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Click Chemistry in Protein Engineering, Design, Detection and Profiling

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13.1 Introduction

The definition of click chemistry as first posited by Kolb, Finn, and Sharpless[1] is a set of high-yielding, energetically favorable, highly modular organic transformations that can occur in benign solvents, particularly water. Though originally conceived as a novel philosophy for synthetic organic chemistry (specifically the construction of small molecule libraries), many of the characteristics of a click reaction are desirable in other branches of chemistry, as evidenced by the wide array of topics covered in this book. Specifically, high-yielding reactions that can be carried out in water, but also at physiological temperatures, are of much interest in biology. The Huisgen [3 + 2] cycloaddition between azides and alkynes[2] is mentioned by Kolb *et al*. as being 'as good as a reaction can get', but it typically requires temperatures well above what can be tolerated by cells and proteins, limiting its use in biological applications. All of this changed once it was discovered that Cu(I) could catalyze the azide–alkyne cycloaddition.[3,4] The copper-catalyzed version of the reaction works well at ambient temperatures (and at even lower temperatures), can be run in water and in common buffer systems for biological applications and can tolerate atmospheric oxygen. Taken together, these aspects render the copper-catalyzed azide–alkyne cycloaddition (CuAAC) nearly ideal for bioconjugation applications. CuAAC has subsequently been used for the functionalization of many classes of biomolecules including sugars,[5] nucleic acids[6] and proteins, the topic of this chapter.

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The array of molecules that can be conjugated to proteins using CuAAC is nearly limitless in large part because of the high selectivity and synthetic simplicity of adding azides or alkynes to a molecule of interest. A survey of the literature indicates that CuAAC has been used to decorate proteins with molecules including affinity handles such as biotin,[7] sugars,[8] DNA,[9] fluorophores,[10] polymers,[11] as well as more exotic molecules like gadolinium complexes useful in magnetic resonance imaging.[12] The other side of creating bioconjugates using CuAAC is the necessity to introduce the azide or alkyne moiety into the protein of interest. Neither the azide nor the alkyne is present in the canonical set of proteinogenic amino acids, which is simultaneously a blessing and a curse. On one hand, azides or alkynes must be grafted onto proteins in some fashion before bioconjugation via CuAAC can occur. On the other hand, if the azides or alkynes can be added in a controlled or site-specific fashion, there is the potential to create protein conjugates with a high degree of specificity. The specificity of CuAAC and its 'bioorthogonality' are what sets CuAAC apart from canonical bioconjugation techniques such as reacting lysines with activated esters or reacting thiols with maleimides.

In the first part of the chapter, the posttranslational addition of azides and alkynes to proteins will be discussed (Figure 13.1). Both chemical and enzymatic methods that add these functional groups to proteins will be detailed as well as the applications of the protein conjugates produced by these methods. The second segment of this chapter will focus on the cotranslational addition of azides and alkynes into proteins via the *in vivo* incorporation of unnatural amino acids (Figure 13.1). Finally, the last section of this chapter will detail the BONCAT technology, which uses CuAAC to label and analyze the newly synthesized pool of proteins of the cell in a time-resolved fashion.

13.2 Posttranslational Functionalization of Proteins with Azides and Alkynes

The first use of CuAAC for bioconjugation was carried out by Wang *et al*. to produce uniformly labeled cowpea mosaic virus (CPMV) particles.[13] The protein capsid of CPMV consists of 60 identical two-protein units forming a rigid, icosahedral, 30 nm particle that the Finn group has thoroughly explored as a functional nanomaterial.[14] Wild-type CPMV contains a single surface-exposed lysine residue per asymmetrical unit with exceptional reactivity toward electrophiles.[15] Alternatively, a genetically engineered version of CPMV with a single exposed cysteine residue (per asymmetric unit) has been designed and produced.[16] These particles present 60 reactive handles in the form of either amine or a thiol groups, the workhorses of traditional protein conjugation. Reactive linkers were added to the amines or thiols in a quantitative fashion resulting in particles labeled with 60 azides or alkynes. Finally, these functionalized particles were further reacted under CuAAC conditions with fluorescein derivatives functionalized with the appropriate reaction partner. The fluorophore-labeled particles were analyzed using quantitative chromatography. Complete labeling of all 60 reactive sites was achieved with azide-labeled particles after 16 h at 4 °C. A reaction with the opposite polarity (alkyne-labeled particles reacting with azide-labeled dye) was less efficient, but could still be driven to near completion by increasing the concentration of the copper catalyst.

