Chapter 3

ENZYMATIC N-TERMINAL ADDITION OF NON-CANONICAL AMINO ACIDS TO PROTEINS

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Abstract

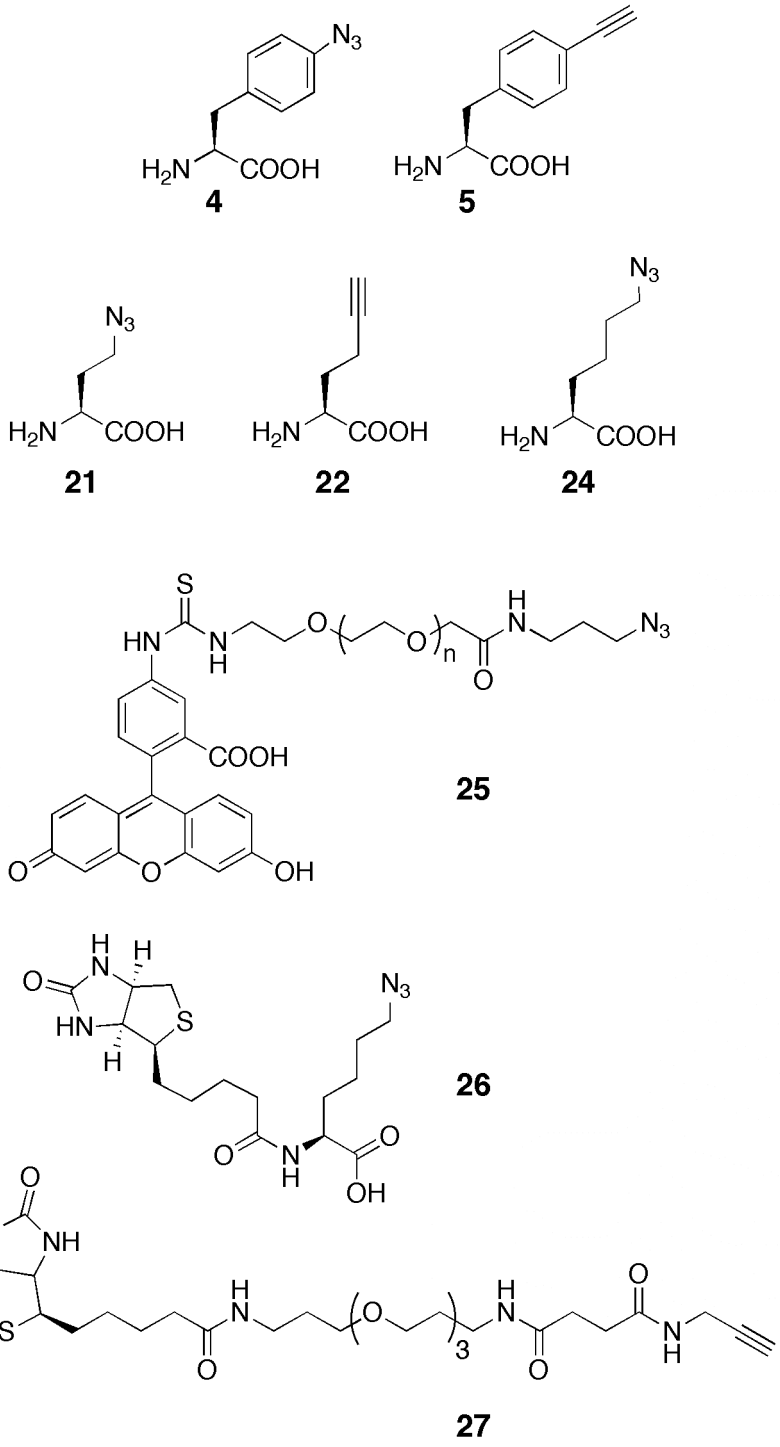
The necessity of modifying therapeutic proteins to enhance efficacy and lengthen half-live *in vivo* is a challenge to the widespread use of protein therapeutics. Although several amino acids can be modified with well-established chemistry, these methods result in global alteration of the amino acid throughout a target protein. Global alteration often results in decreased efficacy of the protein therapeutic due to perturbance of structure or active site. It has been shown that modification with polyethylene glycol at one specific location on a protein's surface can result in extending its *in vivo* half-life while preserving its efficacy. [1, 2] Ideally, such a modification would be effected post-translationally, thus allowing the unmodified protein to fold into its native structure without interference. Proteins bearing non-methionine N-termini, generated using a ubiquitin fusion technique, were modified with non-canonical amino acids using the *E. coli* L,F-transferase and various aminoacyl-tRNA synthetases. The modification was specific to the N-terminus and was available for ligation using [3+2] copper-catalyzed cycloaddition.

