Deacetylase inhibitors & Histone inheritance

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Chapter 5

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Bioorthogonal metabolic labelling with acyl-CoA reporters: targeting protein acylation.

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Bioorthogonal metabolic labeling

The analysis of endogenous biological activity faces serious challenges due to the complexity of life at the molecular level. Nevertheless, metabolic labeling techniques in combination with bioorthogonal chemistry have paved the way for the elucidation of numerous biochemical processes [1]. Towards this aim, a wide variety of small synthetic molecules, coupled to functionalized moieties, was developed to function as structural analogues of cellular metabolites. As a first step, these reporters are enzymatically incorporated into protein-targets via metabolic pathways. In the case of tracking new proteins, structural analogues of amino acids that contain a chemical reporter group can be incorporated into the structure of proteins during translation of mRNA into the protein's amino acid sequence by the ribosome. As long as the chemical reporter group does not change the overall properties of the amino acid too much the enzyme that couples the amino acid to the respective tRNA, called an aminoacyl-tRNA synthetase, is unable to distinguish between a normal amino acid and the modified version. Adding such an unnatural amino acid or completely substituting a natural amino acid with an unnatural analogue in the culture medium of cells thus allows their incorporation into a protein. Similar to the fairground duck fishing game it is like adding a loop to a protein, which allows you to later fish the proteins containing that loop out of thousands of other proteins. Incorporation of the unnatural amino acid, the loop, does not necessarily harm cellular processes, although this requires careful selection of the experimental conditions. As a second step, labeled proteins are visualized and identified via a reaction between the chemical reporter and a (much larger) detection tag. This reaction must be site-specific, rapid and compatible with a biological environment, called bioorthogonal. This two-step strategy of metabolic labeling in combination with biorthogonal chemistry has found great application in tracking new proteins [2].

Bioorthogonal chemistry in context

One of the oldest conventional methods for the detection of endogenous biomolecules is autoradiography, which revolves around the use of specific radioisotope-labelled substances. For instance, addition of radiolabeled precursors of acetyl-CoA allowed researchers to understand the mechanisms behind lipogenesis [3–5] and p53 acetylation in vivo [6]. However, this technique is frequently hampered by practical limitations and is often incompatible with modern proteomic approaches.

More commonly used research methodologies in the field of proteomics make use of protein-specific antibodies, such as immunoprecipitation [7], Western blot [8] and ELISA [9]. While antibodies have become the standard choice for studying proteins in complex biological matrices, a lack of antibody selectivity due to cross reactivity with other epitopes and loss of target proteins due to poor affinity are in some cases problematic [10, 11]. Moreover, for certain protein modifications antibodies do not exist. An important example is the absence of antibodies against lipid protein modifications, such as myristoylation and palmitoylation.

Some limitations of antibodies can be circumvented by using bioorthogonal chemical labeling strategies instead. One of the first bioorthogonal reactions was presented by Bertozzi in 2000 (before she coined the term bioorthogonal in 2003) and she humbly called it the Staudinger ligation [12]. Deviating from the Staudinger reduction [13], where an azide reacts with a triaryl phosphine to form a water-labile aza-ylide intermediate, the now-named Staudinger-Bertozzi ligation yields a stable amide bond through a triaryl phosphine carrying an electrophilic trap. The trap, a methoxycarbonyl at the ortho-position, stabilizes the intermediate by intramolecular cyclization [14]. Proof of principle was provided by the coupling of biotinylated phosphine molecules to cell-surface azido sialic acid chemical reporters [12]. The abiotic nature of both reaction partners, the mild reaction conditions and the favorable reaction kinetics allowed chemo selective ligation to occur for the first time in a variety of biological systems. Since the pioneering work of Bertozzi, she and

others massively expanded the bioorthogonal toolkit. Over the years, some of the newer reactions show marked improvements with regard to reaction kinetics and nature of the chemical reporter, among which the "click" reaction has a central position [15, 16].

Selecting a suitable reaction for a given application is not necessarily an easy task and requires several aspects to be taken into account [17]. One important consideration is whether a reaction can be carried out in vitro or in vivo, and correspondingly, whether or not performing a reaction in vivo is truly necessary. For instance, in two-step labeling procedures with the goal of detecting proteins, the first step, comprising metabolic incorporation of chemical reporters using a cell's own metabolic machinery, typically needs to occur in vivo. The second step, however, may be performed in vitro, since proteins of interest are then already labelled. This is only feasible when metabolic labeling yields a stable chemical reporter-to-protein bond, and when adequate measures have been taken to reduce or control for background noise due to side-reactivity or toxicity of the selected reaction. Taking these requirements into consideration, we can argue that in vivo labeling with the appropriate probes and in vitro bioconjugation of isolated proteins are sufficient for monitoring the desired biological target especially when toxicity of the coupling reagents is a concerning issue.

