., 2004).

In vivo target modification by activity-based probes

Only few activity-based probes are hydrophobic and small enough for fast permeation across the cell membrane. Only these compounds can be directly used for cell labeling in vivo to localize the active enzymes by fluorescence/laser scanning microscopy. HL-60 cells were treated with 5(6)-carboxyfluoresceinyl-L-phenylalanyl-chloromethyl ketone to detect activation of apoptotic serine proteases, which in analogy to caspases are called serpases (Grabarek et al., 2002). Greenbaum et al. (2002a) reported that living dendritic cells treated with a cell permeable fluorescent activity-based probe specific for the papain family of cystein proteases, Green-DCG-04, showed a fluorescence staining pattern characteristic of lysosomal compartments when visualized by fluorescence microscopy. Baruch et al. (2001) demonstrated by activity-based profiling with a membrane permeant biotinylated DCG-04 derivative that the activity of the calcium-dependent cysteine protease Lp82 correlated spatially and temporally with cataract formation. During cataract formation the abundant lens protein γ-crystallin is proteolytically processed and consequently forms insoluble aggregates causing lens opacity.

Unfortunately, most activity-based probes are too large and too hydrophilic. These compounds are not able to pass across cell membranes. Especially the reporter tag increases the size and polarity of the probe. Therefore, activity recognition probes have been designed that are coupled to the reporter after an enzyme-ligand binding event (Speers et al., 2003; Ovaa et al., 2003; Speers and Cravatt, 2004b). These novel activity recognition probes resemble the natural substrates more closely and are able to cross the membrane of living cells. Thus active enzymes can be tagged in intact living cells (in vivo) and not only in cell homogenates. Even enzymes in animals may be labeled with these probes. In addition activities in cells and tissues can be localized by fluorescence microscopy allowing measurements of changes in activity and in subcellular localization. Introduction of the reporter tag after enzyme-inhibitor binding also circumvents possible reporter tag-based discrimination of enzyme inhibition (Speers et al., 2003).

Azide and alkyne labeled sulfonate-based probes were used for the non-directed identification of proteins in complex proteomes and in living cells using azide-alkyne [3 + 2] cyclo-addition using alkyne or azide labeled Rhodamine or biotin tags (Speers et al., 2003; Speers and Cravatt, 2004b). The authors observed superior sensitivity and improved signal to noise ratio, but much lower reaction times if alkyne-labeled enzyme probes were reacted with azide-labeled tags instead of azide-probes and alkyne-tags. The authors suggested that this was due to nonspecific labeling of the proteome by the alkyne-reporter tag. When the authors compared in vivo versus in vitro enzyme activity profiles of human breast cancer cell lines, they identified enzymes that were predominantly or exclusively labeled in living cells but not in cell homogenates, possibly due to several reasons. Protein activity may be sensitive to posttranslational forms of regulation or localization to specific compartments. The authors also used the probes to quantify inhibition of aldehyde dehydrogenase 1 by the active site-directed agent disulfiram in living

mice, indicating that this method may be used to assess inhibitor activity in vivo.

A similar approach was used to identify protein targets of bioactive compounds in vivo (Evans et al., 2005). For instance, fumagillin, luminacin D and FR901464 contain an electrophilic 1-oxa-spiro[2.5]octane substructure capable of covalently modifying the active sites of enzymes. Based on this structural motif a library of structurally diverse, protein-reactive compounds was synthesized that contain the spiroepoxide electrophile, a variable binding group and an alkyne for coupling the probe to azide modified reporter tags by azide-alkyne $[3+2]$ cycloaddition. Cell-based screening identified a compound, MJE3, which inhibits proliferation of breast cancer cell. In vivo proteome reactivity profiling revealed that the glycolytic enzyme phosphoglycerate mutase 1 (PGAM1) was covalently labeled by MJE3 but not by other compounds. Importantly, PGAM1 inhibition and labeling by MJE3 was observed exclusively in intact cells. Thus the authors suggest that cancer cell viability depends on glycolysis and that PGAM1 may be a potential therapeutic target. Similarly, an alkyne modified carbamate inhibitor of fatty acid amide hydrolase (FAAH) was synthesized to evaluate proteome reactivity of FAAH-directed carbamates in vivo (Alexander and Cravatt, 2005). Although the inhibitor was specific for FAAH in the nervous system, several other enzymes, including carboxylesterase 6 (CES6) and esterase 31 (ES31), also reacted with the inhibitor.

