

# Activity-Based Proteomics of Lipolytic Enzymes

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**Abstract:** Lipases play fundamental roles in biological processes since hydrolysis of triacylglycerols and cholesteryl esters is a key event in energy homeostasis of animals. Perturbations in the metabolism and the cellular retention of lipids result in common diseases such as obesity, type 2 diabetes, and atherosclerosis. The introduction of active site-directed chemical probes for enzymatic activity profiling in complex mixtures, known as activity-based proteomics, has greatly facilitated and accelerated global analysis and functional annotation of lipolytic proteins. Here we review probe design and application for discovery and discrimination of lipolytic and esterolytic enzymes. These probes are usually detected by their fluorescent or affinity tags and their protein targets are analyzed using established proteomics techniques. Moreover, microarray technologies can be applied for higher throughput screenings of enzyme or probe specificity.

**Key Words:** Activity-based proteomics, functional proteomics, enzymatic activity profiling, active site-directed chemical probes, lipases, lipolytic enzymes.

## LIPID-ASSOCIATED DISEASES AND LIPASES AS DRUG TARGETS

Perturbations in the metabolism and the cellular retention of lipids are major risk factors for the development of obesity, type 2 diabetes mellitus and cardiovascular disease [1]. Obesity and its associated disorders are reaching epidemic proportions in the modern, industrialized world [2]. Despite the widely held view that obesity and other lipid-associated disorders are largely the result of misbehavior, it is increasingly evident that overeating in humans is actually “hard-wired” [3]. Indeed, adipose tissue mass is genetically determined [4]. Numerous twin/adoption and family studies confirm a major contribution of genes to the development of obesity. The basic reduction in energy expenditure by an increasingly urbanized and sedentary life style and the increase in caloric intake by easy access to food have contributed to what is now termed the “obesogenic” environment. The combination of genetic predisposition and “obesogenic” environment, results in the tremendous increase in obesity and related disorders in today’s world.

Lipid storage is a general process to deposit excess nutritional lipid substrates for later use as energy substrates, membrane components or metabolic or signaling precursors. Mammals store triacylglycerols in adipose tissue as the primary source of energy during periods of starvation and increased energy demand. Another important function of lipid storage is the detoxification of otherwise cytotoxic compounds, such as free fatty acids and unesterified cholesterol and their deposition at high concentrations. Lipid esters are the most common storage forms of fat. For example, increased concentrations of fatty acids, which represent a ma-

major energy substrate in the body, disrupt cell membrane integrity and need to be stored as triacylglycerols [5]. Similarly, cholesterol and retinol are esterified to avoid the harmful effects of excess intracellular concentrations and blood levels. The balance of lipid storage and mobilization is tightly regulated to ensure whole body energy homeostasis. Innumerable molecular components are involved in the regulation of lipid and energy homeostasis in the body. These include the hormonal and neural control of feeding behavior, the regulation of food resorption in the gut, the partitioning of energy substrates among tissues, and the cellular metabolism and catabolism of lipid substrates [5]. Despite the enormous need to understand these physiological processes and to develop strategies for the treatment of lipid-associated disorders, many basic mechanisms that govern the energy balance and lipid metabolism are incompletely understood. Stored fat is mobilized by activation of lipolytic enzymes, which degrade adipose triacylglycerols and cholesterol esters and release non-esterified (free) fatty acids into the circulation. Variation in the concentration of circulating free fatty acids is an established risk factor for the development of insulin resistance in type 2 diabetes and related disorders [6-9].

Triacylglycerol lipases and cholesteryl esterases are key enzymes in lipid absorption and mobilization and respective lipolytic activities have been described in various tissues. However, not all of them have been identified on the molecular level. Many characterized lipases, such as vascular lipases, lipoprotein lipase (LPL) [10], hepatic lipase (HL) [11] and endothelial lipase (EL) [12], and the digestive lipases, namely pancreatic lipase (PL) [13] and gastric lipase (GL) [14-17], are secretory proteins, whereas few identified lipases are intracellular enzymes, such as hormone sensitive lipase (HSL) [18], monoglyceride lipase (MGL) [19], adipose triglyceride lipase (ATGL) [20] and triacylglycerol hydrolase (TGH) [21]. PL and GL are the key enzymes involved in fat digestion. Lipids are partly digested by GL,

