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Detection of Dihydrofolate Reductase Conformational Change by FRET Using Two Fluorescent Amino Acids

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Supporting Information

ABSTRACT: Two fluorescent amino acids, including the novel fluorescent species 4-biphenyl-L-phenylalanine (**1**), have been incorporated at positions 17 and 115 of dihydrofolate reductase (DHFR) to enable a study of conformational changes associated with inhibitor binding. Unlike most studies involving fluorescently labeled proteins, the fluorophores were incorporated into the amino acid side chains, and both probes [**1** and L-(7-hydroxycoumarin-4-yl)ethylglycine (**2**)] were smaller than fluorophores typically used for such studies. The DHFR positions were chosen as potentially useful for Förster resonance energy transfer (FRET) measurements on the basis of their estimated separation (17−18 Å) and the expected change in distance along the reaction coordinate. Also of interest was the steric accessibility of the two sites: Glu17 is on the surface of DHFR, while Ile115 is within a folded region of the protein. Modified DHFR I (**1** at position 17; **2** at position 115) and DHFR II (**2** at position 17; **1** at position 115) were both catalytically competent. However, DHFR II containing the potentially rotatable biphenylphenylalanine moiety at sterically encumbered position 115 was significantly more active than DHFR I. Irradiation of the modified DHFRs at 280 nm effected excitation of **1**, energy transfer to **2**, and emission by **2** at 450 nm. However, the energy transfer was substantially more efficient in DHFR II. The effect of inhibitor binding was also measured. Trimethoprim mediated concentration-dependent diminution of the emission observed at 450 nm for DHFR II but not for DHFR I. These findings demonstrate that amino acids containing small fluorophores can be introduced into DHFR with minimal disruption of function and in a fashion that enables sensitive monitoring of changes in DHFR conformation.

Förster resonance energy transfer (FRET) has been utilized extensively to monitor conformational changes in macromolecules such as nucleic acids and proteins[1] as well as intermolecular association between macromolecules by measuring changes in the efficiency of energy transfer between a donor and acceptor:[2] the fluorophore donor is excited by irradiation, and the absorbed energy is transferred to the (fluorescent or quenching) acceptor in a nonradiative process. Distance measurements made by FRET typically range from 41 to 73 Å, although the use of dye−quencher pairs has enabled the measured distance to be ∼23 Å.[3] The fluorophores are typically large polycyclic aromatic molecules, and they are generally attached to the macromolecule by flexible tethers, giving them conformational freedom independent of conformational changes in the macromolecule.

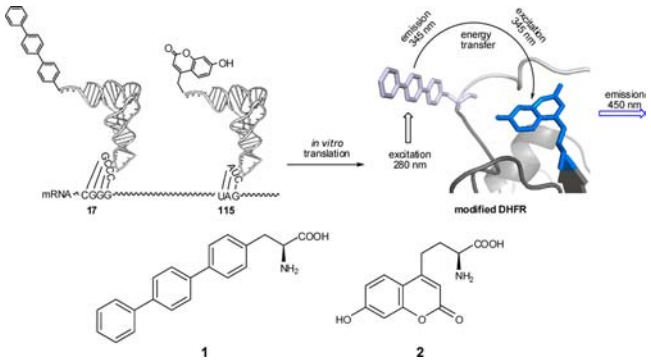
We recently described the use of dihydrofolate reductase (DHFR) containing two pyrenylalanines to measure protein dynamics via the observation of excimer formation.[4] The distance changes measured in DHFR were on the order of several angstroms.[4,5] In an effort to develop a complementary technique enabling the measurement of conformational changes in proteins over somewhat longer distances, we explored the use of FRET. The smaller distances of interest in comparison with those typically measured by FRET argued for the attachment of the dyes close to the protein backbone with fewer degrees of conformational freedom. This in turn would necessitate the use of acceptors and donors sterically similar to the side chains of proteinogenic amino acids. The use of fluorescent acceptors provides qualitative assurance of energy transfer through the longer-wavelength emission from the acceptor fluorophore[6] and has been reported to have higher sensitivity than dye−quencher systems and to enable ratiometric analysis, which is generally superior to intensity-based measurements.[7,8]