3.1 Introduction

The preparation of well-defined protein conjugates is essential for many therapeutic and biochemical technologies. The amino terminus is an especially attractive target for conjugation, and selective N-terminal modification has been achieved by a variety of chemical and enzymatic methods.[1, 3-7] Selective conjugation at the N-terminus of proteins can exploit the lower pKa of the terminal amino group[1] or occur through transamination methods using pyridoxal 5-phosphate or glyoxalic acid.[6, 7] Coordinated reactions between the terminal amine group and specific amino acid side chains have been reported for N-terminal serine, threonine, cysteine, and tryptophan.[2, 8-11] N-terminal enzymatic addition of peptides and amino acids has been achieved using transglutaminase, proteases, or modified initiator tRNA.[4, 5, 12] In the previous chapter, the *E. coli* enzyme, the L,F-transferase was shown to utilize a variety of non-canonical amino acids as aminoacyl substrates for the modification of a dipeptide substrate. The L,F-transferase requires only an unstructured and accessible N-terminus and a basic N-terminal residue for modification of a protein substrate. These relaxed conditions are ideal for the post-translational introduction of non-canonical amino acids at the N-termini of therapeutic and biochemically interesting proteins.

The addition of non-canonical amino acids, such as those shown in Scheme 3-1 allows the presentation of non-proteinogenic chemical functionality that can be used to make bioconjugates through the attachment of polymers such as polyethylene glycol, fluorescent dyes, cofactors, or other desired materials. These macromolecules are best made through bio-orthogonal reactions, which target very specific non-natural moieties to

the exclusion of the native protein chemistry[13, 14]. The specificity and exclusivity of the reactions allow the use of such methods in the complex milieu of both lysates and living biological systems. The Staudinger ligation, one of the first bio-orthogonal reactions to be applied to biological problems, uses the same azide functionality as presented in amino acids, Aha and Anl. A triarylphosphine is reacted with the azide to form an aza-ylide that decomposes into a phosphine oxide. The aza-ylide is then caught in a strategically placed electrophilic trap to form a covalent linkage.[15] Recently, the Huisgen or azide-alkyne [3+2] copper-catalyzed cycloaddition has been expanded for use in aqueous solution under very mild conditions.[16, 17] Work by Meldal and Sharpless has refined the reaction and allowed its application to numerous biological systems such as proteomics, protein evolution, fluorescent labeling of cells, and protein immobilization.[18-23] The robust yield of the reaction under a variety of conditions and its compatibility with commonly used buffer systems is unparalleled in bio-orthogonal chemistry. The copper catalyst can be delivered as a copper (I) salt either from copper bromide directly or from copper sulfate in conjunction with a reducing agent.[24] The cycloaddition has been expanded to a catalyst-free system by the Bertozzi group, where the usual terminal alkyne is replaced by a strained alkyne, a cyclooctyne.[25] The strain-catalyzed cycloaddition is particularly useful for any system sensitive to copper, which includes any real-time monitoring or diagnosis of live cells and organisms. This method of cycloaddition has been used to label azide-bearing carbohydrate analogs for dynamic *in vivo* labeling of glycan processing.[26]



Scheme 3-1. Chemical structures of amino acids and affinity tags used to modify *E. coli* dihydrofolate reductase.The amino acids are *p*-azidophenylalanine (**4**), *p*-ethynylphenylalanine (**5**), azidohomoalanine (**21**), homopropargylglycine (**22**), and azidonorleucine (**23**).An azide-polyethylyene glycol- fluorescein conjugate (**24**) and azidonorleucine- biotin (**25**) are shown.

