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Tuning probe selectivity for chemical proteomics applications

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Covalent chemical probes enable investigation of a desired fraction of the proteome. It is possible to adjust the selectivity of these probes, so they either react with a certain amino acid in all proteins, a class of proteins or only a single protein species. A combination of specific reactive groups with additional recognition elements can fine tune probes to hit the desired proteins, even in the presence of related family members. Using probes of lower or higher selectivity, screening experiments for inhibitor discovery and imaging experiments for localization studies can be performed, showing only a fraction of the power of covalent small molecule probes.

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Introduction

The daunting task of proteomics does not only include the identification of all members of the proteome, but also the assignment of protein function, the detection of protein localization and the mapping of regulatory pathways and networks. The analysis of the proteome can be significantly enhanced by chemical techniques that allow quantitative or qualitative analysis of a fraction of the proteome. Some chemical proteomics approaches use affinity resins with molecules that reversibly bind target proteins [1]. Another approach is the covalent modification of proteins by small molecules. In contrast to reversible binders, covalent chemical probing allows, for example, in-gel detection of probe-protein complexes and mass spectrometry-based identification of the binding site. Covalent probes can be divided into different types: Molecules incorporated by metabolic labeling (through the usage of endogenous enzymes), electrophiles (probes with intrinsic reactivity) and photocrosslinkers (affinity labeling). The available covalent chemical probes display a variety of selectivity and specificity properties.

Within the scientific community the words selectivity and specificity are often used in a mutually exchangeable way. In order to make this review as clear as possible, we have defined the main terminology in Box 1. Although the phrase 'highly selective' has positive connotations, selective probes are not always required. In this review we focus on the question of which strategies can be used to tune the selectivity of a covalent probe. We illustrate the usage of selective and non-selective probes with recent developments in chemical proteomics applications.

Global targeting of the proteome

Modification of the proteome with reagents that display no selectivity, but high specificity, has proven especially useful for purposes of protein quantification. Both ICAT [2] (isotope-coded affinity tagging) and iTRAQ [3] (isobaric tags for relative and absolute quantification) make use of reactive groups that specifically and non-selectively modify sulfhydryl or amino groups on proteins or tryptic peptides. In this way, every member of the proteome containing a cysteine or a free amino group can be analyzed and quantified in comparison to other samples.

The identification of post-translational modifications (PTMs) on proteins is possible by the use of probes that get incorporated by the metabolic machinery of the cell. These probes mimic the structures of the PTM building blocks, such as lipid or carbohydrate monomers, and contain a small azide or alkyne tag for downstream visualization using tandem labeling strategies. The metabolic enzymes govern the selectivity of the labeling. Generally, a given PTM occurs on a wide variety of proteins, but the modification sites are highly specific. A landmark in PTM analysis by metabolic labeling was the detection of azido-acetyl containing sialic acids in cell surface glycoproteins by feeding cells per-acetylated *N*-azidoacetyl-mannosamine (1; ManNAz; Figure 1a-1) followed by biotinylation through a Staudinger ligation [4]. This research has launched the investigation of PTMs using a combination of metabolic labeling and bioorthogonal chemistry. An interesting recent application of metabolic labeling with ManNAz is the discovery of BACE1 protease substrates. BACE1 is an Alzheimer related protease that sheds membrane anchored proteins from the cell surface. Since most cell surface proteins contain glycans with sialic acid, this strategy could be

Box 1 Glossary of terminology

Activity-based probe: An activity-based probe (ABP) is a small molecule that covalently binds to the catalytic center of its active target enzyme. The resulting complex can be analyzed by means of an affinity or visualization handle on the ABP.

Chemical proteomics aims to chemically separate or modify the proteome in order to facilitate quantitative or qualitative analysis of a fraction of proteins within the whole proteome.

Selectivity: The word selectivity is derived from the Latin verb *selegere*, which means to choose. The fewer proteins a probe will 'choose' to bind, the higher its selectivity is. Hence, the ultimate probe selectivity is the modification of one single protein species. A highly selective probe can be desirable for some purposes, but other applications may require less selective probes.