Proteins containing fluorescent amino acids have been reported,[9] and a few studies have employed proteins with two such amino acids to record large changes in the proximity of the fluorescent amino acids resulting from protein backbone cleavage[10] or extensive reorganization of protein structure.[11] In the present work, the fluorescent amino acids 4-biphenyl-L-phenylalanine (**1**) and L-(7-hydroxycoumarin-4-yl)ethylglycine (**2**)[12] were used to explore more subtle conformational changes in *Escherichia coli* DHFR. These amino acids were of interest because of the relatively small sizes and complementary shapes of their side chains. **1** is a para-substituted phenylalanine having two biphenyl linkages, which should permit some conformational mobility for inclusion within folded protein structures. **2** has a bicyclic side chain whose dimensions do not differ dramatically from those of tryptophan, and it has been reported to have a high fluorescence quantum yield and to be sensitive to pH and solvent polarity.[13] Critically, the excitation and emission maxima for **1**

Received: March 25, 2013
Published: August 13, 2013

were found to be at 280 and 345 nm, respectively, while the comparable values for **2** are 345 and 450 nm. This suggested that it should be possible to selectively excite the 4-biphenyl-L-phenylalanine moiety in a modified DHFR containing both species by irradiation at 280 nm and to observe fluorescence from the coumarin moiety at 450 nm due to FRET (Scheme 1).

Scheme 1. Strategy Employed for Incorporation of 1 and 2 into DHFR at Positions 17 and 115, respectively



The amino acid residues chosen for substitution in DHFR were Glu17 and Ile115, which were estimated to be 17.7 Å apart and potentially could undergo a change in relative distance of ~1.1 Å along the collective reaction coordinate.[14] The calculated FRET R_0 value for **1** and **2** was 22 Å. Additionally, as may be appreciated readily from the crystal structure of DHFR (Figure 1), Glu17 is at the enzyme surface and has been shown to tolerate

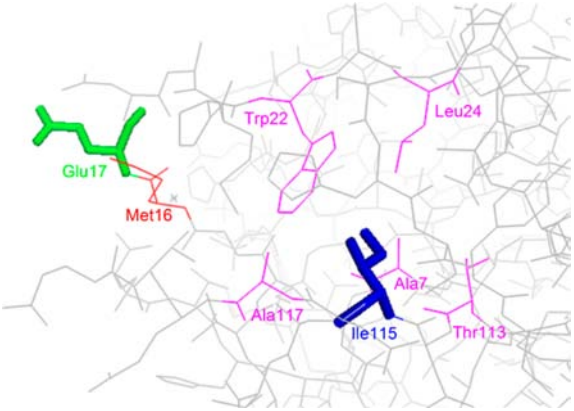
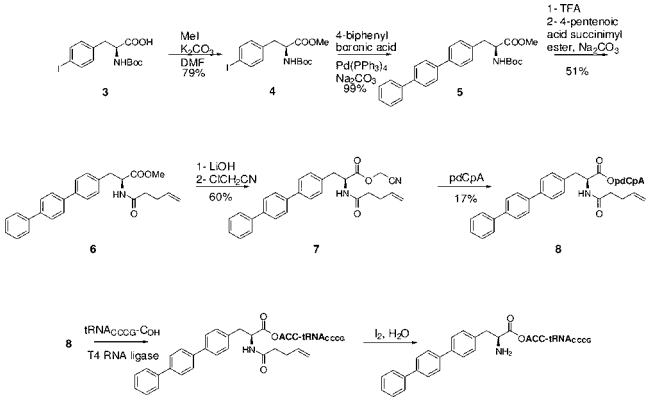


Figure 1. Structure of wild-type *E. coli* DHFR (PBD entry 1RA1), with Glu17 shown in green and Ile115 in blue. Met16, which is spatially close to the Glu17 side chain (≤4 Å), is shown in red, and Ala7, Trp22, Leu24, Thr113, and Ala117, which are close to the Ile115 side chain (≤4 Å), are shown in magenta.

the attachment of a fluorescence quencher,[15] while Ile115 is within a folded region of the protein where changes in side-chain structure might be expected to affect the protein folding to varying degrees. Nonetheless, it was anticipated that structural changes could be made without dramatically affecting the structure or function of DHFR.