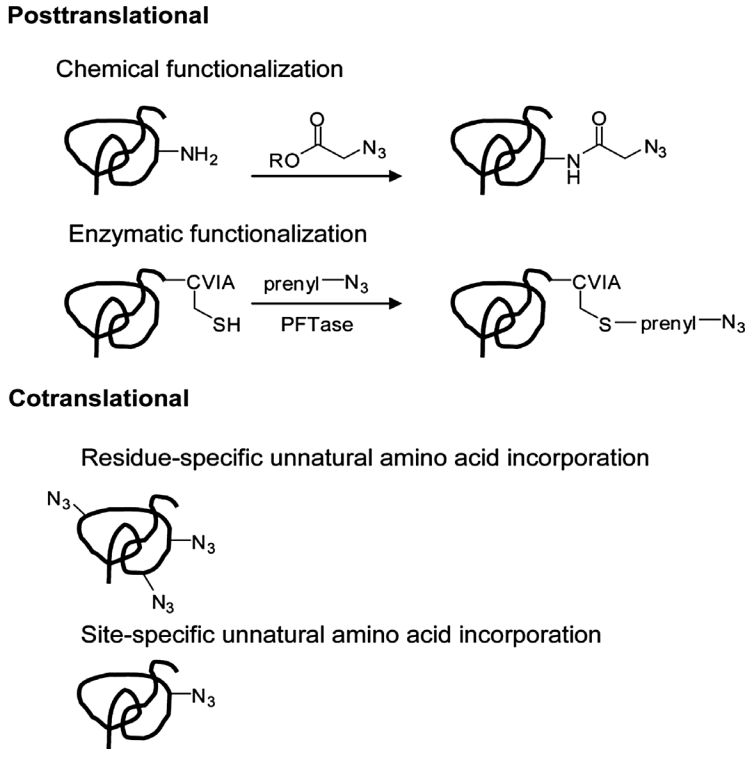

Figure 13.1 *A sampling of methods for the covalent introduction of azides and alkynes into proteins. Methods can be classified as either posttranslational or cotranslational. Examples are given for azide incorporation into proteins; the same methods can be used for introduction of alkynes. From top: reaction of azide-bearing activated ester with lysine residue, functionalization of the C-terminus of a protein via protein farnesyltransferase (PFTase), multiple site incorporation of azide-bearing unnatural amino acids and site-specific (single site) incorporation of azide-bearing unnatural amino acids.*

Although the output of these seminal experiments (fluorophore-labeled virus particles) could have been obtained more easily using traditional protein bioconjugation, this first example of protein bioconjugation using CuAAC is significant for several reasons. The authors claim that the kinetics of CuAAC bioconjugation are comparable to other bioconjugation reactions, such as the conjugation of thiols to maleimides, thus establishing CuAAC as a viable bioconjugation method. This report also demonstrated that the conditions required for CuAAC are gentle enough for the maintenance of tertiary (and even quaternary) protein structure. Lastly, this report introduced the *tris*-triazolylamine family of ligands. These ligands, particularly the benzyl derivative TBTA,[17] serve the dual purpose of both activating the Cu(I) ion for catalysis and protecting it from disproportionation. The TBTA ligand thus further improves the kinetics of CuAAC bioconjugations and also allows for long reaction times required in some applications. Its use has proved critical for the success of many subsequent applications of CuAAC for protein labeling.

Another seminal use of CuAAC in protein conjugation was carried out by Speers and Cravatt.[18] CuAAC was used to advance the field of activity-based protein profiling (ABPP). In ABPP, reactive substrate analogs are incubated with complex proteomic mixtures in order to covalently label enzymes at their active site.[19] The promise of ABPP is to rapidly assign functions to proteins, one of the main challenges in the postgenomic era. Traditional ABPP used probes in which the substrate analog was directly conjugated to a fluorescent dye or affinity handle such as biotin. The large size of the dye or biotin, however, was viewed as an impediment to ABPP since these molecules may sterically interfere with the insertion of the probe into the active site. To circumvent this limitation, the authors synthesized a phenyl sulfonate probe derivatized with an azide group, which consists of only three atoms and is much smaller than the dyes typically used in ABPP probes. The phenyl sulfonate probe is known to specifically label the active site of several enzymes including a glutathione-*S*-transferase (GSTO 1-1), aldehyde dehydrogenases (ALDH-1) and enoyl CoA hydratases (ECH-1). The probe was incubated with several complex proteomic mixtures including mammalian cell lysates and mouse tissue homogenates. Following this incubation, the entire protein mixture was subjected to CuAAC conditions with an alkyne-tagged rhodamine dye. Following separation of the proteins by electrophoresis, the labeled proteins were readily detected by fluorescence scanning of the gel. In addition to these *in vitro* demonstrations of CuAAC-enabled ABPP, the authors also injected the azide-labeled phenyl sulfonate probe into live mice to test whether CuAAC-enabled ABPP could function *in vivo*. Remarkably, following treatment of homogenates of the heart tissue from these mice with CuAAC conditions and the rhodamine alkyne dye, labeled ECH-1 protein was readily detected via gel electrophoresis and fluorescence scanning. These results underscore the 'silence' of the azide group toward biomolecules since the probe was able to survive the metabolism of a live animal and find its protein target. Moreover, the probe demonstrated no apparent toxicity for the animal. This work also demonstrates the incredible tolerance of the CuAAC reaction. The reactions were successful despite being carried out in a cellular milieu composed of thousands of different molecules. In a subsequent report,[20] the same authors present several important refinements to CuAAC-enabled ABPP including the interesting observation that switching the polarity of the reaction such that the probe carries the alkyne group and the dye carries the azide leads to lower levels of background labeling in proteomic samples. In this report, the importance of being able to perform ABPP *in vivo* is also underscored as the authors report several proteins that are labeled by the probe in intact cells but not in cell lysates.

