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Cyanotryptophans as Novel Fluorescent Probes for Studying Protein Conformational Changes and DNA−Protein Interaction

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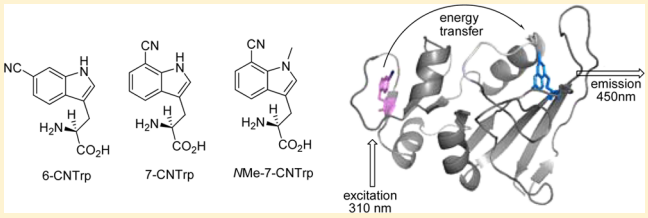
Supporting Information

ABSTRACT: Described herein are the syntheses and photophysical characterization of three novel cyanotryptophans, and their efficient incorporation into proteins as fluorescent probes. Photophysical characteristics indicated that each was significantly brighter and red-shifted in fluorescence emission relative to tryptophan. Each analogue was used to activate a suppressor tRNA transcript and was incorporated with good efficiency into two different positions (Trp22 and Trp74) of *Escherichia coli* dihydrofolate reductase (*ec*DHFR). The Trp analogues could be monitored selectively in the presence of multiple native Trp residues in DHFR. 6-CNTrp (**A**) formed an efficient Förster resonance energy transfer (FRET) pair with L-(7-hydroxycoumarin-4-yl)ethylglycine (HCO, **D**) at position 17. Further, 6-CNTrp (**A**) was incorporated into two DNA binding proteins, including the Klenow fragment of DNA polymerase I and an RNA recognition motif (RRM2) of heterogeneous nuclear ribonucleoprotein L-like (hnRNP LL). Using these proteins, we demonstrated the use of FRET involving **A** as a fluorescence donor and benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione 2′-deoxyriboside (T_f) or 4-aminobenzo[*g*]quinazoline-2-one 2′-deoxyriboside (C_f) as fluorescent acceptors to study the binding interaction of the Klenow fragment with duplex DNA oligomers (labeled with T_f), or the domain-specific association between hnRNP LL and the *BCL2* i-motif DNA (labeled with C_f). Thus, the non-natural amino acid could be used as a FRET partner for studying protein−nucleic acid interactions. Together, these findings demonstrate the potential utility of 6-CNTrp (**A**) as a fluorescence donor for the study of protein conformational events.



Understanding protein structural dynamics is currently one of the great challenges in biochemistry. Fluorescence measurements can be made on a nanosecond time scale, and the sensitivity of fluorescence techniques allows the study of biophysical changes in proteins.[1,2] Distance-dependent phenomena, such as Förster resonance energy transfer (FRET), have greatly facilitated the study of protein conformation and function by allowing the measurement of changes in the efficiency of energy transfer between a donor and an acceptor.[1,2] Acceptor excitation requires that the donor emission wavelength overlap with the excitation wavelength of the acceptor. The acceptor may be a fluorescent molecule, leading to another longer wavelength emission, or a quencher.[2−6] FRET systems used to monitor protein motions may include variants of green fluorescent protein (GFP),[1,7−9] or large polycyclic aromatic molecules.[10,11] However, because of the large size of the fluorophores typically employed, it may not be possible to observe modest conformational changes in the labeled proteins. Additionally, the large polycyclic aromatic fluorophores/quenchers are often attached to the proteins via flexible linkers[10,11] that provide the reporter molecules with increased conformational freedom even in the absence of any actual protein conformational change. This additional flexibility of the probe thus reduces the ability to measure subtle conformational changes in proteins, such as those involved in the catalytic cycle of many enzymes. If optical probes could be made sufficiently small, they could provide the resolution necessary to analyze protein motions reliably. Thus, it is important to develop smaller fluorophores/quenchers to allow the measurement of small conformational changes in proteins and to incorporate them within the normal peptide backbone, thereby limiting their degrees of freedom unrelated to changes in protein conformation.

