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Residue-specific incorporation of non□canonical amino acids into proteins: recent developments and applications

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Abstract

Residue-specific incorporation of non-canonical amino acids into proteins allows facile alteration and enhancement of protein properties. In this review we describe recent technical developments and applications of residue-specific incorporation to problems ranging from elucidation of biochemical mechanisms to engineering of protein-based biomaterials. We hope to inform the reader of the ease and broad utility of residue-specific non-canonical amino acid incorporation with the goal of inspiring investigators outside the field to consider applying this tool to their own research.

Introduction

Evolutionary processes have selected a strikingly small set of amino acids to generate natural proteins. These amino acids must achieve a delicate balance: their side chains must be stable under biological conditions, yet reactive when needed for selective catalysis. They must engage in molecular interactions (hydrogen bonds, disulfide bonds, electrostatic and cation-pi interactions, etc.) of sufficient diversity to generate well-defined, three-dimensional structures and intricate molecular complexes. But there are many things that the natural amino acids cannot do, and their limitations provide important opportunities for the chemist and chemical biologist.

Cohen and coworkers set the stage for work on non-canonical amino acids (ncAAs), when they showed in the 1950s that selenomethionine (Se-Met, **1**, Figure 1) could be quantitatively incorporated into bacterial proteins in place of methionine [1]. Many years later, through the work of Hendrickson and coworkers, this simple transformation revolutionized protein X-ray crystallography [2]. Protein chemists and engineers have now developed methods for incorporating hundreds of ncAAs into proteins in either residue-specific or site-specific fashion. These methods have been reviewed recently [3–9]. Here we focus on recent examples of the use of residue-specific methods to effect selective protein labeling with the goal of answering important biological questions or generating proteins with novel properties.

Residue-specific incorporation vs. site-specific incorporation

Residue-specific and site-specific methods for incorporating ncAAs into proteins are in many ways complementary to one another. Site-specific methods are ideal for introducing point mutations into proteins with minimal perturbation of structure; the utility of such methods for elucidating details of protein structure and function is unrivaled [10••,11]. On the other hand, genetic manipulation of the target sequence is required (unknown protein targets cannot be labeled) and incorporation of more than one ncAA (or more than one copy of a single ncAA) is difficult [12]. Opportunities to change global protein properties are therefore limited at present. Methodological advances may soon overcome this limitation [13].

Residue-specific methods allow partial to quantitative replacement of canonical amino acids by their non-canonical analogues (Figure 1). Since ncAAs are incorporated at multiple sites, the resulting protein may display substantially altered physical and chemical properties as compared to natural proteins. Depending on what the investigator wants to do, such changes may be the point of the experiment or an unacceptable complication. The method typically involves exchange of a natural amino acid with the ncAA of interest in the growth medium; the use of amino acid auxotrophs as expression hosts is generally required for high-level replacement of the natural amino acid in the target protein. Residue-specific methods are ideal when one wishes to generate globally modified proteins with novel chemical and physical properties. No genetic manipulation is required and all cellular proteins can be modified (e.g., in proteomic studies as described below).

