Chapter 2

SELECTIVE DYE-LABELING OF NEWLY SYNTHESIZED PROTEINS IN BACTERIAL CELLS

2.1 Abstract

In this chapter, I describe a method for fluorescence labeling of newly synthesized proteins in *Escherichia coli* cells by means of Cu(I)-catalyzed cycloaddition between alkynyl amino acid side chains and the fluorogenic dye 3-azido-7-hydroxycoumarin. The method involves co-translational labeling of proteins by the non-natural amino acids homopropargylglycine (Hpg) or ethynylphenylalanine (Eth) followed by treatment with the dye. As a demonstration, the model protein barstar was expressed and treated overnight with Cu(I) and 3-azido-7-hydroxycoumarin. Examination of treated cells by confocal microscopy revealed that strong fluorescence enhancement was observed only for alkynyl-barstar treated with Cu(I) and the reactive dye. The cellular fluorescence was punctate, and gel electrophoresis confirmed that labeled barstar was localized in inclusion bodies. Other proteins showed little fluorescence. Examination of treated cells by fluorimetry demonstrated that cultures supplemented with Eth or Hpg showed an 8- to 14-fold enhancement in fluorescence intensity after labeling. Addition of a protein synthesis inhibitor reduced the emission intensity to levels slightly above background, confirming selective labeling of newly synthesized proteins in the bacterial cell.

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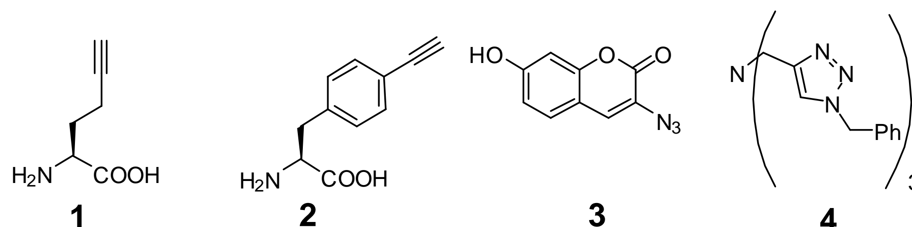
2.2 Introduction

Fluorescence labeling of proteins has become a central tool in the study of biological systems. Most labeling methods rely on fusion of an enzyme, fluorescent protein, or peptide tag to the protein of interest.[1, 2] Green fluorescent protein (GFP) is the most common fusion partner for monitoring cellular interactions and protein localization.[3] While it is likely that GFP and its many variants will continue to find widespread use, the size of GFP and the requirement for genetic manipulation of the target protein place limits on its utility. Tsien and coworkers have reported a less invasive strategy in which a tetracysteine motif is added to the protein of interest to allow site-specific labeling by fluorogenic bisarsenical dyes.[2] This strategy demonstrates some of the most important traits of a useful fluorogenic label: small size, membrane permeability, low background, and selective reactivity. For some purposes, however, it would be desirable to circumvent the need for genetic introduction of fluorescent reporters or labeling sites. For example, spatially regulated protein translation is believed to contribute to synaptic plasticity, but the products of local protein synthesis have not yet been fully identified.[4] We describe here an approach that involves co-translational labeling of proteins by the non-natural amino acids homopropargylglycine (Hpg, **1**) or ethynylphenylalanine (Eth, **2**) to provide alkynyl sites for attachment of fluorogenic dyes. The method permits fluorescence labeling of newly synthesized proteins in *Escherichia*



coli cells by means of a Cu(I)-catalyzed cycloaddition between the alkynyl amino acid side chains and the dye 3-azido-7-hydroxycoumarin (**3**).

The azide-alkyne cycloaddition has been used in many bioconjugation reactions, including identification of enzyme inhibitors, labeling of the *E. coli* cell surface, profiling of enzyme activities, and labeling of glycoproteins on the surface of mammalian cells.[5-8] Recently, the azide-alkyne cycloaddition has been used to activate weakly fluorescent coumarins to give intensely fluorescent triazole products.[9] Desirable features of **3**, as well as other quenched coumarin probes, include small size, membrane permeability, and intense fluorescence upon activation. The azido functionality of **3** is essentially bioorthogonal and allows selective *in vivo* labeling only when the alkynyl coupling partner is accessible in the cell.[10]

Previous work has demonstrated that alkynyl amino acids can be incorporated easily into recombinant proteins in a residue-specific manner. Hpg serves effectively as a methionine (Met) surrogate even without modification of the translational machinery of the host, and Eth acts as a phenylalanine (Phe) analogue in strains that over-express appropriately designed mutant phenylalanyl-tRNA synthetases.[11, 12] We show here that these two analogues allow efficient labeling and visualization of newly synthesized proteins in bacterial cells.