The proteinogenic amino acid tryptophan (Trp) is commonly used as a probe for fluorescence analysis.[12] However, in proteins containing more than one tryptophan residue, spectral isolation of individual Trps is typically difficult. In such cases, it would be desirable to substitute tryptophan with a fluorophore having different spectroscopic properties (quantum yield and excitation and emission wavelengths) while at the same time maintaining the dimensions of Trp and the native protein conformation. This study investigated the fluorescence properties of the Trp

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analogues 6-cyanotryptophan (6-CNTrp, **A**) and 7-cyanotryptophan (7-CNTrp, **B**). N-Methylated 7-cyanotryptophan (*N*Me-7-CNTrp, **C**) was also studied, as recent findings suggest that N-methylation enhances the fluorescence of 7-azatryptophan.[13] As it is more hydrophobic than **B**, analogue **C** may also stabilize the hydrophobic core of proteins. The asymmetric syntheses of the Trp analogues were accomplished by a stereoselective strategy utilizing the Schöllkopf chiral reagent,[14,15] and the photophysical properties of these Trp analogues were characterized. They possess many photophysical properties that differ from those of Trp itself, suggesting that they may be useful to selectively monitor a targeted conformational change even in the presence of multiple native Trp residues (Table 1). A biosynthetic method involving the use of misacylated suppressor tRNAs was employed to incorporate the new Trp analogues into three proteins in a site-specific fashion.[16−21]

Table 1. Absorption and Fluorescence Properties of N-Acetylated Methyl Esters of Trp Analogues in MeOH

	$\lambda_{abs,max}$ (nm)	ε (M^{-1} cm^{-1})	λ_{ex} (nm)	λ_{em} (nm)	Φ_F	brightness
tryptophan	280	6900	280	340	0.18	1240
A	290	10300	290	370	0.53	5460
B	310	8100	310	390	0.40	3240
C	315	8000	315	395	0.41	3280

50nm, 0.23%

MATERIALS AND METHODS

The chemicals used for synthesis were purchased from Aldrich Chemical Co., Sigma Chemical Co., or Combi-Blocks. THF was distilled under argon from sodium-benzophenone ketyl, and CH_2Cl_2 was distilled under argon from calcium hydride. Ni-NTA agarose was obtained from Qiagen Inc. DNA oligonucleotides were purchased from Integrated DNA Technologies. DEAE-Sepharose, ammonium persulfate, acrylamide, *N*,*N*-methylene-bis-acrylamide, acetic acid, potassium glutamate, ammonium acetate, dithiothreitol, magnesium acetate, phosphoenolpyruvate, *Escherichia coli* tRNA, isopropyl β-D-thiogalactopyranoside (IPTG), ATP, GTP, CTP, UTP, cAMP, amino acids, rifampicin, formamide, Tris, and SDS were obtained from Sigma-Aldrich. [^{35}S]Methionine (1000 Ci/mmol, 10 μCi/μL) was purchased from PerkinElmer Inc. Protease inhibitor (complete, EDTA free) was obtained from Boehringer Mannheim Corp. T4 DNA ligase, T4 RNA ligase, T4 polynucleotide kinase, deep vent DNA polymerase, calf intestinal alkaline phosphatase, and restriction endonucleases *Nde*I and *Bam*HI were purchased from New England Biolabs Inc. Plasmid pET-28a(+) was from EMD Millipore.

Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 instrument equipped with ImageQuant version 5.2 from Molecular Dynamics. UV spectral measurements were taken using a Cary 60 UV/vis spectrometer. Fluorescence was monitored using a Varian Cary Eclipse Fluorescence Spectrophotometer.

Synthesis of pdCpA and Phosphoramidite Derivatives. The route employed for the synthesis of the pdCpA derivative of amino acid **A** is outlined in Scheme 1. The syntheses of the pdCpA derivatives of amino acids **B** and **C** are outlined in Schemes S1 and S2, respectively, and the experimental procedures and compound characterizations are provided in the Supporting Information. The synthesis of the aminoacylated pdCpA derivative of **D** has been reported previously,[22] as have the syntheses of T_f and C_f.[23] For incorporation into the requisite DNA sequences, their respective phosphoramidite derivatives[23] were prepared.

*3-Formyl-1-tosyl-1H-indole-6-carbonitrile (**2**).* Oxalyl chloride (0.60 mL, 0.89 g, 7.00 mmol) was added in a dropwise manner to 3 mL of cooled (ice bath) DMF while it was being stirred. The reaction mixture was then stirred at 0 °C for 1 h. A solution of 1.00 g (7.03 mmol) of 6-cyanoindole in 3 mL of DMF was then added to the reaction mixture in a dropwise manner. The resulting reaction mixture was stirred at room temperature for 5 h. A 2 N aqueous solution of 2 mL of NaOH was then added, and the reaction mixture was heated at 100 °C for 10 min. The cooled reaction mixture was diluted with 50 mL of brine. The mixture was then extracted with two 50 mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure to give 3-formyl-1*H*-indole-6-carbonitrile (**1**) as a yellow solid, which was used directly in the next step without further purification.