A model protein substrate, *E. coli* dihydrofolate reductase (eDHFR), was produced as a ubiquitin fusion protein and then cleaved with ubiquitin protease from *S. cerevisiae* to release either an arginine or glycine residue at the N-terminus of DHFR. The ubiquitin method of producing proteins with non-methionyl N-termini was pioneered by Varshavsky and co-workers and refined with *in vitro* application of the ubiquitin protease by Catanzariti et al. [27, 28]

This model protein was modified with each of the amino acids shown in Scheme 3-1 using the L,F-transferase and an appropriate aminoacyl-tRNA synthetase. The *in vitro* transfer of the reactive amino acids to the model protein was detected through the further modification of the azide or alkyne moieties with affinity tags **26** and **27** using copper-catalyzed [3+2] cycloaddition. These affinity tags present a biotin molecule that allows detection through a conjugate of its binding partner, streptavidin. A rough time course of the extent of modification of R-eDHFR with **5** was determined by Edman degradation of protein samples after 2, 3, and 4 hours of modification.

After the addition of *p*-ethnynylphenylalanine to the model protein, polyethylene glycol conjugate **25** was ligated to the alkyne group through click chemistry. The extent of reaction was determined by densitometry of the modified and unmodified DHFR bands after Western analysis. We show that the L,F-transferase can be used to efficiently modify proteins *in vitro* with non-canonical amino acids, and that subsequent ligation through copper-catalyzed [3+2] cycloaddition can be achieved with high yield.

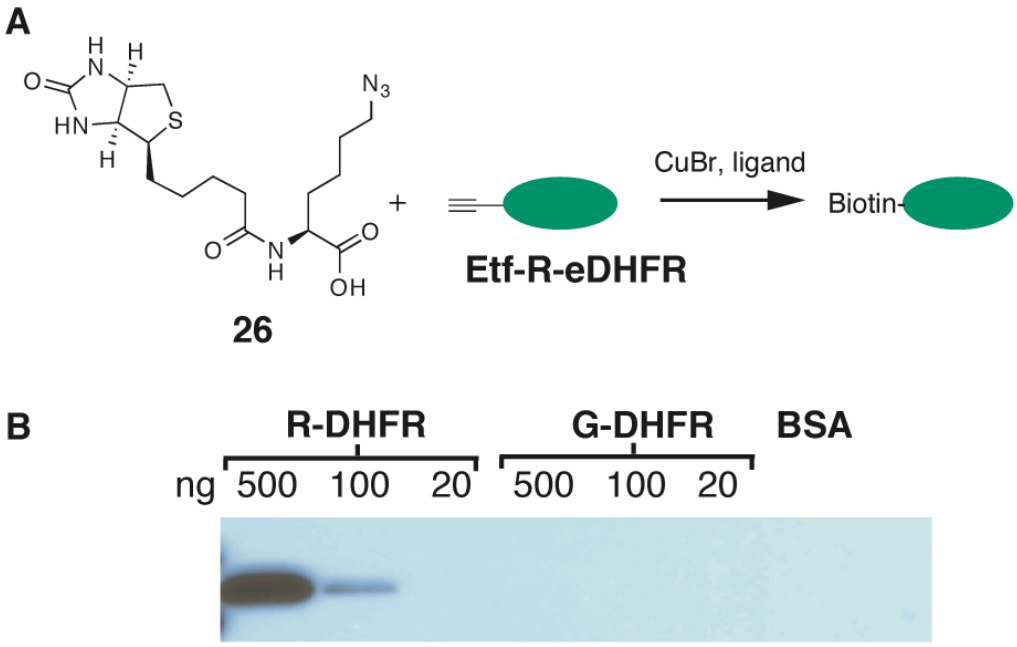


Figure 3-1. N-terminal modification of e-DHFR. A) After N-terminal addition of Etf, the alkyne moiety was conjugated to azide-biotin probe **26**. B) Immunoblot analysis of transfer reactions using both R-eDHFR and G-eDHFR as substrate proteins and their subsequent modification with azidonorlueucine-biotin using azide-alkyne [3+2] copper-catalyzed cycloaddition. Detection with streptavidin-HRP gives no signal in either the BSA or G-DHFR negative controls.