Ovaa et al. (2003) developed probes for in vivo labeling of the catalytically active subunits of proteasomes. A peptide-based vinyl sulfonate, $AdaAhx_3L_3VS$, was modified with an azide group, which neither interfered with the inhibition profile nor with the cell permeability of the probe. After labeling of whole EL-4 cells with this probe, followed by cell lysis and denaturation of cellular protein, the azido groups were subjected to a modified Staudinger ligation with a biotinylated phosphane reagent. In vivo labeling appeared to be more effective than labeling of cell lysates, thus indicating a more efficient targeting of proteasomes in living cells. This may be due to partial dissociation of the proteasome particle during cell lysis and storage.

Combined computational and activity-based proteomics methods for target identification

A combination of the activity-based proteomics approach using either biotinylated or Rhodamine-labeled fluorophosphonates and computational analysis of active site structures of serine hydrolases was used to identify such enzymes in yeast (Baxter et al., 2004). For the latter approach, a set of serine hydrolase fuzzy functional forms, structural motifs for identification of functional sites, was obtained by combining physicochemical and structural data from the protein data base and activity information from the literature. By computational analysis and by activity-based proteomics 52 and 23 serine hydrolases were assigned, respectively. 15 proteins, however, were identified by both methods and thus designated as high-confidence identifications. Eight of these resembled so far uncharacterized proteins, and within these a novel family of serine hydrolases, designated as Fsh, was discovered by the authors. The study emphasizes the advantage of using a combination of complimentary, large scale methods that provide different types of functional information for extracting valuable biological information that will help to decipher protein function in complex pathways.

b. Active site profiling

Mechanism-based activity-recognition probes are bound to the catalytically active amino acid residue in the active site of an enzyme. Thus the covalent tag can be used to identify the active amino acid. Radioiodinated peptides containing vinyl sulfone residues were employed as active site probes for mechanistic studies of the catalytic β subunits of proteasomes in different tissues and cells (Bogyo et al., 1997, 1998; Nazif and Bogyo, 2001; Kessler et al., 2001; Wang et al., 2003a). Labeled active subunits of the proteasome were identified by comparing radioactive spots on 2D-gels with the known pattern of proteasomal subunits.

More recently, Adam et al. (2004) established a method for profiling enzyme active sites in whole proteomes that utilizes activity-based chemical probes coupled to a gel-free analysis platform. After treatment with a Rhodamine-tagged phenyl sulfonate ester, proteomes were denatured and their thiols were reduced with dithiothreitol followed by alkylation with iodoacetamide and digestion with trypsin. The resultant peptide mixture was then incubated with anti-Rhodamine antibodies to affinity capture the probe-labeled peptides before analysis by LC-MS/MS. Probe labeling was found to occur on a conserved active site residue of different enzyme classes, including catalytic nucleophiles, e.g. the Cys32 in glutathione S-transferase omega, and catalytic bases/acids, e.g. Glu269 of aldehyde dehydrogenase 1 and Asp204 of enoyl CoA-hydratase 1, as well as residues of unknown function, such as Asp127 in 3β-hydroxysteroid dehydrogenase/isomerase 1. These results revealed that sulfonate ester probes are capable of labeling a diversity of catalytic residues in a range of

mechanistically distinct enzymes. By a similar approach sialic acid 9-O-acetylesterase, which is selectively expressed in melanoma cell lines, was targeted with a Rhodamine-tagged fluorophosphonate (Jessani et al., 2005c). A single peptide was labeled by the probe with a predicted site of probe modification at a conserved serine Ser127. The residue was essential for catalysis as determined by site specific mutagenesis. Thus, although the enzyme shared no sequence homology to serine hydrolases or any other enzyme class, it could be assigned to the serine hydrolase superfamily.

Hekmat et al. (2005) used a similar method to identify the active site peptides of β-endoglycosidases in complex proteomes. The secreted proteome of the soil bacterium *Cellulumonas fini* was labeled with a biotinylated, cleavable 2-deoxy-2-fluoroxylobioside probe. The labeled proteins were proteolysed and the biotin-labeled peptides were enriched on streptavidin resin. A disulfide linker between the biotin and the reactive group was used as reductive cleavage site for elution of labeled peptides by tris(2-carboxyethyl)phosphine. The active site of a newly identified glycanase containing the catalytic nucleophile was characterized in this manner.