In the context of metabolic tagging, chemical reporters must meet several criteria. The most important being the size of the chemical motif, with only small chemical motifs being widely compatible with the cellular biosynthetic machinery. This may be particularly true for smaller probes, like modified amino acids, where the relative contribution of the chemical motif to the overall probe-size becomes larger. Prime examples of small, non-perturbing bioorthogonal functional groups are terminal alkenes and alkynes [18]. Terminal alkynes and alkenes consist of only two carbons, are metabolically stable and do not naturally exist in cells, making them ideal chemical reporters. Furthermore, these unsaturated hydrocarbons can be modified with a range of bioorthogonal reactions.

Bioorthogonal reactions with terminal alkynes and alkenes

Alkyne-Azide: "Click"

The first bioorthogonal reaction to involve a terminal alkyne, is the copper-catalysed alkyne-azide cycloaddition (CuAAC), known as "click" reaction (Figure 1A). Based on the azide-alkyne Huisgen [3+2] 1,3 dipolar cycloaddition [19], Meldal and Sharpless independently discovered that addition of copper(I)-salts increases the rate of triazole formation [20, 21]. Meeting most requirements for bioorthogonality, Wang and others fine-tuned the CuAAC to conjugate dye-alkynes to azide-labelled virus particles [22]. One of the adjustments needed in the context of bioconjugation was the use of TCEP (tris(2-carboxyethyl)phosphine) instead of ascorbate, since the latter induced substantial disassembly of the virus capsid. It is now understood that this may be caused by ascorbate's ability to form dehydroascorbate byproducts with arginine residues [23]. Despite of this, ascorbate is the preferred reducing agent, since the azide-reducing and copper-binding properties of phosphine TCEP causes it to interfere with the CuAAC reaction. To suppress ascorbate-related side-reactions, aminoguanidine may be added or hydroxylamine can be used as the reductant [24].

While exchanging ascorbate with TCEP turned out to be disadvantageous, an adjustment that did enhance the reaction in general was the addition of a tris(triazolyl)amine ligand [25]. Chan and others observed that synthesis of polytriazoles with the CuAAC occurs at an unusual high rate (through autocatalysis). The stabilizing effect of polytriazole ligands on the Cu(I) oxidation state not only leads to an increased reaction rate, but may also protect biomolecules from hydrolysis by Cu(II) species. Additionally, polytriazoles may act as scavengers of reactive oxygen species resulting from copper or ascorbate related side-reactions, thereby minimizing oxidation of histidines and other amino acid residues.

The main advantage of the CuAAC reaction is the relatively fast reaction rate. Compared to the Bertozzi-Staudinger ligation, for instance, a 25-fold faster rate has been reported [1]. On the other hand, ascorbate's side reactivity [26] and the need for additional reagents to keep copper at the reduced state represent substantial downsides.

Alkyne-Halide: Sonogashira

Another interesting reaction that employs terminal alkynes is the Sonogashira cross-coupling reaction [27]. Here, alkynes react with aryl or vinylhalides to yield a carbon-carbon bond. Traditionally, the reaction proceeds at room temperature in organic solvents, employing a ligand-bound palladium(0)catalyst, a copper(I) co-catalyst and a base. Yet, Kodama and others found that the reaction also proceeds in aqueous media, buffered to a slightly basic pH, with the addition of sodium ascorbate and 12% DMSO [28]. Unfortunately, the required use of ascorbate to assure reductive conditions entails that the same CuAAC related drawbacks apply.

Therefore, Li and others set out to develop a copper-free Sonogashira cross-coupling reaction [29]. They succeeded with an efficient water-soluble palladium–2-amino-4,6-dihydroxypyrimidine (ADHP) complex and selectively coupled fluorescein iodide to alkyne-functionalized proteins. Moreover, Chen's group recently reported a ligand-free Sonogashira coupling method (Figure 1B) [30]. They found that $Pd(NO_3)_2$ as a catalyst sufficiently accelerates cross-coupling of rhodamine-labelled phenyl iodide to alkyne-encoded GFP. It is worth mentioning that they demonstrated that their cross-coupling method is suitable for protein labeling inside bacterial cells without apparent toxicity of the palladium catalyst. On the other hand, non-specific metal binding to proteins has been observed, resulting in the requisite use of a metal chelator [31].

