Proteomics

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Hypersensitive Response to Over-reactive Cysteines

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Over the last few years, the field of proteomics has made considerable progress thanks to the development of new techniques for the analysis of protein expression, function, and activity in complex biological samples. These approaches enable not only the identification and characterization of proteins but also the accurate quantification of protein levels and activity in native proteomes. Quantitative proteomics techniques make use of either stable-isotope labeling (e.g. ICAT, SILAC)[1] or label-free techniques based on mass spectrometry (MS)[2] to identify proteins and quantitatively analyze expression levels (Figure 1a), which may differ significantly between different cell types and tissues, and under various conditions. In addition, most proteins are subject to posttranslational modifications, such as oxidative processes, that influence their function and stability. These modifications as well as the differences between different proteomes, for example, healthy versus diseased, can be analyzed by quantitative proteomics.

In the case of proteins with catalytic activity, protein abundance studies are not sufficient to provide insight into the enzymatic activity, which is usually subject to many levels of regulation. Hence, methods are required that directly monitor protein activity irrespective of protein expression levels. Activity-based protein profiling (ABPP) enables the direct quantification of enzymatic activity with the use of small-molecule activity-based probes (ABPs).[3] These interact specifically with the catalytically active form of target enzymes and may be equipped with an affinity tag for isolation and identification by mass spectrometry. While traditional ABPP experiments are aimed at identification of either the complement of the labeled proteins or the active-site fragments targeted by the probe, a tandem orthogonal proteolysis/activity-based protein profiling (TOP-ABPP) method was recently developed to simultaneously identify tagged proteins and sites of modification (Figure 1b).[4]

In general, the reactivity of amino acid side chains, being either catalytic activity or susceptibility to posttranslational modification, is largely dependent on the local protein microenvironment. However, no consensus sequences are known that systematically identify highly reactive amino acid residues and distinguish them from their nonreactive counterparts. This complicates the global identification of reactive sites in the proteome as well as the annotation of newly discovered proteins. ABPP and quantitative proteomics techniques both target a specific subset of reactive amino acid residues, but none of the approaches fully covers the total "reactivity profile" of the proteome.

Of all naturally occurring amino acid residues the free thiol group of cysteine is considered the most reactive group, since it is highly nucleophilic and very sensitive to oxidative modification. Various thiol-specific labeling reagents are available to study cysteine residues. Among these, iodoacetamide (IA) is frequently used in quantitative proteomics, where cysteine residues in two different proteomes are labeled with a stoichiometric amount of light or heavy probe and the differences analyzed (Figure 1a).[1]

Alternatively, ABPP experiments often make use of ABPs that exclusively target catalytically active cysteine residues in a specific class or subclass of enzymes. Selectivity is accomplished by tuning the reactivity of the ABP in such a way that it reacts only at specific sites in the proteome and leaves other functionalities unaffected. An example hereof is the activity-based probe DCG-04 which selectively labels the cysteine protease cathepsins.[5,6] In addition, the nucleophilicity of particular cysteine residues is determined by measuring pK_a values or the rate of alkylation by specific electrophiles, but this is possible only with purified proteins,[7] which presents an obvious limitation.

Recently, Weerapana et al.[8] designed a strategy for the direct quantification of amino acid side chain reactivity, in particular that of cysteine residues, on a global scale in native biological samples. This approach combines the advantages of TOP-ABPP and quantitative proteomics in the search for functional cysteines in complex proteomes. In this method, termed isoTOP-ABPP (isotopic TOP-ABPP, Figure 1c), the alkyne-iodoacetamide (IA probe) is "clicked" to either a heavy or light azido-TEV-biotin tag. Based on the hypothesis that functionality is reflected by the nucleophilicity and therefore the "hyperreactivity" of cysteine residues, it was reasoned that hyperreactive cysteine residues are labeled to completion with low concentrations of IA probe, whereas the less-reactive cysteine residues are labeled in a concentration-dependent manner. Hence, treatment of a proteome with a low concentration of the heavy IA probe and increasing concentrations of the light IA probe, followed by enrichment of tagged proteins by streptavidin pull-down, trypsin and

