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Multiply labeling proteins for studies of folding and stability

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Fluorescence spectroscopy is a powerful method for monitoring protein folding in real-time with high resolution and sensitivity, but requires the site-specific introduction of labels into the protein. The ability to genetically incorporate unnatural amino acids (Uaas) allows for the efficient synthesis of fluorescently labeled proteins with minimally perturbing fluorophores. Here, we describe recent uses of labeled proteins in dynamic structure determination experiments and advances in unnatural amino acid incorporation for dual site-specific fluorescent labeling. The advent of increasingly sophisticated bioorthogonal chemistry reactions and the diversity of Uaas available for incorporation will greatly enable protein folding and stability studies.

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Introduction

Fluorescence spectroscopy can be used to study protein structural dynamics by harnessing two distance-dependent interactions between chromophores, Förster resonance energy transfer (FRET) and photoinduced electron transfer (PET) [1,2]. FRET and PET studies of protein conformational change offer a combination of structural and temporal resolution that is difficult to achieve using other methods. FRET interactions depend on the geometries and spectral characteristics of donor and acceptor chromophores, allowing for distance measurements on the 1–10 nm scale. PET quenching interactions occur on shorter length scales (0.5–2 nm). Recent developments in double site-specific labeling of proteins have dramatically increased the ease with which one can introduce the probes necessary for FRET and PET experiments.

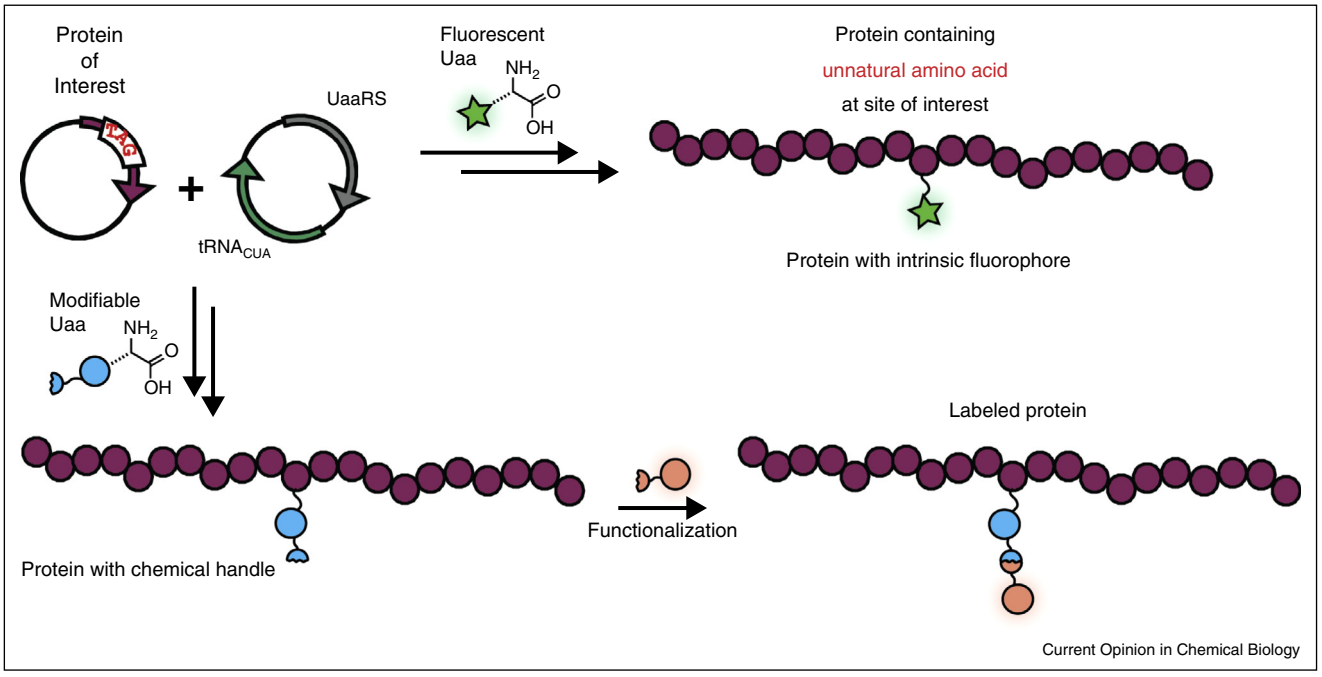
Fusions of intrinsically fluorescent proteins or of tag constructs such as HaloTag [3], SNAP-tag [4], CLIP-tag [5], and TMP-tag [6] have been widely applied in FRET-based studies of protein folding, protein–protein interactions, and protease activity [7]. However, protein tags can be perturbing to the processes being studied, owing to the size of the label relative to the protein of interest [8,9]. Smaller label strategies include fluorogenic bisarsenical dyes that are selectively chelated by tetracysteine motifs and chemoenzymatic labeling using enzymatic recognition of short peptide sequences [10–12]. Such methods rely on insertion of a 5–15 amino acid sequence, which may still be disruptive to native protein structure and function.