The strategy employed to prepare the modified DHFRs is illustrated for DHFR I in Scheme 1. Plasmids expressing the mRNA for DHFR were modified to contain the four-base codon CGGG[16] in lieu of the Glu17 codon GAA, and the nonsense codon UAG in place of the Ile115 codon AUC. 4-Biphenyl-L-phenylalanyl-$tRNA_{CCCG}$ was used to suppress the CGGG codon, while L-(7-hydroxycoumarin-4-yl)ethylglycinyl-$tRNA_{CUA}$ was employed for UAG codon suppression. In the synthesis of 4-biphenyl-L-phenylalanyl-$tRNA_{CCCG}$ (Scheme 2), the key step

Scheme 2



was Suzuki coupling of 4-biphenylboronic acid and protected *p*-iodophenylalanine (**4**) in the presence of $Pd(PPh_3)_4$, which afforded *N*-Boc-4-biphenyl-L-phenylalanine methyl ester (**5**) in 99% yield. Subsequent replacement of the Boc protecting group with an *N*-pentenoyl group afforded **6** in 51% yield, and replacement of the methyl ester with a cyanomethyl ester gave *N*-(4-pentenoyl)-4-(1′,1″-biphenyl-4′-yl)-L-phenylalanine cyanomethyl ester (**7**) in 60% yield. **7** was then treated with 5′-phosphoro-2′-deoxycytidylyl[3′→5′]adenosine (pdCpA),[17] affording aminoacylated dinucleotide **8**. Incubation of this dinucleotide with an abbreviated $tRNA_{CCCG}$-C_{OH} transcript lacking the 3′-terminal nucleotides C and A in the presence of T4 RNA ligase[18] afforded 4-biphenyl-L-phenylalanyl-$tRNA_{CCCG}$; the course of the ligation reaction was monitored by polyacrylamide gel electrophoresis under acidic conditions.[19] The preparation of L-(7-hydroxycoumarin-4-yl)ethylglycinyl-$tRNA_{CUA}$ employed the same overall strategy; the synthesis of the requisite aminoacyl-pdCpA intermediate **11** was carried out as shown in Scheme S1 in the Supporting Information (SI) by modification of a reported[12] procedure.

The first modified DHFR to be synthesized was DHFR I containing **1** at position 17 and **2** at position 115 (Figure S1 in the SI). Also prepared were the two DHFRs containing only one of the two modified amino acids at the same position. The modified DHFRs were purified by successive chromatography on Ni-NTA and then DEAE-Sepharose columns. The ability of these modified enzymes to effect the reduction of dihydrofolate to tetrahydrofolate was studied to ensure that the isolated proteins were properly folded. Substitution of position 17 with **1** was well-tolerated, reducing the activity to 76% relative to the wild-type enzyme (Table S1 and Figure S2). In contrast, the introduction of **2** at position 115 reduced the consumption of NADPH to 23% relative to the wild-type enzyme. The doubly modified DHFR was 20% as active as the wild-type enzyme.

The singly and doubly modified DHFRs were then employed to determine whether FRET between the two fluorescent amino acids could be observed. Upon irradiation at 280 nm, the singly modified DHFR containing **1** at position 17 exhibited an emission spectrum centered at 345 nm (Figure 2, green curve). In contrast, the singly modified DHFR containing **2** at position 115 failed to emit significantly at any wavelength when irradiated at 280 nm (black curve) but gave a strong emission at 450 nm