Time-resolved and cell-selective methods for analysis of protein synthesis

Residue-specific incorporation of ncAAs is operationally similar to conventional pulse-labeling with radioactive amino acids, without the complications associated with the handling of radioisotopes. Given a robust technique for selective, post-translational modification of the non-canonical side chain, residue-specific incorporation of ncAAs allows global analysis of protein synthesis, localization, and degradation, even when the proteins of interest are not yet identified. As a result, considerable effort has been focused on the development of bio-orthogonal labeling strategies for proteins containing ncAAs. Many recent examples of residue-specific incorporation use ncAAs that carry reactive functional groups such as azides, alkynes, olefins and ketones for selective protein labeling. Among the most widely used reactive ncAAs are azidohomoalanine (Aha, **2**) and homopropargylglycine (Hpg, **3**) both of which are susceptible to selective modification via the 3+2 azide-alkyne cycloaddition (“click chemistry”). Dieterich and coworkers used Aha to develop a method, “bio-orthogonal non-canonical amino acid tagging“ (BONCAT, Figure 2), to enrich and identify newly synthesized cellular proteins [14••]. In the BONCAT method, cells or tissues are pulse-labeled with a reactive ncAA that can be selectively ligated to an affinity tag for subsequent enrichment and identification. In a promising alternative approach, the de Koster and Bertozzi groups have recently developed a strain-promoted azide-cyclooctyne on-bead methodology for direct enrichment of Aha-labeled proteins prior to MS analysis [15]. This work may enable more rapid identification of azide-modified proteins from complex mixtures. Newly synthesized proteins have also been selectively labeled with reactive dyes and visualized by fluorescence microscopy. Beatty *et. al* demonstrated this approach in bacterial and mammalian[16] cells and more recently labeled two temporally-defined protein populations in mammalian cells using both Aha and Hpg [17]. Recently, Schuman and coworkers introduced the term FUNCAT (fluorescent non-canonical amino acid tagging) to describe this technology [18].

Applications of time-resolved residue-specific protein labeling

The power of the BONCAT/FUNCAT methodologies lies in their potential for quantifying proteome-wide responses to different stimuli; the resulting information illuminates fundamental biochemical processes and may provide new insight into the nature of disease. A few recent examples are highlighted here. Flanagan *et. al* used residue-specific incorporation of Aha followed by fluorescence labeling with an alkyne-dye to visualize newly synthesized proteins in neuronal axons and dendrites [19••. The authors showed that DCC, a transmembrane receptor that regulates axon growth and guidance, accumulates in the same regions as newly synthesized proteins and regulates translation in response to external stimuli. Hang and coworkers developed a tandem labeling and fluorescence imaging method for monitoring dynamic S-palmitoylation of proteins. They used a model S-acylated protein, H-Ras^{G12V}, to correlate protein synthesis with reversible palmitate cycling and determined a palmitate half-life of ~50 min [20••]. This value agreed with literature precedent derived from radiolabeling studies; S-acylation dynamics are often vastly different in diseased cells compared to healthy cells and the authors suggest that their method could be utilized to measure the rate of S-acylation in any protein of interest without the need for radioactive probes. Schuman and coworkers recently employed Aha and Hpg to visualize newly synthesized proteins in rat neurons [18]. The authors appended quantum dots (QDs) to Aha-labeled proteins in living cells and monitored QD diffusion to gain insight into protein localization dynamics.

Expanding the scope of residue-specific incorporation: development of novel aminoacyl-tRNA synthetases (aaRSs)

When the wild-type translation apparatus does not support incorporation of ncAAs, alteration of the biosynthetic machinery is required. Sometimes, simply over-expressing the wild-type aaRS is sufficient [21]. Typically, however, mutation of either the aaRS editing domain [22] or the amino acid binding pocket [23] is required. Link and coworkers developed a rapid flow cytometry-based screen for the discovery of new aaRS activities using cell surface display of outer membrane protein C (OmpC) containing reactive ncAAs [24]. The authors found three MetRS mutants, all sharing the L13G mutation, that enabled near quantitative incorporation of the long-chain methionine analogue, azidonorleucine (Anl, **4**) into cellular proteins. The single-site L13G mutant (MetRS*) efficiently incorporated Anl in the absence of Met, but was less effective when equimolar mixtures of Met and Anl were used. Tanrikulu and coworkers conducted a more thorough screen of aaRS mutants and identified MetRS variants that prefer Anl to Met [23]. One mutant, the NLL-MetRS, was later used by Ngo *et. al* for cell-selective labeling of proteins [25••]. Working with mixed cell populations, Ngo and coworkers demonstrated selective Anl incorporation, dye labeling, and fluorescence imaging of newly synthesized proteins in *E. coli* cells bearing the NLL-MetRS plasmid. This work illustrates the potential of the BONCAT method for proteomic analysis of a single cell type in complex multicellular systems, and suggests the possibility of selectively analyzing the proteomes of selected tissues in higher organisms. In a complementary approach, Link and coworkers showed that incorporation of a single genomic copy of MetRS* into *E. coli* allowed robust cell survival and Anl incorporation with no need for a wild-type MetRS; the MetRS* incorporates both Met and Anl for cell-survival and labeling, respectively [26]. The authors suggest that these genetically modified organisms may be useful for studying host-pathogen interactions without the need for introducing a mutant MetRS plasmid.