In theory, all these strategies can be used to simultaneously identify the activity probe-labeled proteins and their sites of modification. However, since the methods are based on enrichment of the probe-labeled peptides and the rest of the proteome digest is discarded, a lot of information is lost and protein assignments are rendered less statistically significant. Thus a proteolysis strategy for parallel characterization of probe-labeled proteins and sites of probe modification was designed by Speers and Cravatt (2005). After labeling of a mouse heart proteome with an alkynyl phenyl-sulfonate ester probe, the labeled proteins were coupled by click chemistry to an azide group of a biotin tag containing a tobacco etch virus protease (TEV) cleavage site. Tagged proteins were subjected to enrichment on streptavidin before on-bead trypsin digestion. The supernatant was isolated by filtration while the probe-labeled peptides were eluted from the beads by incubation with TEV. The supernatant and the eluted peptides were then analyzed in sequential MudPIT experiments to characterize probe-labeled proteins and sites of probe modification, respectively.

c. Inhibitor/drug discovery

Activity-based probes can be used to screen for (unlabeled!) inhibitors of drug targets since the probes compete with the inhibitors for the active site. Inhibitor discovery by activity-based probes offers several advantages over conventional screening methods: Enzymes have not to be overexpressed and purified for this kind of analysis since they can be analyzed directly in their parent proteomes. Consequently, the inhibitors are tested with multiple enzymes in parallel and thus their potencies and selectivities are concurrently evaluated. Moreover, novel enzymes lacking known substrates for conventional substrate assays are also amenable to analysis.

Irreversible inhibitors can be screened by pretreating proteomes with libraries of the respective compounds followed by labeling with an activity-based probe. Labeling intensity of each target relative to the control untreated sample can be used to generate percent competition values. The resulting data is then clustered and visualized using programs designed for analysis of microarray data. Specific inhibitors for the cysteine protease falcipain 1, which was shown to be involved in host cell invasion by the human malaria parasite *Plasmodium falciparum*, were identified by screening of libraries containing positional isomers of peptide epoxides in crude *Plasmodium falciparum* cell extracts (Greenbaum et al., 2002c). Inhibition potency and selectivity of the compounds was analyzed by labeling with the radioactive cysteine protease activity-based probe ^{125}I-DCG-04. The identified falcipain-specific inhibitors blocked parasite invasion of host erythrocytes, thus the authors established falcipain 1 as a potential target for antimalarial drugs.

A similar competitive approach was used in a proteomic screen for targets and inhibitors of prostate cancer. Three covalent inhibitors resembling β-lactones, namely orlistat and ebelactones A and B, were screened against serine hydrolases in prostate tumor cell lines (Kridel et al., 2004). In this screen a new target for orlistat, a covalent inhibitor of digestive lipases and a drug used in the treatment of obesity, was identified. Orlistat was shown to inhibit fluorophosphonate labeling of the thioesterase domain of fatty acid synthase, an enzyme closely linked to tumor progression. Moreover, orlistat halted tumor cell proliferation, induced tumor cell apoptosis and inhibited the growth of PC-3 prostate tumors in nude mice.

A similar approach can be used to screen reversible inhibitors since the presence of such compounds reduces the rate of labeling of an enzyme by activity-based probes. A prerequisite, however, is the optimization of labeling times since reversible inhibitors only affect probe labeling for a restricted period of time, depending on both, the affinity of the inhibitor and the rate of probe reactivity. End-point labeling with activity-based probes would

result in complete tagging of all active enzymes irrespective of the presence of reversible inhibitors. A library of potential serine hydrolase inhibitors resembling electrophilic ketone agents was profiled in competition experiments using a Rhodamine-fluorophosphonate in mouse proteomes (Leung et al., 2003). By this approach, reversible inhibitors of several enzymes, including the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH), triacylglycerol hydrolase (TGH) and a membrane-associated hydrolase (KIAA1363), were identified simultaneously.