dx.doi.org/10.1021/ja403007r | J. Am. Chem. Soc. 2013, 135, 12924−12927

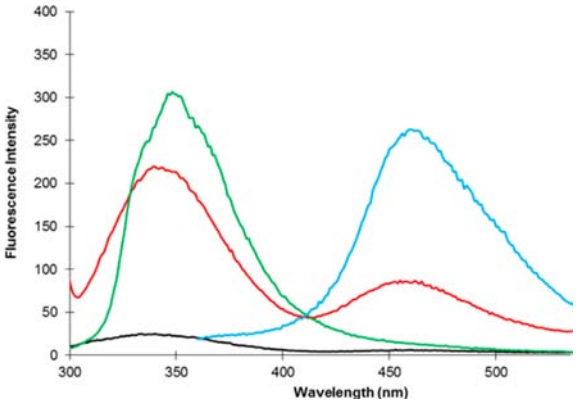


Figure 2. Fluorescence of modified DHFRs (0.5 μM) measured at pH 8.0: modified DHFR containing **1** at position 17 with λ_{ex} = 280 nm (green); DHFR I containing **1** at position 17 and **2** at position 115 with λ_{ex} = 280 nm (red); modified DHFR containing **2** at position 115 with λ_{ex} = 280 nm (black) and λ_{ex} = 340 nm (cyan).

when irradiated at 340 nm (cyan curve). This demonstrated that irradiation at 280 nm in the doubly substituted DHFR should effect excitation only of the 4-biphenyl-L-phenylalanine moiety. When irradiated at 280 nm, doubly modified DHFR I produced emission peaks at 345 and 450 nm (red curve), with the longer wavelength emission being of lesser intensity. This established the successful FRET from excited **1** to **2**.

Also prepared using an analogous strategy was isomeric DHFR II having **2** at position 17 and **1** at position 115. The singly modified DHFRs having one or the other of these amino acids were also prepared. Experiments illustrating activation of the suppressor tRNAs as well as the synthesis and purification of the proteins are shown in Figures S3−S5, respectively. Interestingly, the single modification of DHFR with amino acid **2** at position 17 or amino acid **1** at position 115 had essentially no effect on the activity of DHFR (Table S1 and Figure S2). Doubly substituted DHFR II retained 50% of the activity of the wild-type enzyme.

The use of DHFR II in a FRET experiment gave results qualitatively similar to those obtained with DHFR I (Figure 3). Irradiation at 280 nm to excite the DHFR containing **1** at position 115 gave a single emission centered at 345 nm. Similar excitation of the DHFR containing **2** at position 17 gave minimal emission at 450 nm. Excitation of doubly modified DHFR II gave

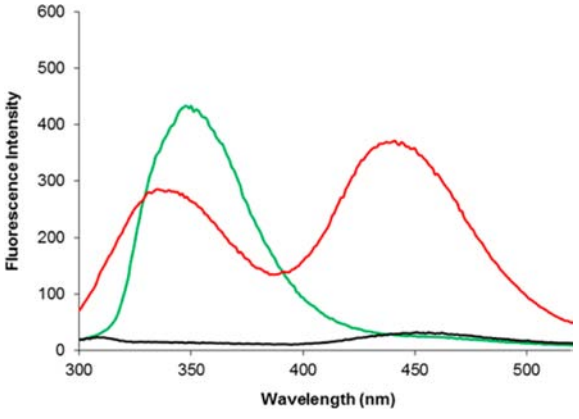


Figure 3. Fluorescence of modified DHFRs (0.5 μM) measured at pH 8.0 with λ_{ex} = 280 nm: singly modified DHFR containing **1** at position 115 (green); DHFR II containing **2** at position 17 and **1** at position 115 (red); singly modified DHFR containing **2** at position 17 (black).

two emission peaks centered at 345 and 450 nm. However, in this case the emission at 450 nm was the stronger of the two. This established both the greater FRET efficiency of DHFR II relative to DHFR I and the value of ratiometric analysis in establishing the relative FRET efficiencies.[8] Essentially the same results were obtained at pH 7.0, but the fluorescence emission was dramatically diminished at pH 6.0 (Figure S6).