The OmpC cell surface display system described above relies on a reaction between the incorporated ncAA and a functionalized probe; more general screening methods are needed to assess activation of non-canonical substrates that lack reactive side-chain functionality.

The Schultz laboratory has developed several powerful screening methods for identifying aaRSs that enable incorporation of ncAAs into proteins in site-specific fashion [27]. Yoo and coworkers have reported complementary methods for use in residue-specific experiments, using a mutant green fluorescent protein (GFP) as a translational reporter [28]. Met sites were removed from the (β-barrel of GFP to ensure that incorporation of Met analogues would not suppress fluorescence. Five new Met sites were engineered into loop regions to ensure that fluorescence would be observed only if a Met-starved cell successfully incorporated an amino acid in response to Met codons. Protein synthesis was induced in *E. coli* cells bearing the mutant GFP gene and an aaRS library in medium supplemented with the ncAA of interest. Cells were separated by fluorescence-activated cell sorting (FACS). Fluorescent cells were isolated and subjected to a negative selection step that removed aaRS variants capable of activating any of the canonical amino acids in lieu of methionine; the population of cells exhibiting reduced fluorescence with 19 amino acids was collected. This study produced a MetRS variant that enabled incorporation of 6,6,6-trifluoronorleucine (**5**) in response to Met codons.

Altering global protein properties

As mentioned above, residue-specific incorporation of ncAAs allows alteration of global protein properties. Yoo *et. al* used this approach to evolve a fluorinated form of GFP (fl-GFP) through global replacement of leucine by 5,5,5-trifluoroleucine (**6**). The fl-GFP mutant displayed folding kinetics and fluorescence properties comparable to or better than those of the parent GFP [29]. Interestingly, when the authors expressed the evolved mutant in medium supplemented with Leu, they found that the folding and fluorescence properties of this protein had also improved, suggesting that evolving proteins with ncAAs may provide a new approach to enhancing the properties of proteins containing only canonical amino acids.

Perturbing proteins with ncAAs can provide important new insight into the connections between protein behavior and the properties of individual amino acids. Raines and coworkers have shown that replacement of proline by 4-fluoroproline (7) in a $(ProProGly)_7$ collagen model peptide raises the melting temperature of the triple helix by more than 50 °C [30]. The folding and stability characteristics of GFP are also altered when prolines are globally replaced by fluorinated analogs (e.g. **7**) that change the *cis-trans* isomerization tendencies of Pro residues [31]. Budisa and coworkers have shown that changing the hydrophobicity of the human prion protein by replacement of methionine with the more hydrophobic norleucine (**8**) or the more hydrophilic methoxinine (**9**) drastically perturbs aggregation behavior [32].

Several laboratories have used ncAAs to engineer the photophysical properties of proteins. The Deo group replaced tyrosine in the chromophore of the red fluorescent protein DsRed with 3-amino-L-tyrosine (NH_2-Tyr, **10**) and with 3-fluoro-L-tyrosine (fl-Tyr, **11**) [33]. The resulting mutants exhibited increased fluorescence quantum yields and shifted fluorescence maxima. The Budisa lab has reported several recent studies in which residue-specific incorporation was used to alter protein fluorescence. They demonstrated that 4-azatryptophan (4-azaTrp, **12**) could be metabolically synthesized from 4-azaindole and incorporated in place of the single Trp residue in human annexin A5 (anxA5) [34••]. The resulting anxA5 mutant exhibited normal folding behavior and inherent blue fluorescence arising from the 4-azaTrp chromophore. A recent review further describes their efforts towards developing novel, inherently blue fluorescent proteins [35]. The same group recently fused a 4-aminoTrp (**13**)-modified enhanced cyan fluorescent protein (termed gold fluorescent protein or GdFP [36]) to anxA5 to visualize anxA5 binding to phosphatidylserine on the surfaces of apoptotic cells [37].