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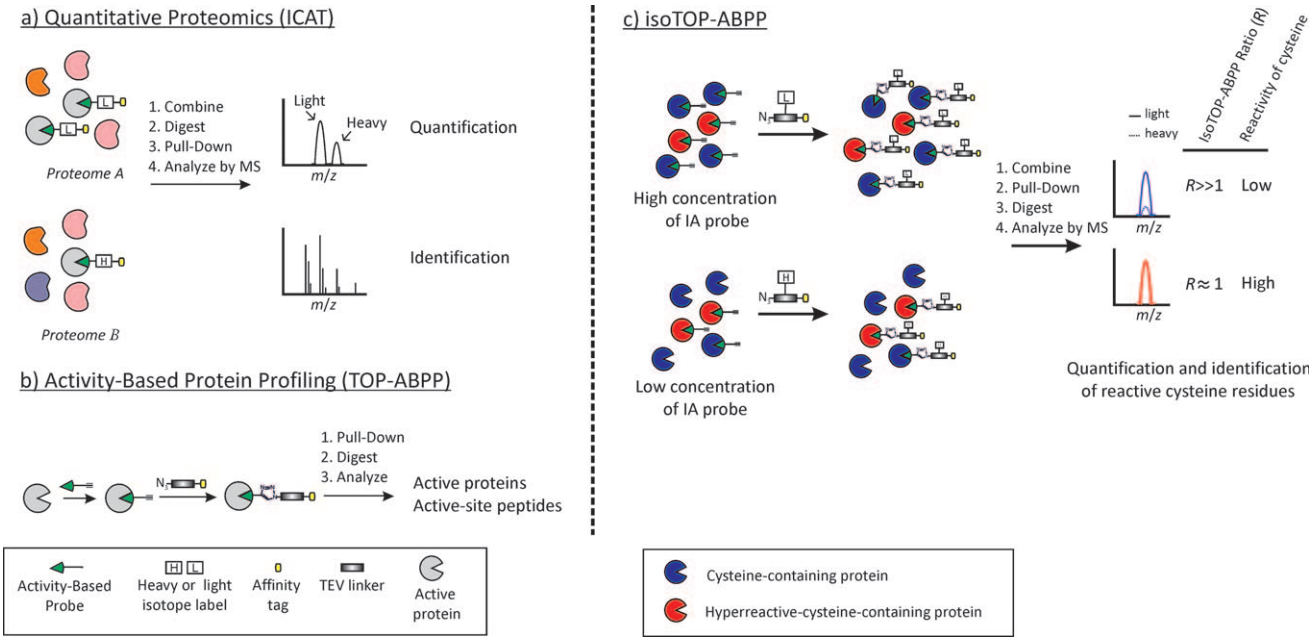

Figure 1. Schematic view of three techniques used in proteomics. a) Quantitative proteomics compares two states of the proteome, mostly with the use of isotope labels. b) Activity-based protein profiling identifies active proteins and/or active-site fragments by using ABPs; TEV = tobacco etch virus. c) The isoTOP-ABPP method identifies functional cysteine residues by quantification of their reactivity towards cysteine-specific probes.

TEV digest, and LC-MS/MS analysis gives a light/heavy ratio for each labeled cysteine-containing peptide. Very reactive cysteine residues are expected to have isoTOP-ABPP ratios $R_{[light]:[heavy]}$ of approximately 1, and for less-reactive cysteine residues $R_{[light:heavy]} \gg 1$. Since this ratio is dependent on reactivity rather than protein abundance and the IA probe is small and cell-permeable, this technique can be used to study functional cysteine residues in native proteomes.

Weerapana et al. show that isoTOP-ABPP ratios for individual cysteine residues are indeed independent of protein abundance and tissue origin. Furthermore, the group of peptides that showed a ratio of $R_{[light:heavy]} < 2$ (for an IA-probe concentration of 10:1; with a tenfold excess of IA probe compared with a heavy IA probe) is enriched in cysteine residues that are annotated as being catalytically active, part of an active site, or subject to posttranslational (oxidative) modifications. A low isoTOP-ABPP ratio of cysteine residues in uncharacterized proteins provides valuable information that may lead to elucidation of their function. Additionally, this method can be used to predict the functionality of computationally designed proteins in a complex mixture.

The results of this study show that with an elegant concentration-based design cysteine reactivity can be correlated to functionality. Instead of lowering the reactivity of the probe in order to achieve selectivity (as in ABPP), a very reactive probe is now used in different concentrations to discriminate between residues of different reactivity. At low probe concentrations cysteine residues compete for reaction with the probe, resulting in labeling differences. A similar approach is used in organic chemistry, for instance to determine the relative reactivity of glycosyl donors by competition against a common activator.[9] However, since labeling of cysteine residues is also largely dependent on abundance, Weerapana et al. make use of the ratio between high and low concentrations of lightand heavy probes rather than absolute labeling quantification.[8]

A shortcoming of the method is that a subset of (functional) cysteine residues might not be labeled by the probe due to sterical reasons. Other cysteine residues might be dependent on cofactors for their activity or have a different mode of action. Therefore, the absence of hyperreactivity does not exclude functionality and only its presence can be used as a strong indication that the residue is involved in a functional process. One could envisage that with a different set of electrophilic probes, targeting a different fraction of the proteome, the scope of this method would be greatly enhanced. Currently, however, very few general reagents for functional groups other than thiols are at hand, so the challenge lies in the development of these. Alternatively, this technique could be expanded to specific ABPs, like DCG-04. Stoichiometric amounts of probe would label the family of enzymes as a whole, whereas lower concentrations of probe could be used to gain insight in the activity of individual members of the family under various conditions. This would for instance give an entry to assess relative cathepsin activities in a setting where their concerted action in the processing of MHC class II antigenic peptides is the subject of study.[10] Future research will show if modest changes in isoTOP-ABPP ratios can be used to quantify even subtle differences in activity.

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