3.2 Materials and Methods

3.2.1 Materials

Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification, unless otherwise noted. Amino acid **4** was purchased from Bachem (Bubendorf, Switzerland) and used as received. PFP-Biotin was purchased from Pierce (Rockford, IL) and mSPA-PEG-5000-fluorescein was obtained from Nektar (San Carlos, CA). Amino acid **23** was prepared by alkylation of diethyl acetamidomalonate with the appropriate tosylate as described previously.[29] Compounds **21** and **24** were synthesized by diazo transfer to the equivalent amine-bearing amino acids;[24] **5** was prepared as previously reported.[30] The *E. coli* phenylalanyl-, leucyl-, and methionyl-tRNA synthetases were produced as described previously.[30-32] The leucyl, phenylalanyl transferase was purified as previously described.[33]

*3.2.2 Synthesis of Azidonorleucine-biotin (**26**)*

Azidonorleucine (12.65 mg, 0.073 mmol) and PFP-biotin (30.1 mg, 0.073 mmol) were dissolved in 1.5 mL of a 9:1:5 mixture of dimethylformamide, dimethylsulfoxide and methanol. Four equivalents of triethylamine were added and the reaction stirred overnight at room temperature. The reaction was purified on a C18 Sep-Pak column (Waters) using a stepwise gradient from 0.1% trifluoroacetic acid to 100% acetonitrile. The fractions containing the desired product were lyophilized to 6.8 mg of a fluffy white powder (23.4% yield). ESI MS (*m/z*): 398.9 $[M+H]^+$ (expected *m/z*: 398.17).

*3.2.3 Synthesis of Azido-PEG5000-Fluorescein (**APF, 25**)*

Azidopropylamine was synthesized as previously described.[34] To prepare the azide-functionalized PEG, mSPA-PEG-fluorescein (29 mg, 6 mmol) was dissolved in azidopropylamine (300 mL, 2.9 mmol) and allowed to react at room temperature for 2 h. The reaction mixture was then added dropwise to 20 mL of diethyl ether which caused the PEG reagents to form a yellow precipitate. The precipitate was collected by centrifugation and the ether supernatant decanted. The precipitate was dissolved in 1 mL of methanol and added dropwise to ether again and the precipitate collected as before. The recovered precipitate was used without further purification. Addition of an azide was confirmed by IR analysis.

3.2.4 Construction of Arg-eDHFR-HA and Gly-eDHFR-HA plasmids

DNA fragments encoding the gene for *E. coli* dihydrofolate reductase were amplified by PCR from *E. coli* genomic DNA with the following pairs of primers: Oligo 298 (5'-AGGCTCCGCGGTGGTCGTAAAATGATCAGTCTGATTGCGGC –3') and Oligo 314 (5'-TTTAAGCTTAGGCGTAATCTGGGACATCGTATGGGTAGCCGCTCCCCCGC CGCTCCAGAATCT –3') for Arg-eDHFR-HA; and Oligo 298 and Oligo 301 (5'-AGGCTCCGCGGTGGTGGTAAAATGATCAGTCTGATTGCGGC-3') for Gly-eDHFR-HA. The resulting fragments were digested with *Sac*II and *Hind*III, and ligated into *Sac*II/*Hind*III-digested pHUE[2] to generate plasmids pKP141 and pKP144, respectively.

3.2.5 Expression and purification of recombinant proteins

Overnight cultures in *E. coli* strain KPS17[35] were subcultured 1:100 into 200 mL Luria broth containing ampicillin and grown to a late exponential phase at 37°C. Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.1 mM. After 3 h, harvested cells were resuspended in 15 mL of buffer L (50 mM Na_2HPO_4/NaH_2PO_4 at pH 7.4, 500 mM NaCl, 10 mM imidazole), with 1 mM phenylmethylsulphonyl fluoride. His_6-tagged recombinant proteins were purified by nickel-affinity chromatography using batch mode under native conditions, based on the QIA*express* protocol (Qiagen). The cells were lysed by sonication (3 x 1 min bursts at 0°C) and the soluble protein fraction recovered by centrifugation (15 min, 4°C, 15,300*g*). To the supernatant, 2 mL of a 50% slurry of nickel-nitrilotriacetic acid (Ni-NTA) agarose in buffer L was added; the mixture was then placed on a rotary wheel at 4°C for 1 h. The lysate/Ni-NTA mixture was centrifuged (5 min at ~550*g*) and the remaining Ni-NTA agarose pellet was washed 5 times in 50 mL buffer W (50 mM Na_2HPO_4/NaH_2PO_4 at pH 7.4, 500 mM NaCl, 30 mM imidazole). The His_6-tagged protein was eluted from the Ni-NTA resin in 1 mL fractions with buffer L containing 300 mM imidazole. Chosen fractions were pooled and dialyzed against buffer L, along with deubiquitylating protease Usp2-cc for 12 h at 4°C. After cleavage by the protease, nickel affinity chromatography was used to separate Usp2-cc and the His_6-tagged ubiquitin domain from the modified DHFR according to published methods.[27]