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which is stable and active at acidic pH in the stomach, where they form large fat globules [22,23]. In the intestinal lumen these fat globules are mixed with bile salts and pancreatic juice, containing digestive lipases to form small fatty droplets. PL is the principal lipolytic enzyme in the small intestine and activated by binding of colipase, which is secreted as a precursor (procolipase) from the pancreas and processed by cleavage [24]. Deletion of PL in mice mainly affects cholesterol but not triacylglycerol absorption suggesting that other enzymes, such as carboxyl ester lipase (CEL) [25] and pancreatic lipase-related protein 2 [26], may significantly contribute to intestinal triacylglycerol hydrolysis [27]. Another pancreatic lipolytic enzyme is pancreatic phospholipase A2, which hydrolyzes intestinal phospholipids [28]. Hydrolysis of Lipids and phospholipids induces the formation of mixed micelles which provide a continuous source of fatty acids, mono- and diacylglycerols, lysophosphatidic acid, cholesterol and fat-soluble vitamins for absorption at the brush-border membranes of enterocytes. In the enterocyte the hydrolysis products are re-acylated to triacylglycerols and cholesterol esters and form chylomicrons that enter circulation through the lymph, where they are transported to the liver. The liver has a key role in lipid metabolism by acting as a buffer for transient energy fluctuations. It temporarily stores fatty acids as triacylglycerol and secretes them as very low density lipoprotein (VLDL) into the serum when the period of maximum lipid load has passed. LPL, HL and EL are vascular triacylglycerol lipases and phospholipases and responsible for lipoprotein hydrolysis. In contrast to HL and EL, LPL is activated by apolipoprotein-CII [12]. In a given tissue, the level of lipoprotein lipase (LPL) expression appears to be the rate limiting process for the uptake of triacylglycerol derived fatty acids [10]. HL has a role in high density lipoprotein (HDL)-metabolism [11]. EL is a better phospholipase than triacylglycerol lipase and efficiently hydrolyzes HDL *in vitro* [12]. Adipocytes are the main sites for fatty acid cycling through lipolysis and re-esterification, thereby securing the energy supply to oxidative tissues, such as skeletal muscle and heart. HSL was shown to hydrolyze cholesteryl esters, diacylglycerols and to a lesser extent triacylglycerols in adipose tissue. Yet, in HSL-knock out mice, triacylglycerol hydrolysis in adipose tissue as well as cholesteryl ester hydrolysis in macrophages were not abolished [18]. MGL is ubiquitously expressed and rather specific for monoacylglycerol hydrolysis [19]. TGH appears to be involved in triacylglycerol mobilization and VLDL secretion in liver and is also highly expressed in adipose tissue [21,29,30]. ATGL has recently been established as a key enzyme responsible for triacylglycerol hydrolysis in mouse adipose tissue [20,31,32].

The active site of lipases and carboxyl esterases typically consists of a catalytic triad formed by Ser-His-Asp/Glu which is common for most serine-hydrolases [33]. A feature specific for many lipases and esterases, however, is the  $\alpha/\beta$ -hydrolase fold consisting of a series of parallel  $\beta$ -sheets and a number of helices that flank the sheets on both sides [34,35]. Most lipases contain a lid controlling the access of substrates to the hydrophobic active site. The same structural features are found in esterases, except for the lid. Therefore, in contrast to lipases, most esterases do not show activation

at lipid/water interfaces. However, it has to be emphasized that the borderline between lipases and esterases is not well defined and some exceptions exist. For the same reasons, analytical discrimination and classification of lipolytic enzymes is difficult.

