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Tryptophan-based fluorophores for studying protein conformational changes

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ABSTRACT

With the continuing interest in deciphering the interplay between protein function and conformational changes, small fluorescence probes will be especially useful for tracking changes in the crowded protein interior space. Presently, we describe the potential utility of six unnatural amino acid fluorescence donors structurally related to tryptophan and show how they can be efficiently incorporated into a protein as fluorescence probes. We also examine the various photophysical properties of the new Trp analogues, which are significantly redshifted in their fluorescence spectra relative to tryptophan. In general, the Trp analogues were well tolerated when inserted into *Escherichia coli* DHFR, and did not perturb enzyme activity, although substitution for Trp22 did result in a diminution in DHFR activity. Further, it was demonstrated that **D** and **E** at position 37 formed efficient FRET pairs with acridon-2-ylalanine (Acd) at position 17. The same was also true for a DHFR construct containing **E** at position 79 and Acd at position 17. Together, these findings demonstrate that these tryptophan analogues can be introduced into DHFR with minimal disruption of function, and that they can be employed for the selective study of targeted conformational changes in proteins, even in the presence of unmodified tryptophans.

1. Introduction

Following the changes in the fluorescence spectrum of a protein is a common technique for monitoring protein conformational and electrostatics transitions due to ligand binding, protein–protein interactions, or chemical transformations. The fluorescence signal can result from naturally occurring amino acids (e.g., tryptophan and tyrosine) or can be introduced chemically (e.g., via thio linked Alexa dyes). The sensitivity of fluorescence techniques allows the monitoring of subtle biophysical changes under equilibrium or non-equilibrium conditions. Therefore, protein conformation and function changes are widely studied through the use of Förster resonance energy transfer (FRET) by measuring changes in the efficiency of energy transfer between a donor and acceptor.[1,2] The acceptor may also be a fluorescent molecule, leading to another longer wavelength emission, or a quencher.[2–6] With the appropriate pairs, FRET can be used to quantitatively determine distance changes that are related to protein processes.

Typically, a FRET system in a single protein may include two different variants of green fluorescent protein (GFP),[1,7–9] or two large polycyclic aromatic molecules.[10,11] Additionally, the large polycyclic aromatic fluorophores/quenchers are often chemically tethered to the proteins via flexible linkers.[10,11] However, the large size of a fluorophore can significantly perturb protein structure and function. This is particularly of concern when trying to monitor conformational changes in a crowded protein interior space, such as an enzyme active site. Moreover, the flexible tethers used for attaching large fluorophores provide the reporter molecules with still more conformational freedom, independent of actual protein conformational changes. In fact, this is a vastly under-appreciated and problematic issue; a recent study has pointed out that many observed reaction-coupled protein motions might be completely disconnected from the actual chemical transformation.[12] Thus, to measure subtle conformational changes in proteins, it is important to develop smaller fluorophores/quenchers that can be efficiently incorporated into the normal peptide backbone, thus limiting their degrees of freedom unrelated to changes in protein structure.

A biosynthetic method has been employed to site-specifically incorporate small fluorescent amino acids into proteins.[13–18] A few studies have incorporated a donor and an acceptor amino acids into a single protein, through decoding a four-base codon CGGG

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and a nonsense codon UAG in the presence of an aminoacyl-$tRNA_{CCCG}$ and an aminoacyl-$tRNA_{CUA}$.[19–22] Such small amino acid FRET pairs have been shown to be useful for studying the reorganization of protein structure[20–22] or for monitoring protein backbone cleavage.[19] In previous studies, we have incorporated a series of biphenyl-L-phenylalanine derivatives into *Escherichia coli* dihydrofolate reductase (DHFR) as a fluorescence donor and L-(7-hydroxycoumarin-4-yl)ethylglycine as an acceptor, to study DHFR conformational changes.[21,22] In these reports, all of the amino acids studied were well tolerated at position 17 of DHFR, which is sterically accessible. However, DHFR displayed a range of sensitivities to the individual amino acids at position 115, which points into the active site of DHFR. This finding demonstrated the importance of identifying fluorescent amino acids that can minimally perturb protein structures to permit study of their subtle conformational changes.

Among the most thoroughly investigated Trp analogues are the azatryptophans, which have proved to be almost ideal isosteric substitutes for natural tryptophan in cellular proteins.[23,24] Among the azaindoles under study, 4- and 7-azaindoles have exhibited the largest Stokes shifts in steady-state fluorescence measurements.[23,24] They are highly biocompatible and as azatryptophans they can be introduced into target protein sequences by ribosomal translation. Recently, N-methylated 4- and 7-azaindoles have been found to have fluorescence properties better than the parent 4- and 7-azaindoles because they retain the pronounced red shift characteristic of the parent 4- and 7-azaindoles, but with more intense fluorescence.[23]