3.2.6 N-terminal protein modification

eDHFR (7.5 mg) was modified with the amino acids shown in Scheme 3-1 in a reaction volume of 75 mL in modified Aat buffer (1 mM ATP, 50 mM Tris pH 8.0, 50 mM β-mercaptoethanol, 10 mM creatine phosphate, 45 mg *E. coli* total tRNA, 20 mg/mL creatine phosphokinase) with aminoacyl-tRNA synthetase (800 nM) and Aat (1.5 mg). For the methionine analogs, Aha and Hpg, the wild-type *E. coli* MetRS was used and for Anl the L13G mutant methionyl-tRNA synthetase was required. For both of the phenylalanine analogs, the single mutant A294G synthetase was used. The reaction mixture was incubated at 37°C for 4 h and then buffer-exchanged twice into phosphate buffered saline (PBS), pH 7.5, by using gel filtration columns (Bio-Spin 6, Bio-Rad).

3.2.6 Edman analysis of p-ethynylphenylalanine-modified eDHFR

For Edman degradation of modified samples, the reaction mixture was treated with 2 volumes of ice-cold acetone to precipitate the proteins. The precipitated proteins were then separated on a 12% gel and transferred to a PVDF membrane. The protein band corresponding to Etf-R-DHFR was excised and analyzed by Edman degradation at the Caltech Peptide and Protein Molecular Analysis Laboratory.

3.2.7 Copper-catalyzed [3+2] cycloaddition reactions

Ethynylphenylalanine-modified R-eDHFR was used directly after buffer exchange into PBS. Aliquots (10 mL) of the filtrate (0.5 mg eDHFR) were diluted into PBS (100 mL) containing CuBr (400 mM), *tris*-(benzyltriazolylmethyl)amine (TBTA, 400 mM) and either azidonorleucine-biotin (25 mM) or azide-PEG-5000-fluorescein (500 nM) for

[3+2] cycloaddition. The reaction mixtures were either treated with 900 mL of 1:8 trichloroacetic acid/acetone for analysis by immunoblotting or used directly for analysis by gel electrophoresis and fluorescence imaging. Biotinylation was detected with streptavidin-HRP (R&D Systems). Fluorescence images were obtained with a Molecular Imager FX from Bio-Rad with an excitation wavelength of 488 nm for detection of fluorescein. For quantitative pegylation, the reaction mixture (25 mL) obtained from modification of eDHFR with **5** (1.25 mg Etf-R-eDHFR) was diluted into PBS (100 mL) with azide-PEG-5000-fluorescein (100 mM), CuBr (400 mM) suspended in water, and TBTA (200 mM). The reaction mixture was incubated for 5 h at 4°C, with an aliquot taken after 2 h. Sodium azide (1 mM) was added to quench the reaction. The reaction mixture (25 mL) was then separated directly by SDS-PAGE and transferred to a nitrocellulose membrane for Western analysis with mouse anti-hemagglutinin and Cy5-anti-mouse immunoglobulin. The Cy5 fluorescence on the blot was detected with a Molecular Imager FX and the extent of pegylation determined by densitometry using ImageJ software.

3.3 Results and Discussion

3.3.1. Production of R-eDHFR and G-eDHFR substrate proteins

To produce full-length protein substrates of Aat, a construct allowing the generation of proteins with N-terminal residues other than Met was required. Such a system, based on the ubiquitin (Ub) fusion technique, was developed for the elucidation of the N-end rule pathway in *E. coli.* In this method, a Ub-X-protein fusion was expressed in *E. coli* and the junctional residue X (any desired residue except Pro) was made N-terminal through the removal of the Ub moiety by a coexpressed eukaryotic deubiquitylating enzyme.[36] A

recent modification of this technique, in which the fusion protein was purified from *E. coli* and the Ub moiety was then removed *in vitro* with a purified deubiquitylating enzyme,[27] yielded *E. coli* dihydrofolate reductase (eDHFR) bearing either N-terminal arginine (R-eDHFR) or glycine (G-eDHFR).