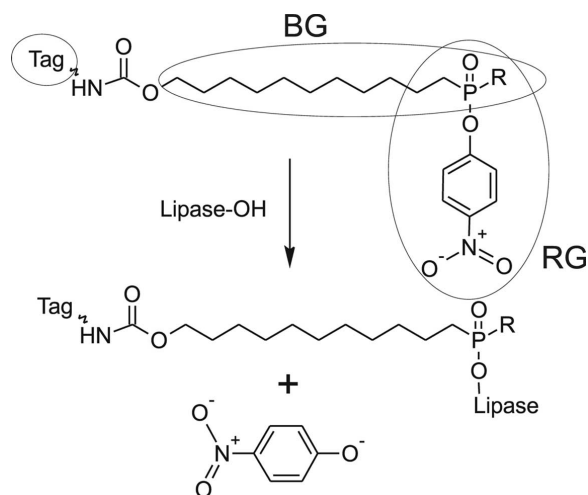
Treatment of obesity has been based on diet, behavior therapy and physical exercise. For patients with morbid obesity (body mass index (defined as weight [kg]/size [m]<sup>2</sup>)  $\geq 40$  kg/m<sup>2</sup>) different surgical treatment modalities have also been developed [36]. The few existing approved drugs for treatment of obesity possess only modest efficacy promoting weight loss in the 2-6 kg range and poor side-effect profiles [37]. As such, there is an urgent need for the development of new pharmacotherapies. One existing strategy for the treatment/prevention of obesity is to affect the neural control of feeding behavior by appetite suppression. An example is Sibutramine (Meridia; Abbott) which blocks presynaptic uptake of norepinephrine and serotonin potentiating the anorectic effects of these neurotransmitters in the central nervous system. Recently, another drug, Rimonabant (Sanofi-Aventis), has been approved in the European Union. Rimonabant blocks endocannabinoid receptors (CB1-blocker), which are involved in reward responses and localized to the central nervous system, but also peripherally to the liver and adipose tissue. Another strategy is to inhibit nutrient digestion and absorption. Orlistat (Xenical; Roche), an approved drug, reduces absorption of dietary fat by inhibiting digestive lipases [36]. Orlistat is a synthetic derivative of lipstatin, a natural product of *Streptomyces toxytricini*, which is a potent inhibitor of intestinal lipases [38], and acts by covalent bonding to the serine residue of the active site of digestive lipases [39-41]. The chemical reactivity of the  $\beta$ -lactone group of lipstatin and its analogues is responsible for their biological effects. Inhibition occurs by nucleophilic attack of the active serine at the carbonyl carbon of the  $\beta$ -lactone and subsequent ring opening. Enzyme activity can be recovered by slow subsequent hydrolysis of the acyl-enzyme intermediate [42]. At therapeutic doses of Orlistat approximately 30% of ingested fat (mainly triacylglycerols) remains unabsorbed [36,37]. Since Orlistat itself has an extremely poor absorption, it does not affect systemic lipid metabolism through inhibiting vascular or other lipases. Its adverse effects are mostly gastrointestinal as a consequence of high fat intake and fat malabsorption. Absorption of cholesterol and fat-soluble vitamins are also reduced, thus the drug is not used for patients with defects in bile acid production and daily intake of multivitamin is recommended to Orlistat users [37]. As the gastrointestinal side effects of Orlistat are largely mechanism related, there might be limited room for further improvement by targeting gastric and pancreatic lipases. Other proteins engaged in lipid transport and absorption might provide better drug targets [43]. Other suggested approaches would be the enhancement of energy expenditure by increasing lipid oxidation or thermogenesis by uncoupling fuel metabolism from the generation of ATP, thereby dissipating energy as heat, or the stimulation of fat mobilization and metabolism to reduce fat mass [43]. The latter approach provides an intriguing possibility for dealing with excessive fat accumulation and body-weight gain. Any increase in fat mobilization achieved either by activation of lipolytic enzymes or inhibition of lipid synthesizing enzymes, however, must be combined with an

increase in lipid metabolism to avoid lipotoxicity of released products, such as fatty acids, cholesterol and diacylglycerols.