Both of the examples discussed so far have relied on chemical derivatization of the protein before carrying out bioconjugation via CuAAC. More recently, there have been several reports of enzymatic or semisynthetic transformations of proteins in order to introduce the requisite azide or alkyne moiety. Kalia and Raines carried out a study[21] in which a variation on the expressed protein ligation[22] protocol was used to install the azide functionality specifically to the C-terminus of RNAse A (Figure 13.2). Expressed protein ligation relies on the formation of an electrophilic thioester during the *N*–*S* acyl shift of the intein protein splicing reaction. The authors evaluated a selection of nucleophiles for their ability to attack a model thioester, and selected the hydrazine moiety for further study. An RNAse A-intein-chitin binding domain tripartite fusion was expressed in *E. coli* and immobilized on chitin beads. Addition of a bifunctional small molecule containing both the azide and hydrazine moieties to the beads effected the cleavage of RNAse A and the addition of

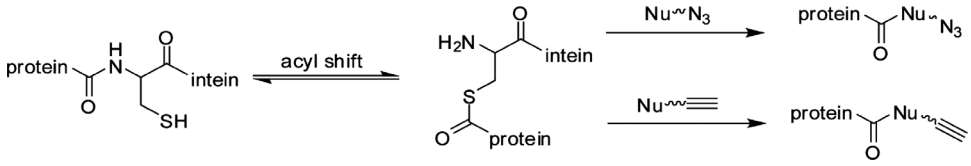

Figure 13.2 *Introduction of azide or alkyne functionality to the C-terminus of a protein via intein-mediated thioester formation. An electrophilic thioester is formed by an N–S acyl shift. The thioester can be attacked by an azide- or alkyne-modified nucleophile, resulting in a single, amide-linked modification on the C-terminus of the protein.*

the azide group to its C-terminus in a single step. The authors report a yield of about 1 mg/l of bacterial culture of the azide-labeled protein, which is exceptional considering the simplicity of the protocol. Lastly, the authors used CuAAC to covalently label azido-RNAse A with alkyne-modified fluorescein. Gel electrophoresis and fluorescence scanning as well as mass spectrometry confirmed that the protein was specifically labeled.

A similar expressed protein ligation strategy was used by Lin *et al*. to label *E. coli* maltose-binding protein (MBP) and green fluorescent protein (GFP) at their C-termini.[23] In contrast to Kalia and Raines, the authors used a nucleophile consisting of either the azide or alkyne linked to the carboxyl group of the amino acid cysteine via an amide bond. Several different azide-labeled small molecules, including fluorescein, biotin, glucosamine, a glycopeptide and a diazide linker, were conjugated to the alkyne-modified MBP using CuAAC. Notably, the authors claim that CuAAC carried out at 4 °C without the TBTA ligand led to poor product yields in these reactions. However, the addition of TBTA to the reaction and raising the temperature to 25 °C led to nearly quantitative modification of the protein in only 6 h. Lin *et al*. also used CuAAC to attach azide- and alkyne-modified proteins to glass surfaces in order to test the potential of CuAAC in the fabrication of protein microarrays. GFP labeled with either an azide or an alkyne was readily attached to surfaces decorated with the appropriate reaction partner, although significantly more protein was attached to the surface when alkynyl-GFP was reacted with an azido surface. Alkynyl-MBP was also covalently linked to the azido glass surface, and binding experiments with biotinyl-maltose indicated that the surface-bound MBP retained activity. The importance of being able to include a single, C-terminal anchoring moiety was underscored in experiments comparing the CuAAC-based protocol to traditional surface anchoring in which proteins are randomly linked to the substrate via surface accessible lysines or arginines. The CuAAC-based surface attachment of MBP led to significantly more active protein (as measured by binding of biotinyl-maltose) on the surface when compared with the conventional random-amide formation method.

Two groups have described an alternative way to specifically introduce an azide or alkyne group near the C-terminus of a protein via posttranslational prenylation catalyzed by the protein farnesyl transferase (PFTase).[24,25] The natural function of PFTase is to add the farnesyl moiety to cysteine residues within a four amino acid motif, CaaX (a = aliphatic amino acid, X = Ala, Ser, Met, Asn), found at the C-terminus of a protein. The yeast PFTase accepts azide and alkyne analogs of farnesyl diphosphate allowing for the facile modification of recombinant proteins with the CaaX motif at the C-terminus. Gauchet *et al*. immobilized alkyne-farnesylated GFP and glutathione reductase (GST) on an azide-modified

glass surface using CuAAC.[25] Duckworth *et al*. immobilized GFP modified with an azidofarnesyl analog on agarose beads that had been modified with alkyne groups. Notably, these authors point out that the endogenous proteome (the entity of all proteins in a cell) of *E. coli* lacks any proteins with the C-terminal CaaX motif, allowing the farnesylation reaction to be carried out in a specific fashion on total cell lysates. The orthogonality of the farnesylation step toward the proteome of *E. coli* coupled with the orthogonality and robustness of the CuAAC chemistry leads to a potent combination that should enable advances in the construction of protein microarrays.

The examples presented in this section demonstrate the diversity of methods to introduce the azide and alkyne moieties into proteins as well as the plethora of applications possible with these modified proteins. All of these approaches, however, rely upon some posttranslational modification of the protein by either chemical or enzymatic means. This 'extra step' in the conjugation of proteins via CuAAC is critical in certain applications, such as ABPP, but its elimination could speed up bioconjugation protocols and place the azide and alkyne on equal footing with amines and thiols, the workhorses of traditional bioconjugation. Research in the past two decades into the incorporation of unnatural amino acids into proteins has facilitated the direct, cotranslational incorporation of azides and alkynes (as well as a host of other functional groups). These technologies and their applications will be discussed in the next section of this chapter.