3.3.2 Transferase-mediated modification of the substrate protein, R-eDHFR with azide and alkyne-bearing amino acids

Purified R-eDHFR was modified *in vitro* with each of the non-canonical amino acids shown in Scheme 3-1. The phenylalanine analogs, **4** and **5**, were delivered to the transferase through charging by the A294G mutant of the *E. coli* phenylalanyl-tRNA synthetase. This versatile enzyme will aminoacylate *E. coli* phenylalanine tRNA with not only phenylalanine, but also a variety of non-canonical amino acids, as reported by Kirshenbaum and coworkers.[30] Similarly, the wild-type methionyl-tRNA synthetase (MetRS) will aminoacylate *E. coli* $tRNA^{Met}$ with methionine analogs such as **21** and **22**. The larger methionine analog, azidonorleucine (**24)** requires a mutant MetRS discovered in the Tirrell laboratory for efficient charging to $tRNA^{Met}$ and subsequent use by the transferase.[19] All of these analogs were transferred successfully to the model substrate protein, eDHFR by the L,F-transferase. To confirm the modification of the substrate protein with these reactive amino acids, the substrate proteins were subjected to further addition through the copper-catalyzed addition of affinity tags, as shown in Figure 3-2. All of the analogs were successfully modified with biotin as detected by Western analysis. Association of the non-canonical phenylalanine analogs **4** and **5** with the L,F-transferase and the PheRS results in signals above the expected molecular weight of R-eDHFR.

3.3.3 Analysis of p-ethynylphenylalanine-eDHFR by Edman degradation

Phenylalanine analog **5** (Etf) was appended to the N-terminus of R-eDHFR to form Etf-R-DHFR and subsequently ligated to azide-biotin probe **26** via copper (I)-catalyzed cycloaddition. As little as 100 ng of biotinylated product could be detected by Western blotting with streptavidin-HRP (Figure 3-1). Under identical conditions, G-eDHFR was unmodified, illustrating the specificity of Aat.

Edman analysis of the Aat-mediated transfer of **5** shows that R-eDHFR was modified almost quantitatively after 3 h, consistent with the results of the peptide assay in the previous chapter. Traces showing the N-terminal residue of the modified eDHFR after Edman degradation are shown in Figure 3-3. The increasing amount of Etf and decreasing amount of N-terminal arginine after each hour of reaction are indicated with the labels “Etf” and “R” over the respective peaks. The extent of reaction was calculated from those traces (Table 3-1).