New materials

Residue-specific incorporation of ncAAs can be highly efficient and can be used to prepare proteins in quantities sufficient for application in materials science. For example, Nowatzki and coworkers prepared thin films of artificial extracellular matrix proteins modified with variable amounts of *p*-azido-Phe (N_3Phe, **14**) [38]. Ultraviolet irradiation yielded crosslinked elastomers with moduli that could be tuned through variation in exposure time and N_3Phe content. These materials were used in conjunction with photolithographic techniques to obtain patterned surfaces [39]. Mammalian cells adhered selectively to regions of the surface patterned with protein, and were absent from the other regions of the material. In a later report, these films were used in conjunction with microfluidic technology to generate protein surface gradients [40]. Photo-initiated protein crosslinking for materials applications can be viewed as a macroscopic analogue of traditional photoaffinity protein labeling; both approaches are facilitated by ncAA incorporation, but the former is particularly suited to residue-specific techniques.

The Finn lab has used residue-specific methods to quantitatively modify nanoscopic virus particles. They demonstrated incorporation of Aha and Hpg into hepatitis B virus (HPV) and bacteriophage Qβ and showed that near quantitative replacement of Met had no adverse affect on formation of the virus capsids [41]. Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reactions were used to chemoselectively modify the Aha and Hpg residues with a variety of tags. In another report, Se-Met and Aha were incorporated into the interior of Qβ coat protein; the Aha mutant was labeled with a Gd-alkyne complex by CuAAC [42••]. The authors utilized inductively coupled plasma optical emission spectroscopy to measure the plasma clearance times for the particles as a function of cationic surface charge.

Attachment of polyethylene glycol (PEG) to proteins is a common strategy for altering the pharmacokinetic properties of protein-based therapeutics. As a result, bioorthogonal methods using ncAAs for chemoselective protein PEGylation are of great interest. The van Hest group coupled a 5 kDa PEG-alkyne to Aha-modified *Candida antarctica* lipase B (CalB) using CuAAC [43]. The authors found ca. 75% of the initial enzymatic activity to be retained after PEGylation. In a later report the same group used CuAAC to couple an Aha-modified elastin to CalB [44••]. The conjugate remained catalytically active and exhibited a lower critical solution temperature (LCST) characteristic of the elastin domain. To avoid concerns regarding Cu(I) chelation and destruction of enzymatic activity, the Nijmegen group recently introduced a new azadibenzocyclooctyne moiety for rapid, selective, copper-free click PEGylation of CalB [45].

In vivo protein function and trafficking are often modulated by post-translational modification (PTM) with fatty acids or sugar residues. Significant research efforts are focused on developing chemoselective ncAA modification strategies for mimicking these common PTMs. The Davis group recently reported a new glycosylation strategy that uses thiol-ene coupling between L-homoallylglycine (L-Hag, **15**) modified proteins and various glycosyl-thiols. The resulting glycoconjugates have potential as artificial immunogens for vaccine development. Future advances in aqueous olefin metathesis may provide yet another orthogonal reaction pathway for olefin-labeled proteins, though this approach requires further optimization [46••,47].

Conclusion

Residue-specific incorporation of ncAAs into cellular proteins has generated new materials with useful properties and has begun to answer important biological questions. Many of the

major technical challenges have been overcome and the field is ripe for application-oriented research. New methods like cell-selective protein labeling, FUNCAT, BONCAT, and new labeling chemistries, will undoubtedly lead to further discovery and to new developments of fundamental and practical value.