d. Activity-based proteomics on microarrays

Microarray-based platforms of different design can be used together with activity-recognition probes. Activity-based proteomics on chip is either based on immobilization of enzymes in their active state on the slide and incubation with an activity-based probe in solution (enzyme chip) or, *vice versa*, spotting of a probe library and incubation with a labeled enzyme in solution (compound chip). Microarray platforms representing the immobilized target enzyme on chip were used to map kinetic constants of enzyme inhibition using activity sensing probes (Funeriu et al., 2005), as well as for enzyme characterization and classification on the basis of suicide inhibitors (Chen et al., 2003; Schmidinger et al., 2006). Microarrays of immobilized activity-recognition probes were used to characterize enzyme activity of lipases transiently overexpressed in COS-7 cells (Schmidinger et al., 2006).

For completeness, it has to be noted that microarray platforms are also utilized as a detection tool for enzymes labeled in solution by activity-based proteomics. For this purpose, addressable oligonucleotide or antibody microarrays were used to ''sort'' the activity-tagged enzymes, respectively. The oligonucleotide microarray system takes advantage of highly specific cathepsin S, L, H, B, C, calpain and caspase-3 inhibitors which are individually encoded by peptidonucleic acid (PNA) tags (Winssinger et al., 2001, 2002). Incubation of these inhibitors with granzyme B activated Jurkat cells, size exclusion and hybridization with oligonucleotide microarrays showed increased activity for caspase-3, whereas intensities for cathepsins remained constant. A PNA-encoded 4000-compounds acrylamidotetrapeptide inhibitor library was used to find novel inhibitors for so far unidentified cysteine proteases in dust mite allergen extract using the same experimental setup (Harris et al., 2004). Antibody microarrays representing antibodies against prostate-specific antigen, urokinase, tissue plasminogen activator and the matrix metalloprotease 9 were hybridized with proteomes previously incubated with activity-recognition probes targeting these enzymes. Fluorescent analysis of such hybridized microarrays nicely represent the activities of the antigens found in the proteome sample (Sieber et al., 2004).

4. Conclusions

Sequencing of the human and other genomes has a large impact on science and medicine. To fully benefit from this rich resource of information, the fields of genomics and proteomics are continuously advanced. The concurrent refinement of a number of proteomics techniques and the application of combinations of disciplines and techniques will probably be necessary to provide the basis for a better understanding of the processes involved in cell function and regulation. Chemical methods are well suited to provide powerful new tools and methods for the functional analysis of proteomes. Activity-based proteomics at the crossroads of chemistry and biology has enjoyed a phase of tremendous technological innovation over the past few years. Recent developments, as highlighted in this review, include the design of probes for in vivo analysis of proteomes, microarray technologies for higher throughput screenings of protein specificity, and the application of activity-based probes for drug screening.

A critical point for broadening the scope and impact of activity-based proteomics will be the development of probes for additional protein classes. This will probably require the synthesis of more structurally diverse libraries of candidate probes. Rather simple compounds may be used for the identification of suitable reactive groups for a certain enzyme/protein class. Increasing the molecular complexity of the probes by introduction of selectivity elements may then be used to generate probes specific for enzyme/protein subclasses. The design of new probes will greatly benefit from the ongoing elucidation of protein structures and enzymatic mechanisms, as well as from the ongoing identification and characterization of targets of natural products with bioactive properties. From the information obtained class selective functional groups may be identified that bind conserved elements in active sites of enzymes.

Another critical point is the fidelity by which the probes report on protein activity. Affinity-based probes that bind to a protein/enzyme class irrespective of enzyme activity are useful for target identification by reducing proteome complexity, as well as for abundance-based proteomics. However, they are not suitable for estimation of protein

activity, mapping of enzyme active sites or competitive inhibitor profiling. While most known activity-based probes modify conserved catalytic nucleophiles in active sites, other probes have been discovered that modify non-catalytic residues in enzyme active sites, like the natural compound microcystin (Drahl et al., 2005). Although such probes are not considered purely activity-based in a mechanistic sense, they still are able to report on the functional state of enzymes if they solely react with active enzymes and act as irreversible inhibitors by sterically blocking the enzyme active site.

In conclusion, we would like to emphasize that the field of activity-based proteomics has a great potential of significantly advancing our understanding of biology by elucidation of protein function and also to speed up drug development in the future.

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