The desire for non-perturbing, single residue labels has brought about a wide array of reagents used for protein conjugation, including classic Cys-selective reagents like maleimides [13–15]. However, such techniques are typically only suitable for systems lacking endogenous cysteine or which are amenable to mutation to obtain a single reactive position. A variety of strategies for introducing labels using unnatural amino acids (Uaas) have been developed, with the techniques pioneered by Schultz now established as the most broadly accessible methods for site-specific incorporation of Uaas [16•,17]. Insertion of a Uaa is accomplished by a mutant aminoacyl tRNA synthetase capable of transferring the Uaa onto a cognate tRNA for ribosomal translation (Figure 1). To be site-specific, this tRNA must recognize an 'unassigned' codon such as a stop codon or a four base codon. The Uaa may either be intrinsically fluorescent [18–21] or modified by a so-called bioorthogonal reaction that is selective in the presence of biological functional groups [15]. The FRET or PET quenching of intrinsic protein fluorescence (e.g. Trp) by incorporation of Uaas has also served as a useful strategy for biophysical characterization of proteins *in vitro* [22].

Several groups have reported useful strategies for dual incorporation of fluorescent probes through selective cysteine conjugation and Uaa incorporation [23–26]. Though readily applicable with a minimum of manipulation for Uaa incorporation, these strategies still have many of the limitations of standard labeling because they use Cys. Here, we focus on recent work in which Uaa mutagenesis has been employed in a sequence-independent manner to obtain site-specific dual incorporation of chromophores for studies of protein folding.[1] While these

[1] Additional excellent developments in protein double labeling have been made by Edward Lemke and coworkers. We have limited our discussion of these articles here, as they are summarized in the chapter by Nikic and Lemke in this volume.

Figure 1



Complementary strategies for generating fluorescently labeled proteins using Uaa mutagenesis that rely on genetic encoding of the protein of interest with a nonsense codon and an orthogonal tRNA/synthetase pair to incorporate the Uaa cotranslationally. *Top*: incorporation of a fluorescent Uaa leads directly to fluorescently labeled protein. *Bottom*: a complementary strategy based on Uaa incorporation and subsequent functionalization allows for modular fluorophore incorporation.

newly developed methods have not yet seen significant application by the protein folding community, we highlight a few recent studies of protein folding and stability to illustrate their potential impact.

Ligation/Uaa labeling

Chemical protein synthesis allows for precise modifications to the resulting protein molecule [27,28]. A combination of Uaa mutagenesis in an expressed protein fragment, along with chemical synthesis of a modified N-terminal or C-terminal sequence, can allow for direct incorporation of two specific modifications through expressed protein ligation (EPL). Our laboratory has focused on the semisynthetic incorporation of a backbone thioamide as a minimal protein alteration for fluorescence-based studies [29]. Thioamides serve as FRET or PET-based quenchers for a variety of fluorophores [30,31]. We have incorporated thioamides into full-length proteins through synthesis of either an N-terminal or C-terminal thiopeptide fragment, which is then ligated to the expressed protein fragment containing a fluorophore (Figure 2). We have applied our techniques to study the misfolding of the Parkinson's disease associated protein α-synuclein (αS) via quenching of tryptophan fluorescence [32] as well as the Uaas *p*-cyanophenylalanine [33••] and acridon-2-ylalanine (unpublished results). The resulting constructs would necessarily contain a cysteine residue at the EPL site, but we have eliminated this restriction through ligation and alkylation to yield a native methionine [34] and through the development of thioamide-compatible desulfurization conditions (unpublished results). The combination of EPL and Uaa mutagenesis is the most general double labeling method, since the synthetic portion may contain groups that cannot be incorporated cotranslationally. However, it can be labor intensive and low-yielding, and efforts to improve efficiency are ongoing in our laboratory.

Dual Uaa labeling

Dual Uaa incorporation into proteins has previously been achieved by cell-free translation systems in response to three and four base codons, allowing for incorporation of fluorophores for proof-of-principle studies in the calcium-binding protein calmodulin (CaM) and the enzyme dihydrofolate reductase [35,36]. Although cell-free systems are amenable to a variety of Uaas, protein yields can be limited, making cell-based expression systems desirable. Cotranslational insertion of two Uaas into a protein in cells requires the use of two mutually orthogonal Uaa synthetase/tRNA pairs which must also be orthogonal to the endogenous translational machinery. Derivatives of the *Methanocaldococcus jannashii* tyrosyl tRNA synthetase and *Methanosarcina barkeri* or *Methanosarcina mazei* pyrrolysl tRNA synthetase fulfill these criteria for expression in