### ACTIVITY-BASED PROTEOMICS FOR TARGET AND DRUG DISCOVERY

Global analysis of changes in gene transcription and translation by abundance-based genomic and proteomic approaches provides only indirect information about protein function since RNA levels do not necessarily correlate with protein abundance and, even more importantly, protein abundance does not necessarily correlate with protein or enzyme activity. Thus other methods are needed to directly assess differences in protein expression, modification and activity. Activity-based proteomics is a quickly evolving technology ideally suited for the global analysis of protein function [44]. So called activity-based probes are employed for detection of specific protein activities. These probes typically contain (i) a reactive group which forms a covalent bond with the target, (ii) a tag for visualization and/or purification of the covalently bound target, and (iii) a binding group, which acts as recognition site and enhances specificity for a certain enzyme class (Fig. 1). The reactive group and the tag are typically 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 activity-dependent manner, leading to irreversible inhibition of the target enzyme. 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. Many researchers have tailored the structure of mechanism-based probes to specifically address enzymes or certain enzyme classes of their interest. To date mechanistic probes have been designed for different enzyme classes, namely serine hydrolases [45-47] including serine proteases [48-51], lipases [52-58] and PAF-acetylhydrolases [59], cysteine proteases including caspases [60,61], papains [62-68] and ubiquitin- and ubiquitin-like specific proteases [69,70], threonine proteases [71-73], tyrosine phosphatases [74,75] and glycosidases [76-79].

In general, fluorophores or affinity groups, predominantly biotin, have been used as reporter tags for activity-based probes. The reporter tag of such probes has to be carefully selected with respect to its polarity, size, charge, structure and chemical reactivity. These factors have a large impact on the reactivity of the inhibitors towards the target enzymes. Especially large reporter tags may have a detrimental effect on the inhibition/recognition profile of the compound. Fluorescent activity-based probes can be detected by their fluorescence after one or two dimensional gel electrophoresis 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 absorbing at short wavelengths because of lower background fluorescence, are rather bulky and polar and may



**Fig. (1).** The principle of activity-based proteomics of lipases: An activity-based probe, which contains a reactive group (RG) for covalent bonding to the serine in the active site of the enzyme, a hydrophobic binding group (BG) as recognition element and a fluorescent/affinity reporter-tag (Tag) for detection/isolation, captures an active lipase in a complex proteome. R = dialkylglycerol, cholesterol etc. (see Table 1). RG is an alkylphosphonate which mimics the tetrahedral transition state of the fatty acid ester substrate after nucleophilic attack by the serine in the active centre of the lipolytic enzyme. *p*-Nitrophenol acts as a leaving group and leads to irreversible covalent bonding of the serine oxygen to the phosphorous.

affect probe recognition by some enzymes. Detection of biotinylated probes involves more time consuming Western blotting procedures using avidin instead of antibodies. In addition, biotin tags are detected with much lower sensitivity compared to fluorescent probes [47]. 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 [46]. 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 fluorescent tags. If available, a suitable antibody against the fluorophore can also be used for affinity isolation of the labeled proteins [80]. 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 [81]. However, the resultant probes are rather large and bulky molecules and the tags may therefore sterically inhibit recognition of the reactive group by some enzymes.

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. The best established 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 [82]. Protein abundance is quantified according to the intensity by which protein spots are stained by various chromogenic or fluorescent protein dyes. For protein identification, in general, spots are excised from the gel, tryptically digested and analyzed by mass spectrometry. Alternatively, tryptically digested crude samples are separated by charge and hydrophobicity during two-dimensional liquid chromatography coupled to tandem mass spectroscopy by a method called multidimensional protein identification technology (MudPIT) [83,84]. However, MudPIT provides only qualitative information. For quantification of protein mass by mass spectrometry chemical modification strategies like isotope-coded-affinity tagging (ICAT) [85] and improved approaches such as iTRAQ™ [86] have to be used. Because of the large dynamic range of protein abundance it is extremely beneficial to produce fractions of the proteome for in depth analysis independent of the protein separation method subsequently used. Since only enzymes of interest are labeled by activity-based probes the detected protein patterns are less complex thus greatly facilitating protein detection and identification.

Microarray-based platforms of different design can also be used together with activity-recognition probes. Activity-based proteomics on chip is either based on immobilization of enzymes in their active state on solid supports followed by 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 [87], as well as for enzyme characterization and classification on the basis of suicide inhibitors [88,89].

A major application of activity-based proteomics 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.

Activity-based probes can also be used to screen for irreversible and even reversible inhibitors of drug targets since the inhibitors compete with the probes for the active site and thus reduce the rate of enzyme labeling by the probes [90]. However, labeling times have to be optimized for reversible inhibitors since they only affect probe labeling for a limited 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. Using activity-based probes for inhibitor screening offers several advantages over conventional methods:

Enzymes have not to be overexpressed and purified. On the contrary, they can be analyzed directly in their parent proteomes. Since the inhibitors are tested with multiple enzymes in parallel their potencies and selectivities are concurrently evaluated. Moreover, novel enzymes lacking known substrates for conventional activity assays can also be subjected to inhibitor screening.