13.3 Cotranslational Functionalization of Proteins with Azides and Alkynes

The language of proteins is the set of 20 canonical amino acids, and a veritable encyclopedia of protein structures and functions have been assembled by Nature using this language. However, curious researchers have not been satisfied with Nature's language for proteins, and have sought to add new words to it in the form of unnatural amino acids. Efforts to add new amino acids to proteins can be classified into two distinct classes. In residue-specific unnatural amino acid incorporation, the codon(s) for one of the 20 natural amino acids is reassigned to an unnatural amino acid.[26] Residue-specific incorporation is also referred to as selective pressure incorporation by Budisa and colleagues.[27] The second class of unnatural amino acid incorporation experiments is referred to as site-specific incorporation in which a twenty-first amino acid is added to the set of proteinogenic amino acids. Site-specific unnatural amino acid incorporation is also a form of codon reassignment, but instead of reassigning sense codons to the unnatural amino acid, one of the three nonsense codons is reclaimed and used to code for a twenty-first amino acid.[28,29]

The residue-specific incorporation technique has been used for more than 50 years[30] and relies on the use of auxotrophic strains of *E. coli* along with a chemically defined growth medium. Site-specific incorporation was first demonstrated as an *in vitro* protein expression technique in which the unnatural amino acid was chemically ligated to a tRNA that could decode the amber nonsense codon.[28] Site-specific incorporation of an unnatural amino acid *in vivo* was first demonstrated by Furter in 1998.[29] The key discovery in this work was that an orthogonal aminoacyl-tRNA synthetase (aaRS)–tRNA pair is required for the incorporation of the twenty-first amino acid. Furter introduced a yeast phenylalanine

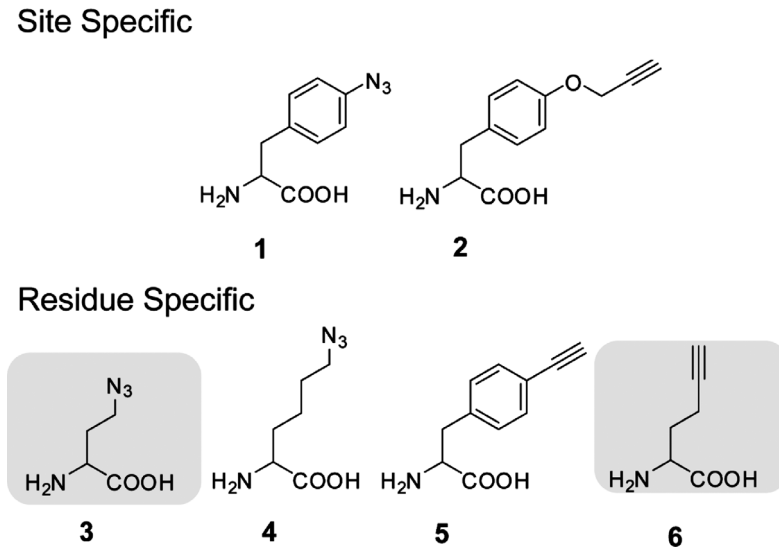

Figure 13.3 *Azido and alkynyl unnatural amino acids that can be incorporated into proteins in vivo.* ***1****,* p-*azidophenylalanine;* ***2****,* O-*propargyltyrosine;* ***3****, azidohomoalanine;* ***4****, homopropargylglycine;* ***5****,* p-*ethynylphenylalanine;* ***6****, azidonorleucine.* ***1*** *and* ***2*** *have been incorporated in a site-specific fashion while* ***3–6*** *have been incorporated in a residue-specific fashion;* ***3*** *and* ***6*** *(boxed) are substrates for the wild-type aminoacyl-tRNA synthetase activity of the cell while* ***1****,* ***2****,* ***4*** *and* ***5*** *require engineered aminoacyl-tRNA synthetase activities.*

tRNA ($tRNA^{Phe}$) with an altered anticodon and the yeast phenylalanyl-tRNA synthetase (PheRS) into *E. coli* cells. The addition of these two components to the cells led to efficient incorporation of fluorophenylalanine in response to a single amber nonsense codon within the test protein, dihydrofolate reductase. Subsequently, Schultz and colleagues have greatly expanded upon this methodology by generating high-throughput screening algorithms for the directed evolution of both the orthogonal tRNA and aaRS.[31] These advances have made possible the site-specific incorporation of dozens of different unnatural amino acids into proteins expressed in *E. coli* and in other organisms.[32] Both residue- and site-specific incorporation techniques have been used to deliver the azide and alkyne functionalities to proteins (Figure 13.3), enabling a host of different applications.