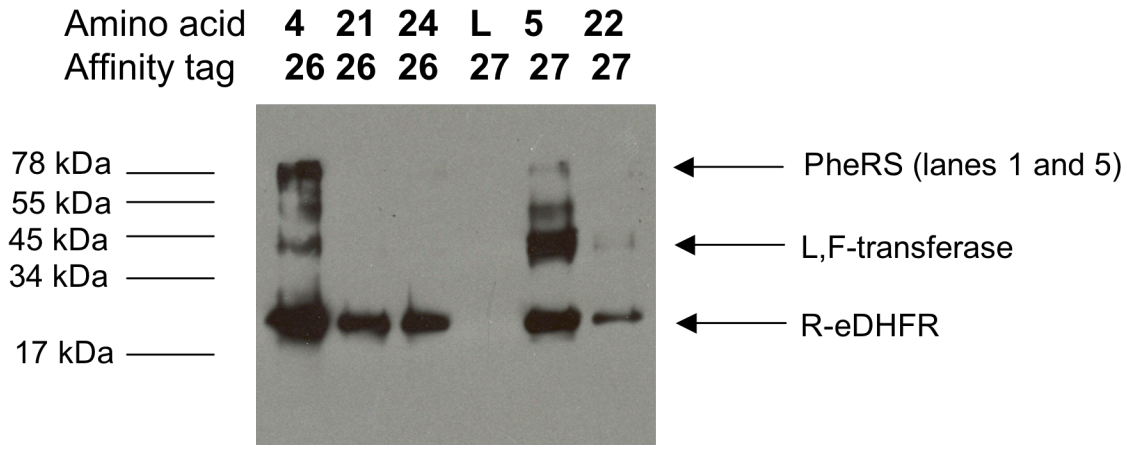


Figure 3-2. Western analysis of R-eDHFR modified with non-canonical amino acids and ligated to affinity tags. Phenylalanine analogs **4** and **5** were appended to eDHFR using a mutant PheRS. Methionine analogs **21** and **22** required wild-type MetRS and analog **24** required mutant MetRS for transfer to the substrate protein. The azide-bearing amino acids **4, 21,** and **24** were ligated to propargyl-PEO-biotin (**26**) using copper-catalyzed [3+2] azide-alkyne cycloaddition. Conversely, the alkyne-bearing amino acids were successfully modified after transfer with azidonorleucine-biotin (**27**). Biotinylation of the substrate protein was detected by Western analysis using streptavidin-HRP. Association of the non-canonical phenylalanine analogs with the PheRS and L,F-transferase results in higher molecular weight signals apparent in lanes 1 and 5.

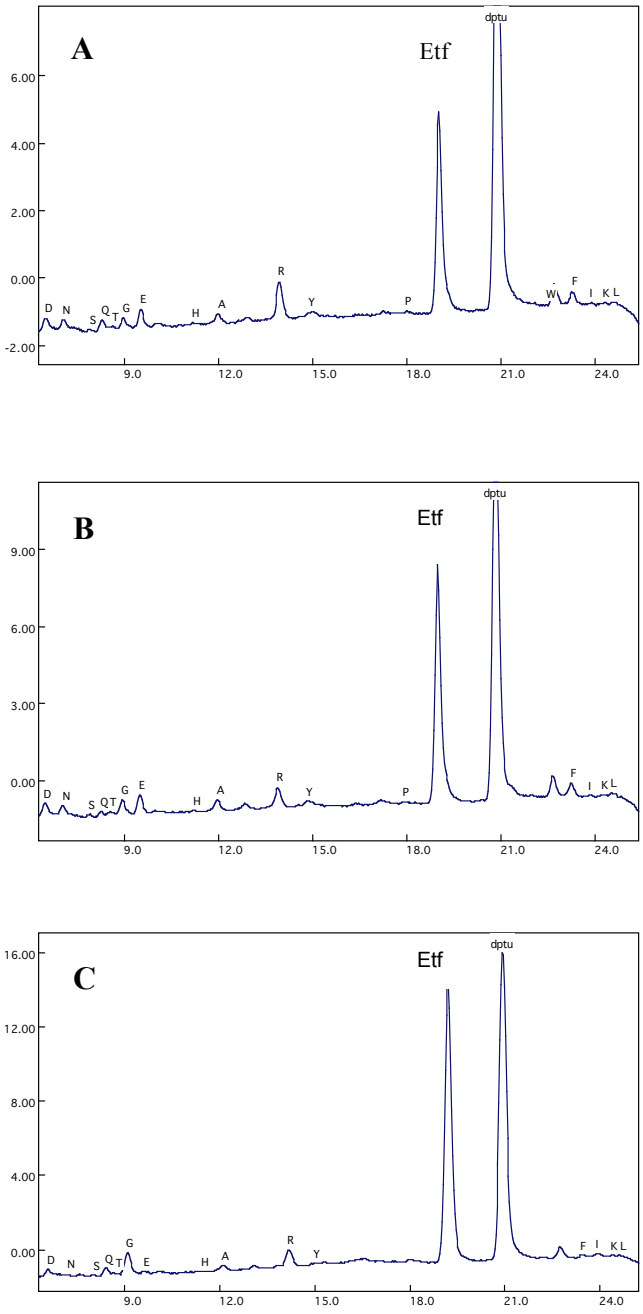


Figure 3-3. Chromatographic analysis of the first residue of Etf-R-eDHFR by Edman degradation after A) 2 h, B) 3 h, and C) 4 h. The peak labeled "R" arises from unmodified protein while the "Etf" peak arises from modified protein.

Table 3-1. The percent of modified protein Etf-R-DHFR after increasing reaction length as determined by comparison of the molar amounts of arginine found by Edman degradation in analyses of the first and second residues. Arginine found as the first residue arises from unmodified protein, whereas arginine found as the second residue arises from modified protein. The reaction is largely complete after 3 h.