To a stirred solution containing 956 mg (5.62 mmol) of **1** in 20 mL of anh DMF at 0 °C was added 450 mg (11.2 mmol) of NaH (60% suspension in mineral oil). The reaction mixture was stirred at 0 °C for 10 min under argon, and then 1.50 g (7.86 mmol) of *p*-toluenesulfonyl chloride was added. The reaction mixture was stirred at 0 °C under argon for 3 h, diluted with 150 mL of brine, and extracted with two 50 mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 cm × 4 cm). Elution with a 4:1 hexanes/ethyl acetate solvent gave the desired product **2** as a yellow solid: yield 1.55 g (68% for two steps); silica gel TLC R_f = 0.82 (1:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.37 (s, 3H), 7.33 (d, 2H, J = 8.4 Hz), 7.56 (d, 1H, J = 8.4 Hz), 7.86 (d, 2H, J = 8.4 Hz), 8.26 (s, 1H), 8.31 (d, 1H, J = 8.4 Hz), 8.42 (s, 1H), 10.10 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.7, 109.4, 117.6, 118.9, 121.6, 123.6, 127.3, 128.0, 129.5, 130.7, 133.7, 134.1, 138.5, 147.0, 184.9; mass spectrum (APCI) m/z 325.0650 $(M + H)^+$ ($C_{17}H_{13}N_2SO_3$ requires m/z 325.0647).

*3-(Hydroxymethyl)-1-tosyl-1H-indole-6-carbonitrile (**3**).* To a suspension of 1.29 g (4.46 mmol) of 3-formyl-1-tosyl-1*H*-indole-6-carbonitrile (**2**) in 15 mL of EtOH was added 339 mg (8.92 mmol) of $NaBH_4$, and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with 100 mL of saturated aqueous $NaHCO_3$, and the formed precipitate was filtered and dried under vacuum. 3-(Hydroxymethyl)-1-tosyl-1*H*-indole-6-carbonitrile (**3**) was obtained as an off-white solid: yield 1.11 g (80%); silica gel TLC R_f = 0.41 (1:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H), 4.68 (s, 2H), 7.18 (d, 2H, J = 8.0 Hz), 7.62 (dd, 1H, J = 8.0 and 1.6 Hz), 7.62 (d, 1H, J = 8.4 Hz), 7.64 (s, 1H), 7.69 (d, 2H, J = 8.8 Hz), 8.20 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.4, 55.8, 107.4, 117.8, 119.4, 121.1, 122.7, 126.2, 126.8, 126.9, 130.2, 132.9, 134.2, 134.5, 145.8; mass spectrum (GCMS), m/z 326.0711 $(M)^+$ ($C_{17}H_{14}N_2SO_3$ requires m/z 326.0725).

*3-(Chloromethyl)-1-tosyl-1H-indole-6-carbonitrile (**4**).* To a cooled (−10 °C) solution containing 1.11 g (3.40 mmol) of **3** in 20 mL of anhydrous CH_2Cl_2 and 2.18 mL (1.58 g, 15.6 mmol) of Et_3N was added dropwise 0.58 mL (947 mg, 7.93 mmol) of $SOCl_2$. The reaction mixture was allowed to warm slowly to room temperature and then stirred for 6 h, diluted with 50 mL of brine, and extracted with two 50 mL portions of EtOAc. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 cm × 2 cm). Elution with a 3:1