As mentioned above, one of the advantages of directly incorporating an azido- or alkynyl-amino acid into a protein is that the protein emerges from the cell ready for bioconjugation. No extra posttranslational modification steps are necessary. Deiters *et al*. used site-specific incorporation to introduce the amino acids *p*-azidophenylalanine (**1**, Figure 13.3) and *O*-propargyltyrosine (**2**, Figure 13.3) into human superoxide dismutase (SOD) expressed in the yeast *S. cerevisiae*.[10] These modified proteins were reacted with azide- or alkyne-modified dansyl or fluorescein dyes and imaged by fluorescence scanning after gel electrophoresis. The same authors demonstrated site-specific modification of human SOD in which *p*-azidophenylalanine had been incorporated with an alkyne-poly(ethylene glycol)

(PEG) reagent.[33] It is noteworthy that *p*-azidophenylalanine can also be incorporated into proteins in a residue-specific fashion using an active-site variant of the *E. coli* phenylalanyl-tRNA synthetase.[34]

Schoffelen *et al*. used residue-specific incorporation of the amino acid azidohomoalanine (AHA, **3**, Figure 13.3) in place of methionine to produce an azide-modified lipase from *Candida antarctica*.[11] AHA has been demonstrated to be an excellent surrogate for methionine in protein synthesis, and near-quantitative replacement of the five methionine residues of the lipase with AHA was readily achieved. Subsequent modification of this enzyme via CuAAC with either an alkyne-dansyl dye or an alkyne-PEG led to protein with only a single modification. Mass spectrometric analysis confirmed that this modification occurred only at the *N*-terminus of the protein despite the fact that there are five methionine residues in the protein. The rationale for this selective modification is that the *N*-terminal methionine is the only methionine residue exposed to the solvent; the other residues are buried in the core of the protein. Furthermore, the modified enzyme retains a significant fraction of its wild-type activity. It is important to point out that the TBTA ligand described earlier was not used in these studies. A sulfonated bathophenanthroline ligand with significantly improved water solubility over TBTA[35] was used instead. Though both site-specific and residue-specific unnatural amino acid incorporation techniques can be used to produce proteins for bioconjugation via CuAAC, protein production via residue-specific incorporation is simpler and does not suffer from the protein yield limitations sometimes observed with site-specific incorporation.[31] The relatively low abundance of methionine in the proteome coupled with its hydrophobicity that often confines it to the core of proteins makes residue-specific incorporation of AHA in place of methionine a promising avenue for 'pseudo-site-specific' modification of proteins via CuAAC.

The ability of CuAAC to tolerate complex biological milieu has been discussed above. In another example of the tolerance of CuAAC to biological conditions, Link and Tirrell set out to use CuAAC to address azide-labeled proteins in their native cellular context.[36,37] As a model system, AHA was incorporated into a variant of the *E. coli* outer membrane OmpC. The modified protein was properly targeted to the outer membrane, and subsequent reaction of the whole cells with a biotin–alkyne reagent under gentle CuAAC conditions led to extensive and specific functionalization of the cell surface with biotin as determined by western blotting experiments on outer membrane fraction of the cells. The biotinylated cells were also readily differentiable from unlabeled cells when stained with fluorescent streptavidin and subjected to flow cytometry. These experiments again demonstrate the versatility of the CuAAC chemistry; the CuAAC reaction is unhindered by the complex environment of the bacterial outer membrane, which contains lipids, proteins, polysaccharides and other molecules. These experiments formed the basis for further investigations by Link *et al*. in which the cell surface display of unnatural amino acids was exploited in a high-throughput screening method for the identification of novel methionyl-tRNA synthetase (MetRS) activity.[38] A saturation mutagenesis library of MetRS was screened for the ability to incorporate the long-chain amino acid azidonorleucine (**4**, Figure 13.3) using a flow cytometric screen, and a variant of MetRS that can activate azidonorleucine efficiently was discovered. In this screening protocol, the copper-catalyzed reaction was eschewed in favor of a cyclooctyne reagent developed in the Bertozzi laboratory[39] because of the observation of toxicity of the copper catalyst toward *E. coli*. The increased sensitivity of the cells to copper in this case may be attributed to perturbations of the outer membrane

of the cells via overexpression of the OmpC protein since wild-type *E. coli* can typically tolerate up to 10 mM concentrations of copper ions. Beatty *et al.* also demonstrated the tolerance of the CuAAC chemistry to a complex cellular environment in experiments in which the unnatural amino acid *p*-ethynylphenylalanine (**5**, Figure 13.3) was incorporated into the recombinant cytosolic protein barstar.[40] Cells expressing the substituted barstar were treated with a fluorogenic azidocoumarin dye[41] and were rendered fluorescent after treatment with CuAAC reagents. Similar experiments were carried out in fixed mammalian cells in which the methionine surrogate homopropargylglycine (HPG, **6**, Figure 13.3) was incorporated throughout the proteome.[42]

The Finn group has also used the residue-specific incorporation of the methionine analogs AHA and HPG in their studies of viral capsids as functional nanomaterials. Prasuhn *et al.* incorporated AHA into genetically engineered mutants of the coat protein of bacteriophage Qb such that the azide groups from AHA are only displayed on the interior of the assembled particle.[12] CuAAC was used to covalently ligate a gadolinium complex to the assembled particles, which were used in a study of the effect of surface charge on the plasma clearance of the particles from mice. The same group also incorporated AHA into a surface-exposed position of the Qb particle and could address 90% (over 300 moieties per particle) of the displayed azide moieties with a fluorescein–alkyne reagent via CuAAC.[43] Despite this heavy functionalization, the recovery of intact particles was efficient, again underscoring the mild nature of the CuAAC reaction conditions.