2.3 Results and Discussion

Histidine-tagged barstar was used as the model protein.[13] Cells expressing recombinant barstar in medium containing the twenty natural amino acids, nineteen amino acids plus Hpg, or nineteen amino acids plus Eth were used for whole cell

fluorescence studies with **3**. Barstar contains two Met sites and two Phe sites, all of which could potentially constitute targets for dye conjugation. Auxotrophic *E. coli* cells harboring a plasmid for inducible expression of barstar were grown to an OD_{600} of 1, washed twice with 0.9% NaCl, and resuspended in medium supplemented with nineteen amino acids plus 1 mM Hpg, 1.5 mM Eth, 0.75 mM Met, or 1.5 mM Phe.[11] Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG). After 3.5 hours, cells were harvested by centrifugation and washed twice with 0.9% NaCl to remove excess alkynyl amino acid. The cells were resuspended in PBS (pH 7.9), and the OD_{600} was adjusted to 1. The cultures were divided into 5 mL aliquots and supplemented with 50 μM **3**. For copper-free reactions, there was no further supplementation. For reaction with $CuSO_4$ catalyst, the aliquots were supplemented with 200 μM $CuSO_4$, 200 μM **4**, and 400 μM TCEP.[6] For reaction with CuBr catalyst, the aliquots were supplemented with 100 μM CuBr and 200 μM **4**.[7] Labeling reactions were allowed to proceed for 14-15 hours at 4 ˚C. Cells were harvested by centrifugation, washed twice, and resuspended in 5 mL PBS (pH 7.9).

Strong fluorescence enhancement was observed only in cells containing alkynyl-barstar reacted overnight with Cu(I) and **3** (**Figure 2.1**, a and b). We examined the fluorescence of individual cells by confocal microscopy; images were obtained by overlaying a fluorescence image (excitation at 800 nm with a 2-photon laser) and a differential interference contrast image. Cells lying out of the plane of focus did not appear fluorescent. In the plane of focus, however, punctate fluorescence was observed, presumably where barstar was localized in inclusion bodies (vide infra). No fluorescent

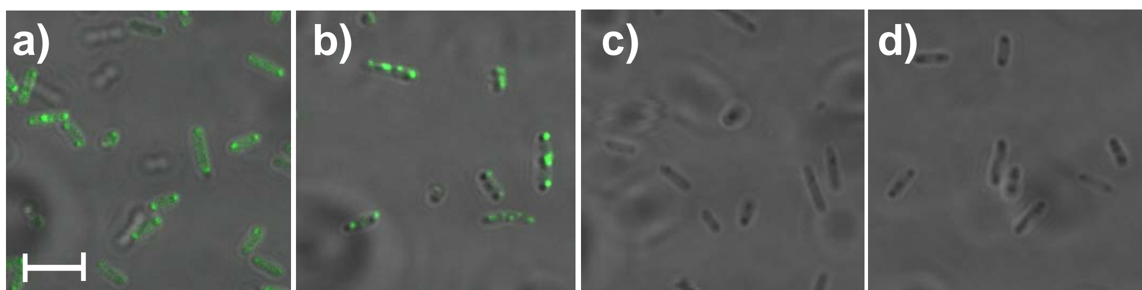


Figure 2.1. Fluorogenic labeling of barstar in *E. coli* cells. Cells were induced in media supplemented with 19 amino acids and one of the following amino acids: Hpg (a); Eth (b); Met (c); Phe (d). Cells were treated overnight with 100 μM CuBr, 200 μM **4**, and 50 μM **3**. Scale bar represents 5 μm.