## ACTIVITY-BASED PROBES FOR LIPASES

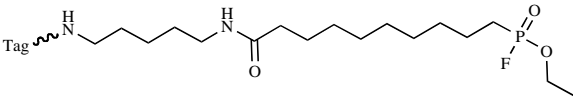
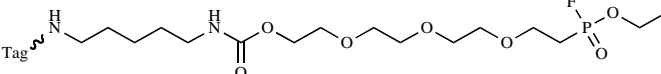
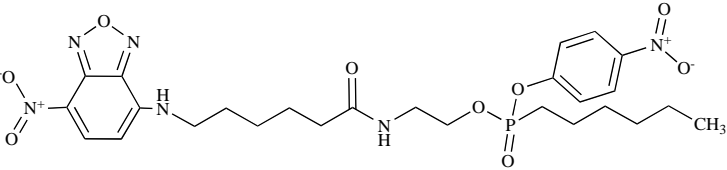
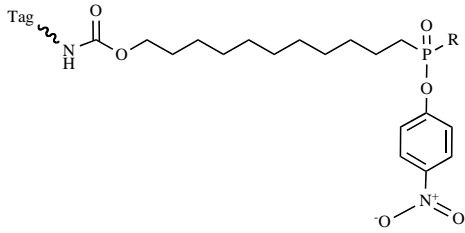
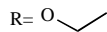
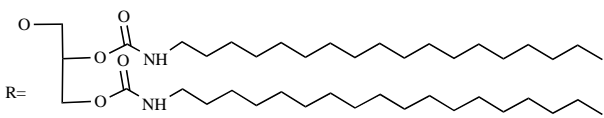
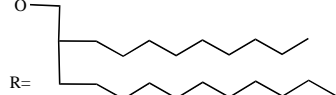
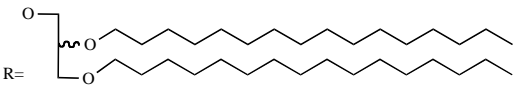
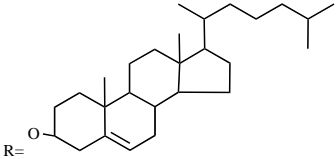
### Probe Design

*p*-Nitrophenyl- and fluorophosphonates have been used extensively to characterize and inhibit serine and cysteine hydrolases. The labeling of these compounds with a reporter tag (biotin and or a fluorophore) makes them excellent probes for activity-based proteomics to identify lipases, esterases and proteases (Table 1). 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 (Fig. 1).

The structure and polarity of the probe may have a large impact on probe specificity. When either a hydrophobic alkyl chain or a hydrophilic polyethyleneglycol moiety were used as a linker in biotinylated fluorophosphonates (Table 1, probes 1 and 2) similar end-point labeled serine hydrolase activity profiles were obtained [45]. However, a kinetic analysis of the proteome-probe reaction by stopping the reaction at different time points revealed that several serine hydrolases reacted at different rates with each probe. Nevertheless, these probes, as well as the probes 3-5, have a rather low specificity and simultaneously detect the whole range of different serine and cysteine hydrolases, including carboxylesterases, thioesterases, serine and cysteine proteases, PAF-acetylhydrolases, (lyso-) phospholipases and lipases. The specificity of the probe can be tuned towards lipolytic enzymes by including hydrophobic elements in the structure. These specificity moieties typically mimic native substrates, such as triacylglycerols or cholesterol esters for lipases and cholesterol esterases, respectively (Table 1, probes 6-9).