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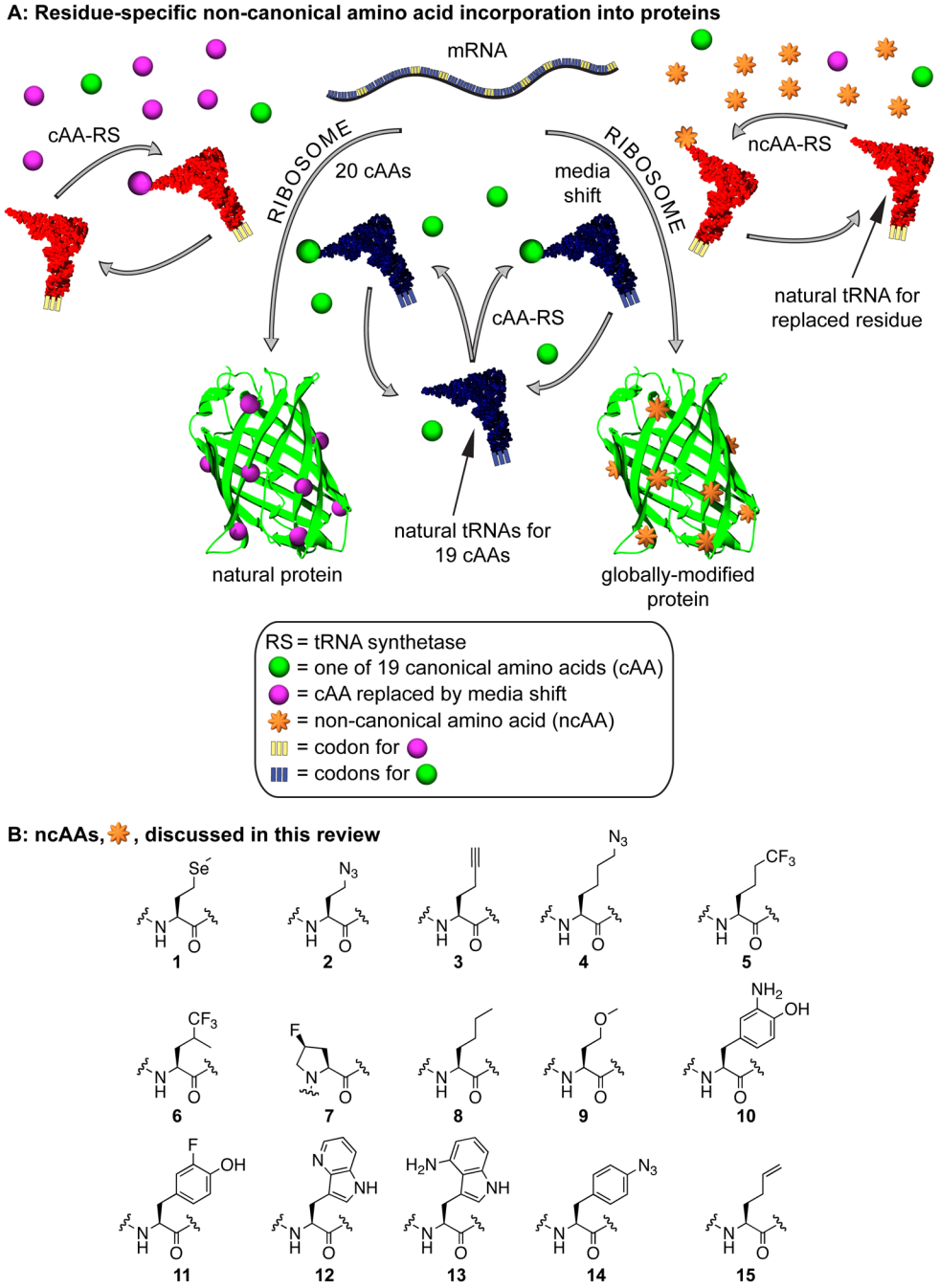

Figure 1.

1A: Schematic for residue-specific incorporation of non-canonical amino acids (ncAAs) into proteins. A natural mRNA contains codons for the 20 canonical amino acids (cAAs). A cAA (purple sphere) assigned to one of those codons (yellow) is replaced with an ncAA (orange star). A medium shift is performed to remove the cAA to be replaced (purple sphere) and to introduce the ncAA (orange star) along with the remaining 19 cAAs (green spheres). The ncAA is charged to the appropriate tRNA (red) by either the wild-type or a mutant aminoacyl-tRNA synthetase (aaRS). The correctly aminoacylated $tRNA^{cAA}$ (blue with green sphere) and the misacylated tRNA (red with orange star) are processed by the

ribosome to give a globally modified protein. The left path depicts normal protein synthesis with 20 cAAs for comparison. 1B: Chemical structures for the ncAAs discussed in the text.