A particularly elegant use of residue-specific incorporation of AHA followed by bioconjugation via CuAAC was described in a recent paper by van Kasteren *et al.*[8] The authors were interested in being able to mimic multiple posttranslational modifications in a site-specific fashion within a protein. To achieve this goal, they generated a mutant version of the SSβG protein, an enzyme with LacZ-type galactosidase activity, in which all of the methionine residues except one were replaced with the nearly isosteric isoleucine. Additionally, one of the cysteine residues found naturally in the protein was mutated to serine creating a protein with a single methionine and a single cysteine. Despite all of the mutations, the mutant SSβG retained its enzymatic activity. The protein was expressed under conditions that led to near-quantitative replacement of methionine with AHA, resulting in a protein with two orthogonal site-specific handles for chemical modification. As a first test, the authors installed glucose at the cysteine thiol via a disulfide-forming reaction and galactose at the azide sidechain of AHA via CuAAC. Both modifications proceeded nearly quantitatively and under gentle conditions. Notably, optimized CuAAC conditions were employed which eliminated the reducing agents used in many applications of CuAAC. These reducing agents, such as ascorbic acid or tris(carboxyethyl) phosphine (TCEP), are incompatible with the thiol chemistry. The authors also generated SSβG with a mimic of tyrosine sulfonation at the cysteine moiety and either a specific trisaccharide or tetrasaccharide at the AHA moiety. The doubly modified protein was designed to be a mimic of the human protein P-selectin-glycoprotein-ligand-1 (PSGL-1). The chemically modified protein was competent in binding to human selectin, and it was demonstrated that both modifications were necessary for optimal binding. Most impressively, the doubly modified SSβG could be used *in vivo* as a sensor of either acute or chronic inflammation in rat cortex. In a similar application, the protein could be used to detect a malarial infection in a mouse model. These studies demonstrate the power of CuAAC-based bioconjugation when used in conjunction with conventional cysteine-based bioconjugation. The techniques described

should be generally useful for biologists interested in mimicking multiple posttranslational modifications to proteins. This is an application for which traditional bioconjugation to lysines and cysteines simply would not work because of the abundance (and functional importance) of lysine residues in proteins. The relatively low abundance of methionine in the proteome along with some clever genetic engineering allowed once again for the 'pseudo-site-specific' incorporation of the azide group into a protein.

13.4 BONCAT: Identification of Newly Synthesized Proteins via Noncanonical Amino Acid Tagging

In the last section of this chapter we want to discuss the application of CuAAC to proteomic profiling of cultured cells and tissues. On the molecular level proteins drive all major cellular functions. Cells, tissues, and living organisms all are dynamic entities responding to perturbations in their environment by changing the set of proteins the proteome they express either through posttranslational modifications of existing proteins or via adjustments in protein synthesis and degradation. For example, the composition of protein complexes and networks, such as the NMDA glutamate receptor complex or signaling cascades, can be regulated by the addition of *de novo* synthesized proteins or removal of existing proteins through changes in the activity pattern. Disruptions or imbalances in cellular protein homeostasis cause severe disorders of which the various types of cancers or fragile X mental retardation are just a few prominent examples. Therefore, a tremendous challenge for researchers and health professionals alike is the comparison of two or more proteomes, for example the cancerous vs noncancerous state, to eventually pinpoint and isolate the exact cellular malfunction.

Currently, various forms of mass spectrometry (MS) based proteomic profiling tools are the prime candidate approaches to characterize expression and functional modification profiles of proteins. Modern proteomics, however, faces several major challenges: first, proteins display an undisputed heterogeneity and cannot be amplified like their genomic counterparts, aggravating their identification in complex mixtures. Second, in-depth identification of a cell's entire proteome, let alone the comparison to another proteome, is unarguably a difficult feat with an estimated number of approximately 10 000 different proteins in a single mammalian cell.[44] Third, copy numbers of proteins from different mammalian cells and tissues vary with a predicted dynamic range of up to six orders of magnitude, and this number is even several orders of magnitude larger in plasma samples. Compared with subfemtomolar sensitivity in the analysis of a single purified protein, the effective identification of low-abundance proteins in complex mixtures is several orders of magnitude lower due to limited dynamic range and sequencing speed of current mass spectrometry instruments.[45] Hence, no single proteome of a mammalian cell or lower eukaryotic microorganism, such as yeast, has been completely characterized so far. A recent proteomic profiling study identified 5111 proteins in murine embryonic stem cells.[46]

How can these obstacles be mastered? How can one achieve in-depth identification and capture temporal and spatial proteome dynamics associated with changes in a cell's activation pattern, or its developmental stage if not all proteins – high or low in abundance – can be identified with equal chances and accuracy?

Biochemical and analytical approaches to reduce proteome complexity and to increase the dynamic range of protein identification use fractionation and affinity-purification tools on protein and peptide levels prior to MS analysis. In particular, fractionation methods of whole organelles (mitochondria[47] and nucleolus[48]) and compartments, such as the postsynaptic density of neurons[49,50] as well as affinity-purified protein complexes,[49,51,52] have been successfully used to enhance in-depth proteomic analysis.

However, not only qualitative knowledge but also quantitative knowledge of a proteome is important to understand a cell's activation state and overall phenotype. Therefore, extensive efforts have been dedicated to the development of differential proteomic profiling approaches to compare proteomes with one another and to obtain relative quantification of individual proteins among samples. These methods include differential 2D gel electrophoresis[53,54] (DIGE), isotope-coded affinity tags[55] (ICAT) or isobaric tags for relative and absolute quantification[56] (iTRAQ), quantitative proteomic analysis using samples from cells grown in ^{14}N- or ^{15}N-media[57] and stable isotope labeling by amino acids in cell culture[48,58] (SILAC).