The FRET efficiency E depends on both the distance between the fluorophores and the dipole orientation factor (κ^2). In the present case, the FRET donor and acceptor do not rotate freely, so dipole orientation can be important to E. We suggest that **2** can be accommodated less well at position 115 than **1**, which has rotatable biphenyl linkages, and that the resulting distortion in the structure of DHFR results in the lower FRET and enzymatic efficiencies of DHFR I compared with DHFR II.[20]

The ability of the modified DHFRs to monitor changes in protein conformation was studied by adding the DHFR inhibitor trimethoprim (TMP). When 0.5 μM DHFR I was treated with excess TMP, the emission intensities at 345 and 450 nm decreased in a concentration-dependent fashion (Figure S9). In contrast, when 0.5 μM DHFR II was treated with TMP, the intensity of the emission at 345 nm remained constant while that at 450 nm decreased in a concentration-dependent fashion (Figure 4), reflecting a decrease in E as a consequence of TMP

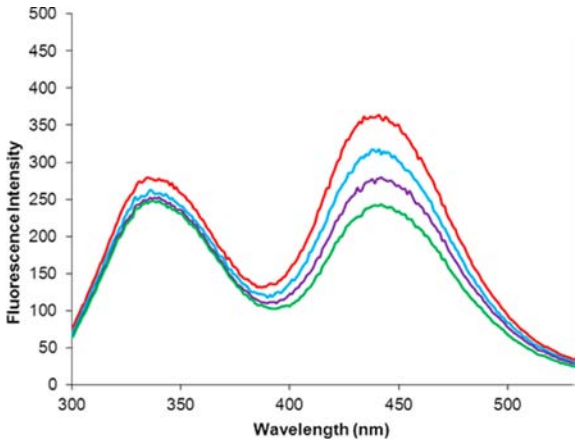


Figure 4. Effect of the DHFR inhibitor trimethoprim on the fluorescence emission spectrum of 0.5 μM modified DHFR II containing **2** at position 17 and **1** at position 115 following excitation at 280 nm. Shown are emission spectra of DHFR II alone (red) and after addition of (blue) 2, (purple) 4, and (green) 6 μM TMP.

addition.[20] Thus, for DHFR II, the change in protein structure occasioned by the addition of TMP can be monitored conveniently by FRET. The binding of TMP and numerous other DHFR inhibitors is known to produce only modest changes in the structure of DHFR,[21,22] far smaller than those ordinarily studied by FRET. Thus, the strategy employed here involving the careful placement of multiple small fluorescent amino acids within a protein holds promise for the monitoring of small conformational changes, such as those that occur during the catalytic cycle.

It may be noted that the use of suppressor tRNAs activated with fluorescent amino acids to introduce fluorophores into proteins has some important advantages relative to other techniques for producing fluorescently labeled proteins. For example, post-translational derivatization of cysteine residues is not position-selective, affording mixtures of products as well as unmodified cysteine residues that failed to undergo derivatiza-

tion with the fluorophore. The latter is also a potential problem with proteins prepared using unnatural amino acids having unique functional groups that can undergo chemoselective modification with fluorophores following translation.[23] In comparison, the use of amino acids with fluorescent side chains small enough to permit efficient utilization by the ribosome enables uniform, site-specific labeling to be verified.[24]

In conclusion, we have demonstrated the specific incorporation of two fluorescent amino acids into *E. coli* dihydrofolate reductase at sites anticipated to undergo a limited change in distance from each other along the reaction coordinate. One of the sites (Glu17) was known from earlier work to tolerate the introduction of a fluorescence quencher without significant loss of enzyme function, while the other (Ile115) was known from crystallographic studies to be more constrained sterically. DHFR I containing fluorescent amino acids **1** and **2** at positions 17 and 115, respectively, retained catalytic competence but exhibited an 80% reduction in the rate of NADPH oxidation. In contrast, the introduction of **2** at position 17 had no effect on the rate of NADPH oxidation, nor did the substitution of **1** at position 115. Doubly modified DHFR II with **2** at position 17 and **1** at position 115 retained 50% of the activity of wild-type DHFR. This verified our hypothesis concerning the need to choose the appropriate amino acid for introduction at position 115 to achieve optimal fluorescent labeling. While both doubly modified DHFRs emitted light at 450 nm when the 4-biphenyl-L-phenylalanine moiety was excited at 280 nm, DHFR II exhibited much more efficient FRET than DHFR I. Additionally, only DHFR II exhibited a measurable, dose-dependent change in FRET when treated with the inhibitor trimethoprim.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the NIH (GM 092946).

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(22) This is illustrated in Figure S10 for the effects of binding of NADPH and methotrexate to *E. coli* DHFR.

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