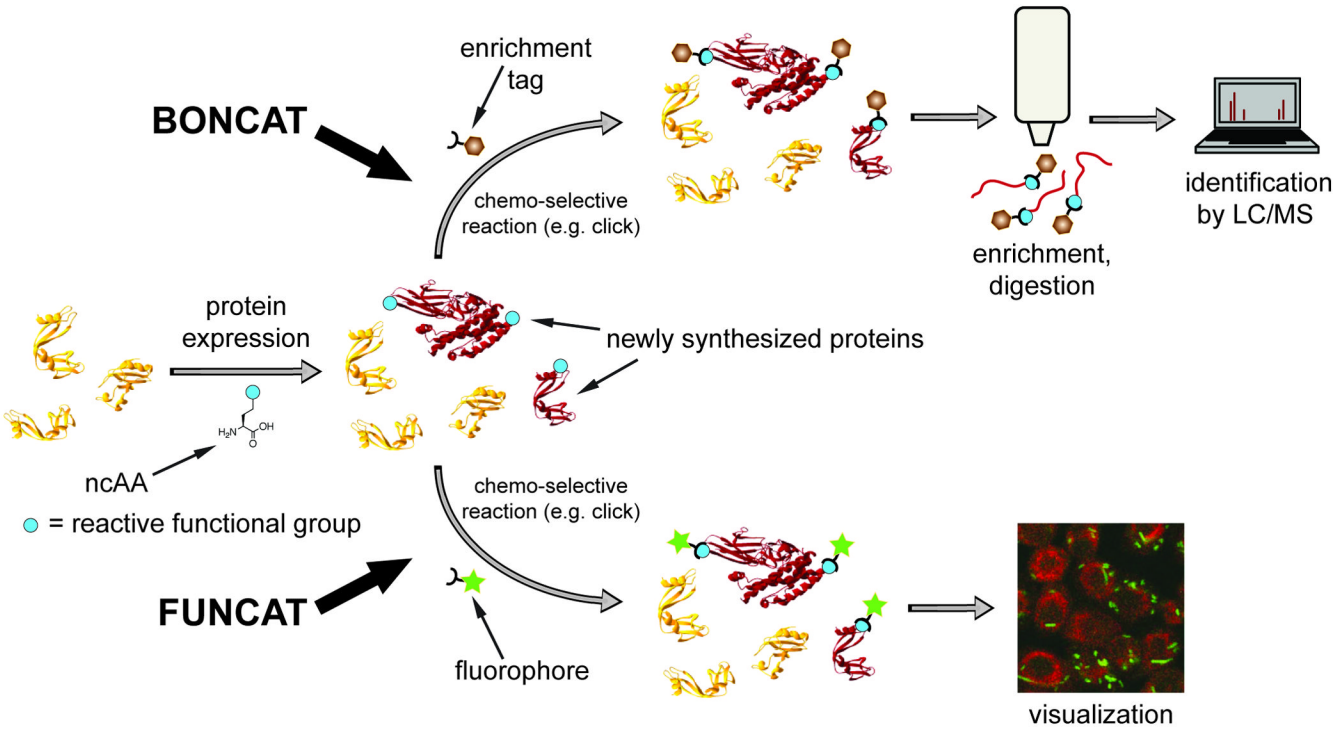

Figure 2.
Schematic depictions of BONCAT and FUNCAT technologies. Both begin with metabolic labeling of newly synthesized proteins using an ncAA bearing a reactive handle (blue sphere). Then, a chemoselective reaction is performed (e.g. click chemistry) to append either an enrichment tag (BONCAT) or a fluorophore for visualization (FUNCAT). Taken together, these technologies allow simultaneous imaging and identification of new proteins in cells; proteomic responses to stimuli (changing conditions, infection, cell-cell interaction, etc.) can be monitored.