cells were observed in experiments in which barstar was expressed in media supplemented with nineteen amino acids plus Met or Phe (**Figure 2.1**, c and d), or in control reactions run in the absence of Cu(I) (data not shown). Although uncatalyzed azide-alkyne reactions proceed favorably under selected conditions, the reaction rate is greatly enhanced by addition of a Cu(I) catalyst.[5, 14]

Measuring the fluorescence emission from a volume of treated cells allowed us to determine the extent of fluorescence enhancement. After washing and resuspension, cells were excited at 395 nm, and emission was monitored from 450 to 500 nm. Significant enhancement of the emission at 470 nm was observed only for cells treated with Hpg or Eth and labeled overnight with **3** and Cu(I) (**Figure 2.2**). To calculate the extent of enhancement, fluorescence from cells expressed in medium supplemented with 19 amino acids and an alkynyl amino acid was compared to background fluorescence from similarly treated cells grown in media supplemented with 19 amino acids and Met or Phe. Cells treated with Hpg and then ligated with **3** and $CuSO_4$ gave an 8.4-fold enhancement

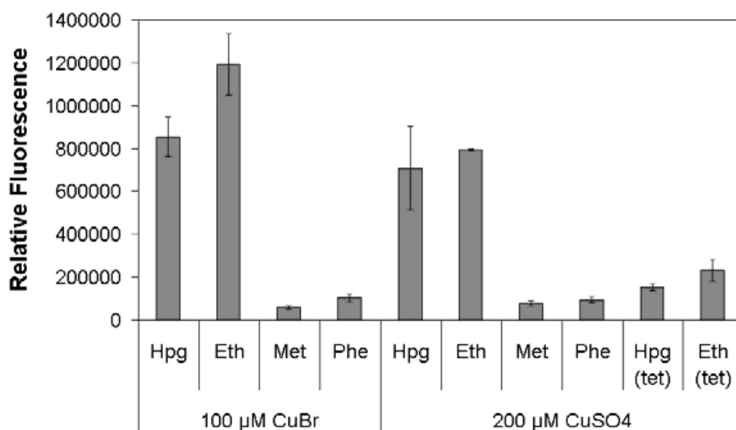


Figure 2.2. Fluorescence of *E. coli* cells after reaction with **3**. Fluorescence was also measured for uninduced cells in media supplemented with tetracycline (tet).

in emission intensity; ligation with CuBr gave a 10.1-fold enhancement. The corresponding enhancements for cells treated with Eth were 9.4- and 14.1-fold, respectively. Addition of tetracycline as an inhibitor of protein synthesis reduced the fluorescence emission to levels slightly above background.

To verify that labeling occurred selectively on newly synthesized proteins (i.e., primarily on barstar), we performed gel electrophoresis on crude bacterial lysates (**Figure 2.3**). Under UV illumination, the most intensely fluorescent band was that of barstar, although low levels of labeling were observed in other proteins, consistent with predominant, but not exclusive, expression of barstar following induction by IPTG. Fluorescence was detectable only in samples that were treated with **3** and a Cu(I) catalyst, confirming that Cu(I) was required for in vivo labeling. Lysates from cultures prepared in media supplemented with 19 amino acids plus Phe or Met lacked detectable fluorescence.

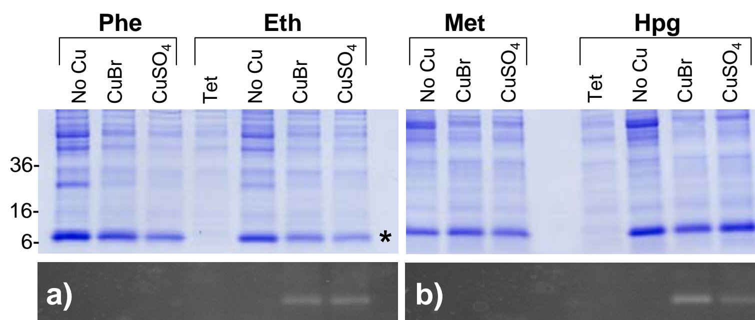


Figure 2.3. Polyacrylamide gel electrophoresis of proteins labeled with **3**. Samples were imaged under UV illumination (bottom) before staining with Coomassie Blue (top). Barstar appears at ~12 kDa (*). Bacterial lysates were from cultures supplemented with (a) Phe or Eth; (b) Met or Hpg.