Incubation Time (hours)	Percent modification
2	80
3	92
4	93

3.3.4 PEGylation of Etf-R-eDHFR

Ligation of an azide-PEG-fluorescein (APF) conjugate to Etf-R-eDHFR was also accomplished in good yield via copper-catalyzed cycloaddition (Figure 3-4). Fluorescence detection at 530 nm confirmed that ligation of APF required prior treatment of the protein with Etf, PheRS, and Aat (i.e., a complete transferase reaction mixture). The two-stage modification was achieved in an overall yield of 80 ± 2%, as determined by densitometric analysis of Western blots detected with Cy3-labeled secondary antibody (Figure 3-4).

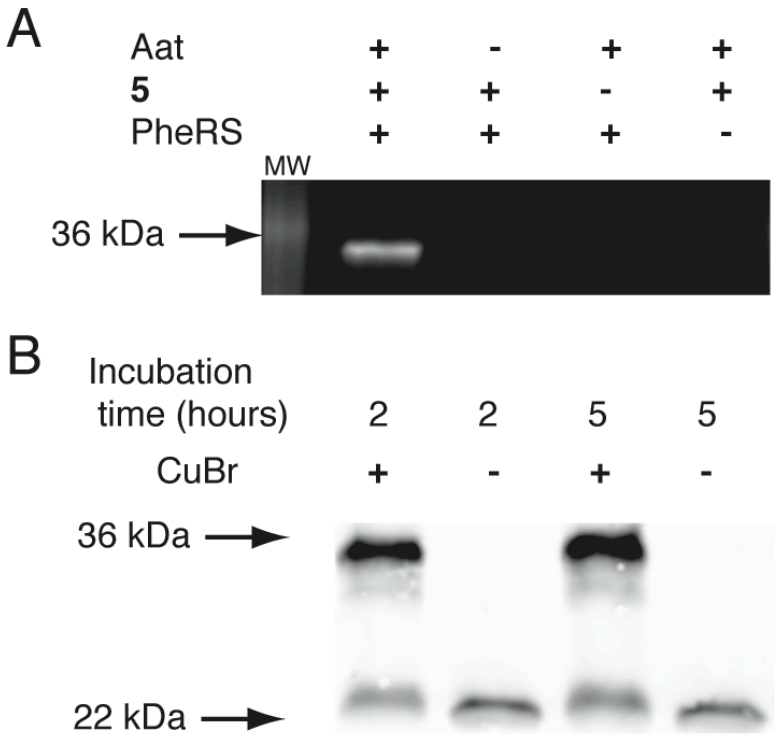


Figure 3-4. Pegylation of Etf-eDHFR with azide-PEG-5000-fluorescein (APF). A) Fluorescence image (detection at 530 nm) of gel separation, showing products of complete and negative-control transfer reactions after treatment with APF. The pegylated protein migrates with an apparent molecular mass of ca. 36 kDa. B) Western blot analysis of eDHFR-HA subsequent to transfer of Etf and subsequent treatment with APF (with or without CuBr, incubated for 2 h or 5 h at 4°C). DHFR-HA bears a C-terminal hemagglutinin (HA) tag, and was detected with Cy5-anti-mouse immunoglobulin and mouse anti-hemagglutinin. The pegylated product migrates with an apparent molecular mass of ca. 36 kDa; unmodified eDHFR-HA with an apparent molecular mass of ca. 22 kDa

3.4 Conclusions

We have used coupled systems of aminoacyl-tRNA synthetases and Aat to modify the N-termini of proteins with a variety of non-canonical amino acids. This modification occurs in good yield for phenylalanine analogs, which is in agreement with the data presented in Chapter 2. This indicates that the peptide reaction can give some guidance as to an expected extent of modification for any given non-canonical amino acid. Increased yield may be achieved through modification of the transfer conditions, such as with the addition of more synthetase or Aat.

Bio-orthogonal reactive functional groups, such as alkenes, alkynes, azides, and ketones, can be transferred to proteins and used to prepare well-defined bio-conjugates in high yield. The method introduced here allows new approaches to the engineering of therapeutic proteins through pegylation; to the study of protein interactions through crosslinking; and to the immobilization of proteins for use in sensors, microarrays, and catalytic systems. This method of modification can be applied to any protein produced with an N-terminal arginine or lysine.

3.5 References

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