Prompted by the need to develop smaller and sensitive fluorophores that can be readily accommodated within a protein without perturbation of the conformation and function of that protein, we have synthesized more hydrophobic N-methylated 4- and 7-azatryptophans and also two tricyclic tryptophan derivatives (Fig. 1).[25] The asymmetric syntheses of the Trp analogues were accomplished by a stereoselective strategy utilizing the Schöllkopf chiral reagent,[25] and the photophysical properties of these Trp analogues were characterized. While size is one important factor of FRET donor/acceptor pairs, intrinsic qualities of the molecules such as their rotational degrees of freedom and propensity to interact with water can also be important. In our previous studies, we have reported a series of hydrophobic amino acids as the fluorescence donor.[21,22] We have now explored a series of derivatives of tryptophan, which acts as an important probe for fluorescence analysis of protein structure and function. Spectral isolation of individual residues is typically difficult in proteins containing more than one tryptophan residue. Thus, it is desirable to substitute tryptophan by a fluorophore having different spectroscopic properties (quantum yield, excitation and emission wavelengths) while at the same time maintaining native protein conformation and function.

These Trp analogues possess many photophysical properties that are unique from normal Trp, suggesting that they can be used to selectively monitor a targeted conformational change even in the presence of multiple native Trp residues (Tables 1 and 2). In general, the Trp analogues induce minimal effects on the enzyme activity when incorporated into DHFR. Two analogues (**D** and **E**) having favorable fluorescence properties were used to demonstrate that these Trp derivatives can function as donors to form efficient FRET pairs with acridon-2-ylalanine (Acd, **G**)[26,27] as the acceptor. Thus, these compounds will have considerable value for a variety of fluorescence studies.

2. Results

2.1. Synthesis of fluorescent aminoacyl pdCpAs esters

The synthesis of the aminoacylated pdCpA derivative of amino acid **D** (Fig. 1) was accomplished starting from commercially available 7-azaindole which was first formylated under Duff reaction conditions[28] to yield aldehyde **1**, the latter of which was subjected to N-tosylation with *p*-toluenesulfonyl chloride (*p*-TsCl) and sodium hydride to afford **2** in 53% overall yield (Scheme 1).[29] Reduction of aldehyde **2** to alcohol **3** using $NaBH_4$ in EtOH proceeded almost quantitatively.[28] Chlorination of alcohol **3** with thionyl chloride afforded **4** in 81% yield.[30] Asymmetric synthesis of the amino acid precursor was carried out using the Schöllkopf reagent ((*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine).[31] Regioselective lithiation (*n*-BuLi, THF, −78 °C) of the chiral auxiliary produced the lithium enolate, which afforded the adduct **5** from **4** with high diastereoselectivity (only one diastereomer was detectable in the ^1H NMR and ^{13}C NMR spectra). N-detosylation of **5** was performed using cesium carbonate in 2:1 THF/MeOH to yield **6** in 39% yield.[32] N-methylation of **6** with methyl iodide and sodium hydride afforded **7** in 83% yield.[29] Mild hydrolysis (2 N HCl) afforded the α-substituted amino acid methyl ester **8**[31] which was protected as the *N*VOC carbamate to yield **9** in 62% overall yield.[21] Methyl ester **9** was then hydrolyzed to afford the free acid which was subsequently treated with chloroacetonitrile to afford the requisite cyanomethyl ester **10** in 71% yield.[21]

In order to evaluate the properties of **D** as a constituent of the protein DHFR, this amino acid was used to activate a suppressor tRNA (Scheme 2). Treatment of cyanomethyl ester **10** with the tris(tetrabutylammonium) salt of pdCpA[33] in anhydrous DMF

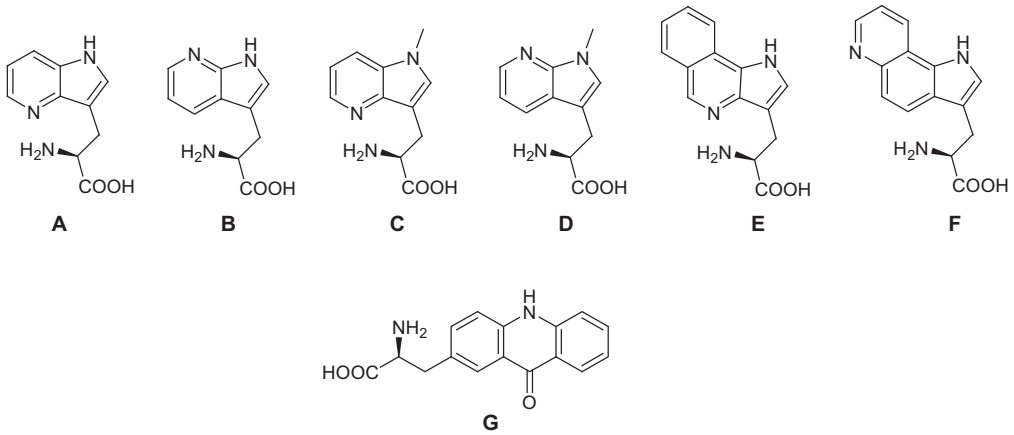

Figure 1. Tryptophan analogues (**A**–**F**) and L-acridon-2-ylalanine (Acd, **G**) which were incorporated into dihydrofolate reductase.