In our laboratory, a set of 14 fluorescently labeled *p*-nitrophenol esters of alkylphosphonates with two selector domains differing in polarity and stereochemistry on both sides of the phosphonate was designed in order to discriminate lipolytic and esterolytic activities [53]. One of the selector domains served at the same time 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 (e.g. Table 1, probes 3 and 4) but also derivatives with high specificity for certain subgroups of lipolytic enzymes, such as lipases and cholesterol esterases, were identified (Table 1, probes 7 and 8). A combination of these probes thus allowed rapid identification and classification of serine hydrolases: While lipases react with both, the low and the high specificity probes, esterases only recognize the low specificity probes. Probe 7 labeled selectively lipases and probe 8 cholesterol esterases. The probes incorporated a rather small uncharged fluorophore, N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amine (NBD),

Table 1. Activity-Based Probes for Lipases

	Structure	Tag	Target	Ref.
1		Biotin-Fluorescein-Rhodamine-	Serine hydrolases	[45,46]
2		Biotin-Fluorescein-Rhodamine-	Serine hydrolases	[45,47,91,93-96,101]
3		NBD-	Serine hydrolases	[52,53,55]
4-8				
4	R= 	NBD-Biotin-S-S-	Serine hydrolases	[53-55,58]
5	R= 	Biotin-S-S-	Lipases	[57]
6	R= 	Biotin-S-S-	Lipases	[57]
7	R= 	NBD-Biotin-S-S-	Lipases	[52-55]
8	R= 	NBD-Biotin-S-S-	Cholesterol esterases	[52-55]

thereby allowing probe recognition by lipases and also ensuring compatibility of the probe with isoelectric focusing, the first dimension of two-dimensional electrophoresis. A set of biotin labeled *p*-nitrophenol esters of alkylphosphonates was synthesized in our laboratory as complementary tool to the fluorescent probes in order to allow affinity-isolation and identification of proteins which are not amendable to analysis by 2D-electrophoresis, such as very large proteins or membrane proteins [55].

The linker between the tag and the reactive group can be used to introduce a third reactive site, e.g. a cleavage site to produce cleavable activity-based probes. Since the non-

covalent interaction between biotin and avidin are very strong 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 designed for directed evolution of lipolytic enzymes [57,58]. These probes (Table 1, probes 4, 5 and 6) were functionally tested for their ability to inhibit purified recombinant Lipolase® and to enrich phages displaying Lipolase® and active mutants thereof [56]. One has to keep in mind, however, that the disulfide bond is rather labile and prone to sulfide exchange reactions which may lead

to unspecific biotin labeling of proteins, especially under reducing buffer conditions and in the presence of free sulfhydryl groups.

### PROTEOMIC APPLICATIONS OF LOW SPECIFICITY PROBES

Cravatt and colleagues applied fluorophosphonates (Table 1, probes 1 and 2) for functional classification of tumors. For this purpose, the probes do not need to be highly specific. On the contrary, they are designed to target the highest possible number of enzyme activities. Serine hydrolase activities were profiled across a panel of cultured human cancer cell lines by one-dimensional gel electrophoresis of rhodamine fluorophosphonate labeled proteomes [91]. The obtained activity profiles were used for classification of cancer cells depending on the tumor, like breast carcinoma or melanoma. Nearly all of these activities were down-regulated in the most invasive cancer lines examined by the authors. However, the activities of 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, were upregulated. The latter protein is also involved in brain detoxification of organophosphorous nerve poisons. Interestingly, this function was discovered just recently by comparing chlorpyrifos oxon, which is a bioactivated metabolite of the insecticide chlorpyrifos, and fluorophosphonate labeled mouse brain proteomes [92]. In a subsequent study, Jessani *et al.* [93] profiled fluorophosphonate-labeled preparations of human cancer lines grown in cell culture or as xenograft, where important host factors may influence tumor biology. For this purpose, orthotopic xenograft tumors of the human breast cancer line MDA-MB-231 were established in the mammary fat pad of immunodeficient mice and their enzyme activity profiles were compared to those of the corresponding cell lines in culture. The authors identified enzyme activities selectively expressed in culture and in xenografts, as well as host enzymes that either infiltrated or were excluded from xenograft tumors. Recently, activity-based protein profiling and MudPIT were combined for the analysis of primary human specimens, such as tumor biopsies, which are heterogeneous and of limited quantity [94]. Serine hydrolase activity profiles obtained by fluorescence scanning of rhodamine fluorophosphonate labeled human breast tumor proteomes separated on one-dimensional gels were hierarchically clustered and used for functional classification of the samples. Representative members of each class were then identified by MudPIT after treatment of proteomes with a biotinylated fluorophosphonate, enrichment of probe-labeled proteins using avidin-conjugated beads and on-bead trypsin digestion. Importantly, when enzyme activity profiles were compared with cDNA microarrays, enzymes were revealed whose activity but not mRNA abundance depicted the tumor class.