Terminal alkynes versus terminal alkenes

While tremendous progress has been made in the development of alkyne-based labeling strategies, there are some anecdotal problems associated with

the introduction of terminal alkynes in living systems. Problems mainly arise due to the relative acidity of the alkynic proton, which affects the stability of terminal alkynes [32]. A closely related issue is that alkyne-labelled carboxylic acids, such as 17-octadecynoic acid, can be converted into highly reactive intermediates by oxidative enzymes, which causes covalent inhibition at 10 μM, whereas metabolic labeling requires the same concentrations [33–35]. Also, alkyne homo-coupling [36] and the covalent binding of terminal alkynes to active site cysteine residues [37] (the thiol-yne reaction) have been described. While the latter may equally apply to alkenes [38, 39], the lower intrinsic reactivity of alkenes makes this less likely to occur.

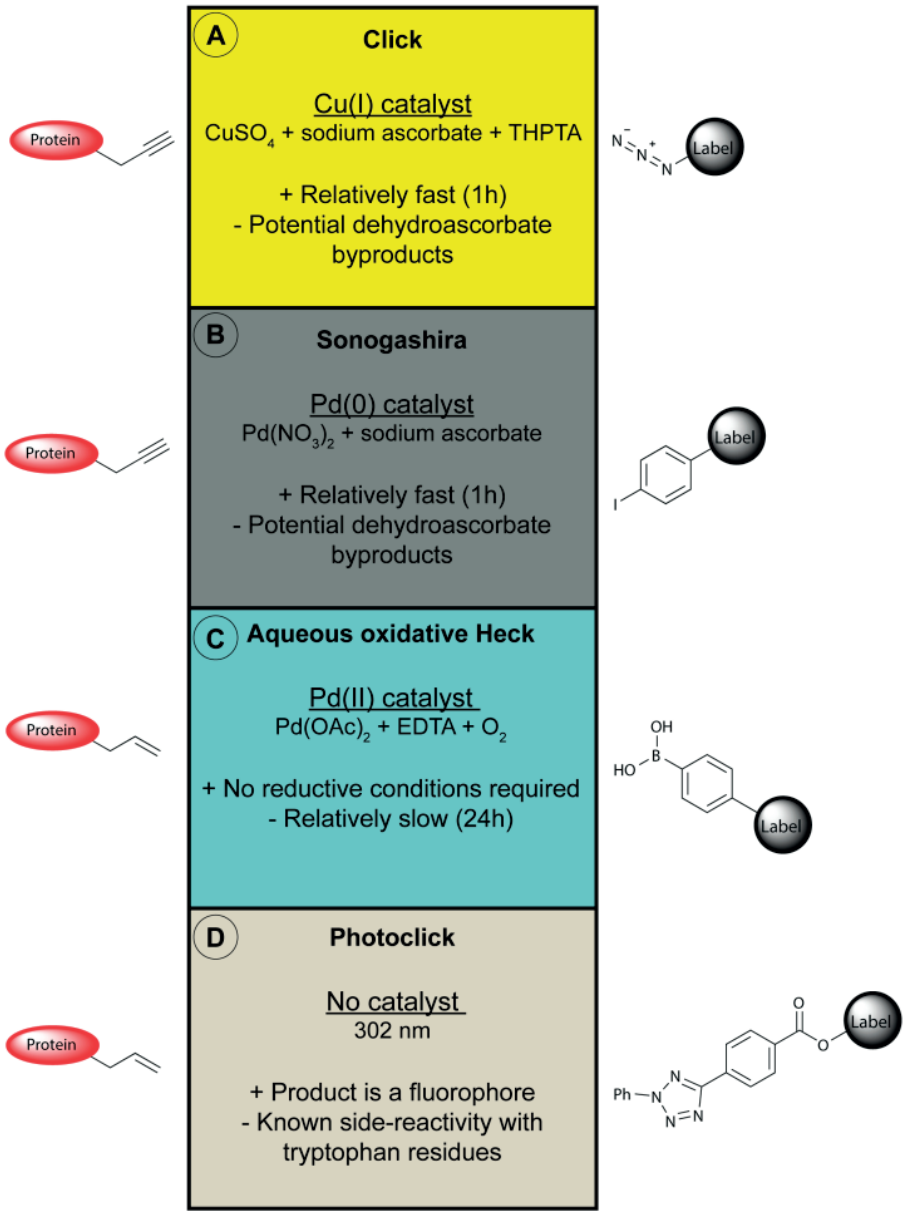

Figure 1. Overview of bioorthogonal reactions with terminal alkyne- or alkene-tagged proteins for in vitro labeling.