Furthermore, the combination of MS with affinity purification for different posttranslational modifications[59–63] decreases sample complexity by enrichment of a specific subpopulation of the proteome. While posttranslational modifications such as phosphorylation or ubiquitination readily provide a suitable handle for enrichment of the 'phosphoproteome' or for proteins destined for degradation, reducing sample complexity by selectively enriching for newly synthesized proteins is troublesome, since all proteins – old and new – share the same pool of 20 amino acids. Nonetheless, the specific enrichment and identification of recently synthesized proteins would complement the range of differential proteomic profiling methods already available, and add another level of separation and simplification of complex protein mixtures, deepening our insights into the spatial and temporal dynamics of proteomes.

To provide the proteomics community with this added feature of selecting for newly synthesized proteins, the BONCAT (bio-orthogonal noncanonical amino acid tagging) technology was developed. The core of the BONCAT technique capitalizes on the manifold potential of small bioorthogonal chemo-selective groups (for a review see Prescher and Bertozzi[64]). In the first step of BONCAT, newly synthesized proteins are labeled using the azide-bearing unnatural amino acid azidohomoalanine (AHA, **3**, Figure 13.3), endowing them with novel azide functionality, which distinguishes them from the pool of pre-existing proteins (Figure 13.4). Employing CuAAC, the reactive azide group of AHA is covalently coupled to an alkyne-bearing affinity-tag in the second step. This tag enables the subsequent detection, affinity purification and MS identification of AHA-labeled proteins. The enrichment for newly synthesized proteins decreases the complexity of the sample, fostering the identification of proteins expressed at low levels.

Although Dieterich *et al*. used an alkyne-biotin-FLAG tag in the original application of BONCAT,[65,66] researchers may wish to substitute the biotin and the FLAG epitope for other affinity moieties. While the biotin moiety is used for avidin-based affinity purification, the FLAG epitope provides sites for trypsin cleavage, allowing immediate proteolysis of proteins on the affinity resin, bypassing the need for a separate elution step. Furthermore, the FLAG epitope can be used as an alternative purification module if native biotinylation of proteins is a concern. In this case affinity-purified AHA-tagged proteins can be eluted using high-salt conditions or competition with the FLAG-peptide. The increasing number

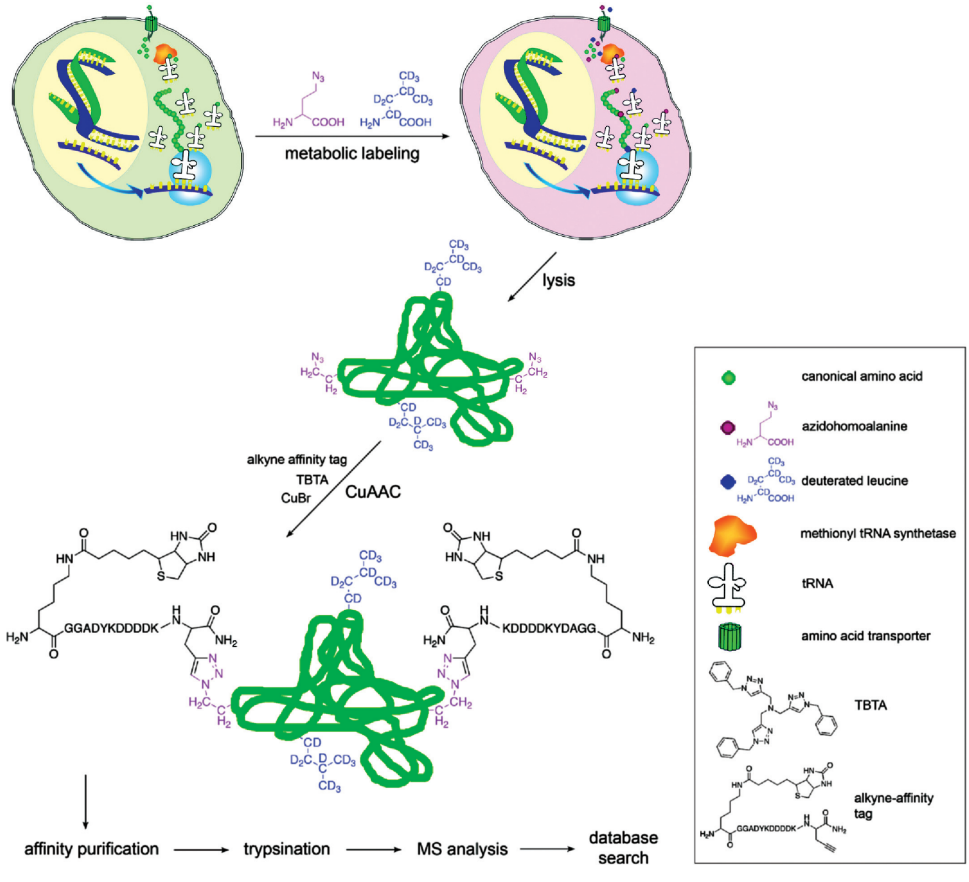

Figure 13.4 *A schematic depiction of the BONCAT approach. Metabolic cotranslational labeling with azidohomoalanine and an isotopically heavy amino acid (shown here is deuterated leucine) confers bioorthogonal functionalization to newly synthesized proteins. After incubation, cells are directly lysed or, alternatively, a subcellular fractionation for biochemical enrichment of specific cellular compartments is performed prior to lysis. Lysates are coupled to an alkyne-bearing affinity tag, followed by affinity purification. Purified proteins are digested with a protease, most commonly trypsin, and the resulting peptides are analyzed by mass spectrometry to obtain experimental spectra. Different search algorithms are used to match the acquired spectra to protein sequences.*

of new functional linkers, such as photocleavable or acid-labile groups, promises to add to the versatility and specificity of affinity tags for BONCAT applications.