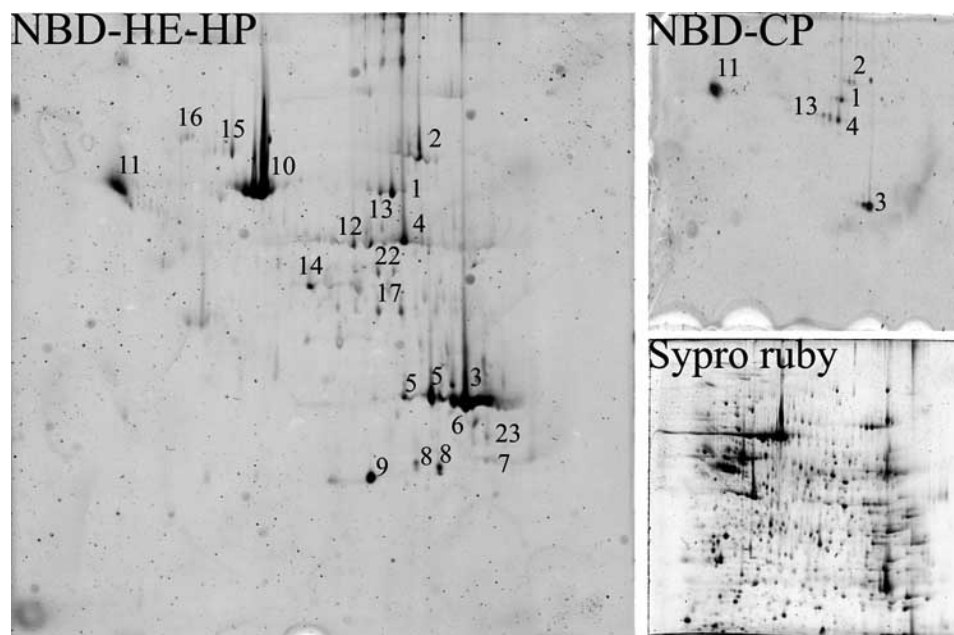
In the same laboratory, irreversible inhibitors were screened by pretreating proteomes with libraries of the respective compounds followed by labeling with an activity-based probe. Labeling intensity of each target relative to the untreated control sample was used to generate percent competition values, which were then clustered using programs designed for analysis of microarray data. Three covalent

inhibitors resembling  $\beta$ -lactones, namely orlistat and ebelactones A and B, were screened against serine hydrolases in prostate tumor cell lines [95]. A new target for orlistat was identified in this screen. Orlistat inhibited fluorophosphonate labeling of the thioesterase domain of fatty acid synthase, which is 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 library of electrophilic ketone agents was profiled in competition experiments using a Rhodamine-fluorophosphonate in mouse proteomes [96]. By this approach, reversible inhibitors of several serine hydrolases, including the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH), TGH and KIAA1363 were identified simultaneously.

In yeast, known and uncharacterized serine hydrolases were identified by 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 [97]. By computational analysis 52 and by activity-based proteomics 23 serine hydrolases were assigned. 15 proteins were identified by both methods, including a novel family of serine hydrolases, designated as Fsh.