Alkene-Boronic acid: Aqueous oxidative Heck

The potential benefits of olefinic chemical reporters are only relevant when suitable alkene-targeted reactions exist. Only some reactions that originate from the realms of organic chemistry exploit the low intrinsic reactivity of alkenes, and even fewer may find an application in proteomics. An especially promising candidate to this end is the (Mizoroki-)Heck reaction, which involves the coupling of a terminal alkene with an unsaturated halide in presence of base and a palladium(0) catalyst to form a substituted alkene [40, 41]. Proof of concept was provided by Kodama and others with the conjugation of vinylated biotin with iodophenyl-functionalized Ras proteins [28]. While they did not observe a significant decrease in the stability of model Ras protein during or after the reaction, the comparatively low yield of 25% is a major shortcoming. Additional work by Uemura and others found that commercially available boronic acids could be used instead of unsaturated halides [42]. Also, Cho and others reported an oxidative palladium(II)-catalysed reaction of boronic acids with alkenes without the need for a base and high temperatures [43]. Further optimization of the catalyst, using BIAN ligands, promoted the reaction of many different arylboronic acids with alkenes [44]. Encouraged by these results, we recently developed a Heck-type reaction to label protein-bound alkenes with arylboronic acid derivatives in vitro [45]. Gratifyingly, we observed that, under the optimized conditions, the oxidative Heck reaction proceeds in high yields and is chemoselective towards terminal olefins, even in complex protein mixtures. Yet, the addition of EDTA after completion of the reaction was found to be essential for chelating palladium out from protein sites. This problem was circumvented with the later development of a water-soluble Pd(II)-EDTA catalyst that effectively catalyses the reaction at room temperature in aqueous environment (Figure 1C) [46]. The reaction does not require any other additives and runs smoothly under oxygen atmosphere. A downside of the oxidative Heck reaction is the relatively long reaction time needed for the cross-coupling (24h).

Alkene-Nitrile imine: "Photoclick"

Lastly, exciting new developments centre around the reaction between a nitrile imine and an alkene through a photoinducible dipolar cycloaddition [47]. Like the CuAAC, the original reaction comes from the group of Huisgen. Recently, Song and others optimized this transformation in bioorthogonal labeling experiments and called it the "photoclick" reaction (Figure 1D) [48]. The reaction relies on the in situ activation of tetrazoles, which rapidly release nitrogen to produce nitrile imines upon exposure to UV-light. Nitrile imines subsequently react with alkenes to form fluorescent pyrazoline cycloadducts. While nitrile imines may form adducts with water, reaction with the dipolarophilic alkene generally occurs at a much faster rate. The reaction has been used in live *E. Coli* cells containing protein-bound alkenes [49]. In this study, a tetrazole was added followed by UV irradiation for 4 min. The bacterial cells were then incubated overnight to allow the cycloaddition to proceed to completion. The next day, alkene-containing cells were visualized by fluorescent imaging. An advantage of the "photoclick" reaction is that nitrile-imines appear to be highly bioorthogonal and only slowly degrade in water. Yet, in the absence of alkene moieties, conjugation of tetrazoles to tryptophan residues is known to occur, depicting a serious drawback [50, 51]. Although more recently developed tetrazoles allow for longer wavelength activation, thereby minimizing the risk of phototoxicity, they have only been shown to react with non-terminal conjugated alkenes via an inverse electron-demand Diels-Alder reaction [52].

Conclusion

Several bioorthogonal reactions involve either alkenes or alkynes as reactions partners. Of these, the click reaction has found the most wide-spread application so far, in part due to its fast reaction kinetics and ease of use. Furthermore, the other reaction partner in the click reaction, an azide, can serve as a chemical reporter in its own right. This allows simultaneous incorporation of both an azide and a terminal alkyne in biomolecules, which gave me the idea to develop a double-click method to study the deposition of new histones on duplicated DNA.

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