After tryptic digestion of avidin-bound or eluted proteins, peptides bearing the tryptic remains of tagged AHA can serve as an immediate validation of candidate proteins. In the event of failed tagging, i.e. unligated AHA, the mass loss of AHA over methionine marks this peptide as derived from a true newly synthesized candidate protein. To increase the chances of detecting metabolically modified peptides co-labeling cells with deuterated

L-leucine (d_{10}L) or any other isotopically heavy amino acid allows, in conjunction with the modification derived from the introduction of AHA, the validation of candidate proteins.

As described in the previous section of this chapter, AHA is an effective surrogate for methionine, and does not require any cellular manipulations to be accepted as a substrate by the methionyl-tRNA synthetase.[7,67] Methionine is an essential amino acid in mammals, and, therefore, the prerequisite for effective depletion and, thus, increased incorporation rates for AHA into newly synthesized proteins are favorable. However, identification of newly synthesized proteins with BONCAT is limited to proteins that possess at least one methionine residue, excluding the 1.02% of all entries in a human protein database, which do not contain a single methionine. Given that 5.08% of the human proteome possess only a single, *N*-terminal methionine and that this residue may be subject to removal by posttranslational processing, at least 94% of the mammalian proteome are candidates for identification by BONCAT.[65] Interestingly, we have found no bias toward methionine-rich proteins in the proteomes characterized so far.

In general, labeling with AHA is very similar to the traditional metabolic labeling with radioactive amino acids (^{35}S-labeled methionine or cysteine) and can be performed in any biology or chemistry laboratory. BONCAT has been tested by us in a variety of cell lines, primary neuronal cells as well as organotypic brain slice cultures (unpublished observations). We found in all systems tested that the presence and incorporation of AHA is nontoxic and does not affect global rates of protein synthesis or degradation. CuAAC can be performed on denatured proteins in the presence of detergents, such as SDS, promoting the identification of diverse classes of proteins, i.e. membranous and soluble, acidic and basic as well as high and low molecular weight proteins. Indeed, the identified proteins display a broad range of functional and biochemical diversity[65] in terms of size or isoelectric point. Since the coupling reaction withstands harsh buffer conditions, even membrane proteins can be tagged and identified. Finally, AHA-tagged newly synthesized proteins show undisturbed subcellular distribution, for example tagged histone proteins could be identified from and found in the nuclear fraction.

Lastly, the BONCAT principle is not restricted to MS-based proteomic profiling, but the spatial fate of newly synthesized proteins can directly be visualized by using fluorescent CuAAC tags as Beatty and coworkers have demonstrated.[42] It also promises the possibility to work in combination with other proteomic approaches to directly compare different proteomes in a single MS experiment or to facilitate the identification of even more specific subpopulations of the proteome. Moreover, subcellular fractionation and immunopurification of protein complexes can be followed by BONCAT to assess the temporal and spatial dynamics of certain subcellular compartments, organelles and protein–protein interaction networks.

13.5 Conclusions and Future Prospects

As evidenced by the breadth of the examples provided in this chapter, the preeminent click chemistry reaction, CuAAC, has done much to advance the fields of protein conjugation, protein engineering and even proteomics. Protein engineers now have a viable alternative to traditional cysteine- and lysine-based bioconjugation strategies that can function in even the most complex biological environments. The relative synthetic ease of introducing the azide and alkyne moieties into small molecules should ensure the continued growth of CuAAC as a bioconjugation method by researchers across many fields of biology.

The use of a copper catalyst is a potential limitation of CuAAC in some biological applications. If CuAAC is being performed on live cells, the concentration of copper needed to effect CuAAC may be toxic to the cells. Similarly, if a protein bioconjugate is being produced for therapeutic use, the removal of all traces of copper is critical. To address these needs, Bertozzi and others[39,68,69] have developed copper-free versions of the azide–alkyne ligation in which the alkyne is activated by ring strain. These conjugation strategies, which are discussed in detail in Chapter 3, promise to enable even more applications in bioconjugation and proteomics.

What is the future of click chemistry in protein engineering? One can imagine that new click reactions beyond CuAAC will come to the forefront and may be used either as a replacement for or in conjunction with CuAAC. In the more immediate future, protein engineers should take advantage of the fact that CuAAC transcends any single discipline, percolating freely through biological chemistry, organic chemistry and materials science. Many of the future uses of CuAAC in protein science may come in interfacing proteins with materials, both soft and hard. Such work is already underway, as evidenced by the use of CuAAC to construct a protein microarray described earlier in this chapter.[23] One could also envision covalently interfacing redox active proteins with electronic materials via CuAAC for sensing applications. On the soft materials front, CuAAC is an ideal chemistry for the attachment of targeting proteins to liposomes or polymeric nanoparticles currently being explored for drug delivery applications. The simplicity and reliability of CuAAC has led to a plethora of uses in protein science described herein in a period of just over five years. These same aspects ensure that CuAAC will continue to be a versatile, well-used tool for protein engineers (and biologists in general) over the next five years and beyond.

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