The punctate fluorescence shown in **Figure 2.1** suggests that the labeled protein is localized predominantly in inclusion bodies. Cells from cultures supplemented with Eth or Hpg were labeled with **3**, and inclusion bodies were isolated and subjected to gel electrophoresis. Fluorescence imaging of the resulting gel was fully consistent with predominant dye-labeling of barstar in the insoluble fraction.

2.4 Conclusion

The results reported here demonstrate the utility of the Cu(I)-catalyzed azide-alkyne cycloaddition to effect selective fluorescence labeling of newly synthesized proteins in bacterial cells. The method exploits the natural scarcity of alkynes and azides in the cellular milieu, and offers a complement to current approaches that require genetic manipulation in order to ensure specific labeling. Susceptibility to labeling is determined not by the identity of the protein, but rather by the extent to which it was translated during the pulse with the alkynyl amino acid. The method is operationally similar to

conventional pulse-labeling with ^{35}S-methionine, but avoids the technical challenges of high resolution autoradiography.[15]

2.5 Materials and Methods

2.5.1 Plasmids and Expression Hosts.

The plasmid pQE30-Barstar contains a gene encoding histidine-tagged barstar under control of a T5 promoter. Briefly, PCR was used to add *Bam*HI and *Hind* III sites to the barstar gene. The gene encodes two mutations (Cys53Ala, Cys95Ala) for improved stability that do not affect barnase binding.[13] We do not believe that cysteine deletion is necessary for effective labeling; we have observed labeling of many cysteine-bearing proteins in companion experiments. After digestion, the gene was inserted into pQE30 between the *Bam*HI and *Hind*III restriction sites. For incorporation of Hpg, pQE30-Barstar was transformed into the *E. coli* Met auxotrophic strain M15-MA to make the expression host M15-MA [pQE30-Barstar].[6a]

For incorporation of Eth, pQE30-Barstar was linearized by digestion with *Nhe*I. The plasmid pQE15-PheRS* contains a mutant *E. coli* phenylalanyl-tRNA synthetase (A294G) under control of a modified *tac* promoter with an abolished *lac* repressor binding site for constitutive expression.[6a, 11b, 16] This plasmid was digested with *Nhe*I, and a 1 kB fragment corresponding to the PheRS* cassette was isolated by agarose gel electrophoresis. This fragment was ligated into pQE30-Barstar to yield the plasmid pQE30-Barstar-PheRS*. This plasmid was transformed into the Phe auxotrophic *E. coli* strain BL21(DE3) containing pLysS-IQ (AF-IQ) to make the expression host AF-IQ [pQE30-Barstar-PheRS*].[17]

2.5.2 Expression of Barstar.

An overnight culture of M15-MA [pQE30-Barstar] was diluted 40-fold into 90 mL of M9 minimal medium containing all twenty natural amino acids (40 mg/L each; 20 mg/L Phe for AF-IQ strain), ampicillin (200 mg/L), and kanamycin (35 mg/L). After reaching an OD_{600} of 1, the culture was sedimented by centrifugation for 5 min (6500*g*) at 4 ˚C. The cell pellets were washed twice with NaCl (0.9 wt %). The culture was resuspended in M9 minimal medium without Met. The culture was divided into 35, 48, and 5 mL aliquots. For protein synthesis inhibition, tetracycline (10 mg/mL) was added to the 5 mL culture. After 15 min at 37 ˚C, the samples were supplemented with either Met (0.75 mM in 35 mL medium) or Hpg (1 mM in 48 mL medium). After 10 min incubation, protein expression was induced for 3.5 h by the addition of IPTG (1 mM). The 5 mL culture supplemented with Hpg and tetracycline was not induced.

The expression of barstar from AF-IQ [pQE30-Barstar-PheRS*] was performed as above, except the M9 minimal medium was supplemented with chloramphenicol (35 mg/L) instead of kanamycin. After reaching an OD_{600} of 1.0, a medium shift was performed as described. The cultures were supplemented with either Phe (1.5 mM) or Eth (1.5 mM). Expression was induced for 3.5 h with IPTG (1 mM).

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