Specificity: The words selectivity and specificity are often used interchangeably. To add to the confusion, the terms have other definitions in different scientific areas. In some disciplines, specificity is used as the ultimate selectivity. However, in our view, this leaves the term specificity hollow and impractical. We here use 'specificity' to indicate the rate of false positives. A highly specific probe gives rise to a low amount of off-targets – regardless of the total number of targets. Maleimide probes, for example, are non-selective, as they modify all cysteine-containing proteins. However, they are specific to this particular residue: amino acids other than cysteine are virtually unaffected. As a result, there are few false positives and the specificity is therefore considered high.

used to modify – in presence or absence of a BACE1 inhibitor – all secreted proteins with biotin, allowing separation from serum proteins and subsequent MS identification. In contrast to earlier ideas that BACE1 only cleaves a few specific proteins, this study showed that it is responsible for the shedding of more than 30 membrane proteins [5•].

PTMs that are very small do not allow direct incorporation of azide or alkyne tags. Examples include S-hydroxylation (SOH) and S-nitrosylation (SNO). These PTMs are often transient and labile, and it is difficult to detect them by MS directly. Covalent labeling of cysteines has provided an indirect way to detect SNO. In the so-called biotin switch assay free thiols are first blocked with reagents such as *N*-ethyl maleimide. Then the SNO modifications are transformed to free thiols with sodium ascorbate. The liberated thiols are subsequently labeled with cysteine reactive biotin tags, which enable MS based identification of the modification sites [6]. This technique has been modified and combined with biotin-SS-NHS to study lysine acetylation [7]. Recent studies have also reported isotope-coded reagents for the relative quantification of SOH and SNO modifications [8,9•].

Although cysteine can be specifically and quantitatively labeled owing to its strong nucleophilicity, not every cysteine residue displays the same reactivity. Cravatt and co-workers added an alkyne handle to an iodoacetamide to make a probe for identification of hyper-reactive cysteines as functional hotspots in proteomes. Not surprisingly, a substantial amount of these hotspots were active site cysteines, redox-active disulfides or sites for post-translational modification [10••].

Increasing the selectivity of probes

Besides cysteine, the proteome contains a variety of other amino acids with nucleophilic side chains: serine, threonine, tyrosine, aspartic acid, glutamic acid and histidine. Weerapana *et al.* demonstrated that different types of carbon electrophiles display distinct reactivities against these residues. Whereas a chloroacetamide and an unsaturated ketone almost exclusively react with cysteine residues, sulfonate esters also form covalent bonds with aspartate, glutamate, histidine and tyrosine [11]. Depending on the chemical environment (e.g. solvent exposed or part of a catalytic center) nucleophilic residues can display different reactivity. Activity-based probes (ABPs; see Box 1) take advantage of this feature by the use of reactive warheads that require an enhanced nucleophilicity of their reaction partner. This prevents general binding to surface residues and leads to a single site-directed modification per enzyme molecule. The nature of the warhead is decisive for the type of target amino acid residue, providing a first degree of selectivity. For example, a fluorophosphonate (2, FP, Figure 1a) reacts specifically with the active site serine residue of many serine hydrolases (SHs) [12••]. Epoxysuccinates (5, Figure 1b) react with cysteine proteases, but need a recognition element (RE; see also later) to provide enough affinity to the protease active site and orchestrate a reaction with the active site cysteine residue [13].

Some ABPs make use of latent electrophiles. These warheads create an electrophile only upon processing by a member of their target enzyme class. The electrophile subsequently reacts with any nucleophile in its proximity, forming a covalent enzyme–probe complex. Most latent electrophiles have been based on mandelic acid derivatives with an α-leaving group which form reactive quinone methides (Figure 1a-2). However, the quinone methides can diffuse from the active site and potentially label off-targets [14•,15,16]. While these probes are mechanistically restricted to hydrolases, another type of latent electrophile was recently developed for monoamine oxidases. The flavin adenosine dinucleoside cofactor of the enzyme oxidizes a tertiary amine on probe 3 (Figure 1b) and creates a Michael acceptor (4), which then reacts with the flavin to form a covalent adduct [17].

An extra degree of selectivity can be added to a probe by the attachment of a RE that will guide the warhead to a smaller set of proteins. Some of these probes have been inspired by natural products. Perhaps the most well-known example is DCG-04 (5, Figure 1b), an ABP for papain-like cysteine proteases based on the cysteine protease inhibitor E-64 from *Aspergillus japonicus* [13].