### PROTEOMIC APPLICATIONS OF HIGH SPECIFICITY PROBES

In our laboratory, some of the probes designed for discrimination of lipolytic and esterolytic enzymes, namely NBD-HE-HP (Table 1, probe 3), which resembles a single chain carboxylic acid ester, enantiomeric trialkylglycerols (NBD-*sn*1-TG, NBD-*sn*3-TG; enantiomeric at the *sn*-2 carbon of probe 7) and a cholesteryl ester (NBD-CP; probe 8), were recently used to map the lipolytic and esterolytic proteome of mouse adipose tissue [52]. When screening enzyme preparations, NBD-HE-HP recognized a wide range of lipolytic and esterolytic enzymes while the *sn*-1 triacylglycerol mimicking inhibitor appeared to be specific for lipases [53]. The *sn*-3 triacylglycerol resembling inhibitor was only recognized very poorly by most enzymes. NBD-CP was the most specific activity tag since it reacted only with known cholesterol esterases. For investigation of the lipolytic proteome of mouse adipose tissue the probe with the lowest specificity, NBD-HE-HP, was used for the major part of the study since it recognized the widest range of enzymes while NBD-*sn*1-TG, NBD-*sn*3-TG and NBD-CP were used for detection of more specific activities. Analysis of the proteome after incubation with these probes by 2D-gel electrophoresis produced a rather complex pattern of serine hydrolases with NBD-HEHP, while NBD-CP labeling further simplified the proteome pattern (Fig. 2). LC-MS/MS of cut out and in-gel digested spots led to the identification of all known intracellular lipases, as well as a number of uncharacterized proteins. One of these proteins was shown to be involved in triacylglycerol mobilization in adipocytes and therefore named ATGL [20]. Functional characterization of transiently expressed enzymes, including HSL, MGL and ATGL, by conventional substrate assays in comparison to inhibitor profiling demonstrated that lipolytic and esterolytic activities could be well discriminated using this small set of



**Fig. (2).** The lipolytic proteome of white mouse adipose tissue. White mouse adipose tissue was labeled with 20  $\mu$ M NBD-HE-HP (Table 1, probe 3) or NBD-CP (Table 1, probe 8) and separated by two-dimensional gel electrophoresis. Fluorescent spots were excised from the gel and digested by trypsin. Peptides were analyzed by nano-HPLC-MS/MS. Identified proteins were 1 Succinate dehydrogenase, 2 HSL, 3 MGL, 4 TGH, 5, Esterase 10, 6 Williams Beuren syndrome critical region 21 (WBSR 21), 7 Esterase 1 homolog (KNP-I), 8 Lysophospholipase 2, 9 Lysophospholipase 1, 10 Albumin, 11 Esterase 1, 12 ATGL, 13 Triacylglycerol hydrolase 2 (TGH2), 14 Protein phosphatase methylesterase 1, 15 Acylpeptide hydrolase, 16 Prothrombin, 17 CGI-58, 22 Very long chain-Acyl-CoA thioesterase, 23 Lysophospholipase-like-1. As negative controls unlabeled samples were scanned to identify 1 Succinate dehydrogenase as autofluorescent protein (not shown). After scanning the fluorescent probes, the 2D-gel was stained with Sypro ruby and scanned again.

structurally differing fluorescent probes (NBD-HEHP, NBD-*sn1*-TG, NBD-*sn3*-TG and NBD-CP). Thus we demonstrated the general applicability of our method for rapid profiling and identification of lipolytic activities in complex biological samples.

In a further study we used the set of NBD-tagged alkylphosphonates as activity sensors for microarray-based characterization of lipolytic enzymes in enzyme preparations [54]. To our knowledge, lipases have not been immobilized in active form on solid support before. Moreover, biotinylated alkylphosphonates were immobilized on streptavidin-coated slides. This inhibitor array was then employed for characterization of selectivity of MGL transiently overexpressed in COS-7 cells.

Possible future developments in this field may be the design of activity-based probes specific for lipases that are suitable for enzyme targeting in living cells. In general, *in vivo* probes have to be small and hydrophobic enough to be able to cross cellular membranes. The largest and most polar part of the probe typically is the bulky reporter tag. Thus tag-free versions of activity-based probes have been designed for proteasomes [98] and non-directed approaches [99,100], whereby the reporter is attached to the probes after labeling of the target enzymes. With these probes active enzymes can be tagged in intact living cells and not only in cell homogenates. Moreover, enzymatic activities can be localized by fluorescence microscopy in cells and tissues allowing observation of changes in activity and in subcellular localization. In addition, “tag-free” lipolytic activity-based probes should resemble the natural substrates more closely. Another inter-

esting development in this field is the possibility to multiplex activity profiles of lipolytic enzymes in the same lane of a one-dimensional gel or in the same two-dimensional gel. In principle, activity-based probes differing solely in the fluorophore can be used to multiplex activity profiles of proteomes [47]. However, possible spectral overlap of the used fluorophores as well as possible probe discrimination in dependence of the fluorophore has to be taken into account. Reporter tag-based discrimination of enzyme inhibition may, of course, be circumvented by introduction of the reporter tag after the enzyme-inhibitor binding event.

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