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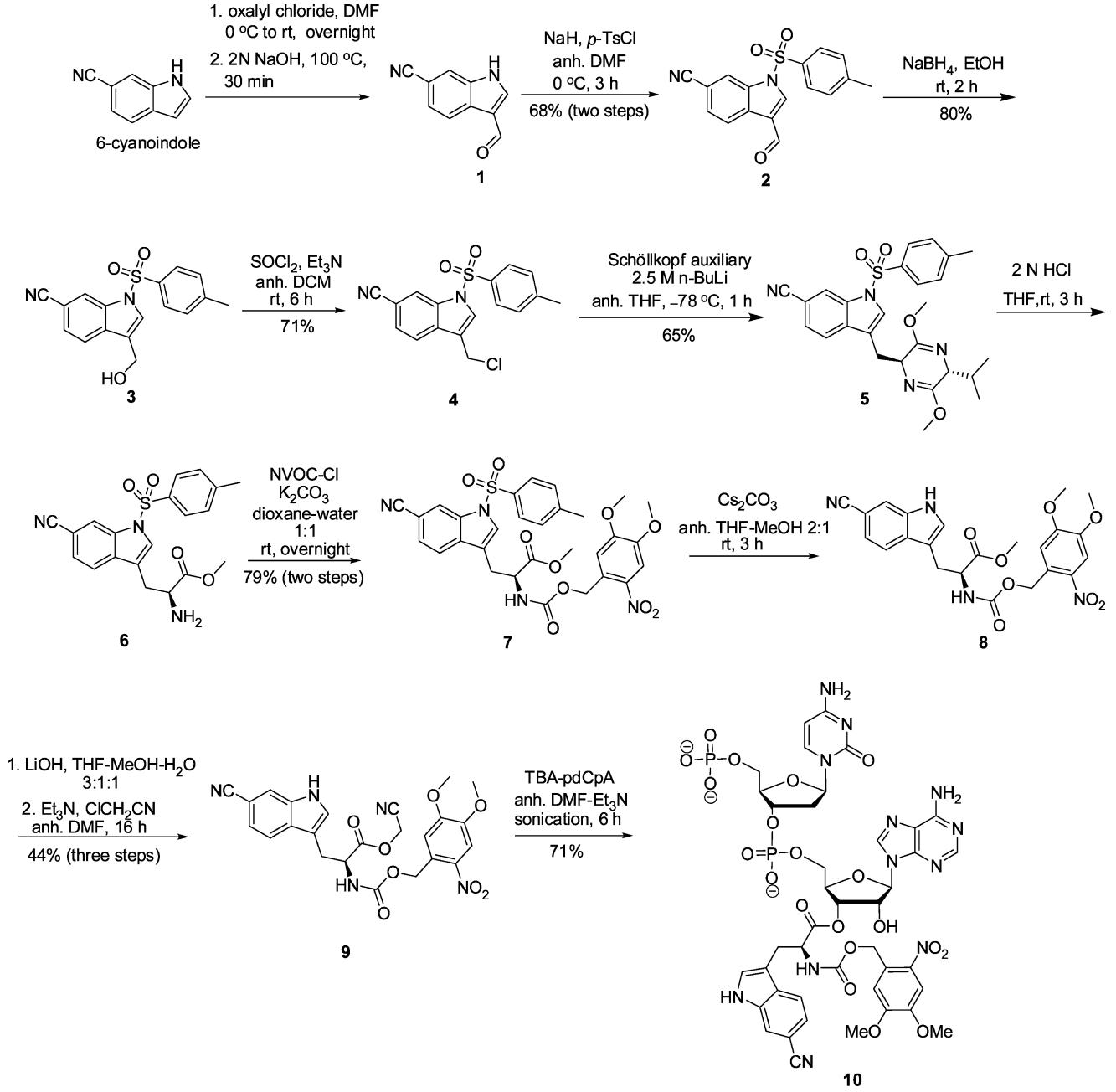
Scheme 1. Synthetic Route Employed for the Preparation of Protected Amino Acid A and Its Aminoacyl-pdCpA



hexanes/ethyl acetate solvent gave the expected product **4** as a yellowish solid: yield 832 mg (71%); silica gel TLC R_f = 0.91 (1:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H), 4.71 (s, 2H), 7.25 (d, 2H, J = 8.0 Hz), 7.48 (dd, 1H, J = 8.4 and 1.2 Hz), 7.69 (d, 1H, J = 8.4 Hz), 7.75 (s, 1H), 7.77 (s, 2H), 8.26 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.6, 36.6, 108.3, 118.0, 118.8, 119.3, 120.9, 126.4, 127.0, 128.2, 130.4, 132.2, 134.2, 134.4, 146.1; mass spectrum (APCI) m/z 345.0463 $(M + H)^+$ ($C_{17}H_{14}N_2O_2SCl$ requires m/z 345.0465).

*3-{[(2S,5R)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl]methyl}-1-tosyl-1H-indole-6-carbonitrile (**5**).* To a stirred solution containing 0.62 mL (650 mg, 3.53 mmol) of Schöllkopf's reagent in 10 mL of anh THF at −78 °C was added 0.45 mL (308 mg, 4.82 mmol) of 2.5 M *n*-BuLi. The reaction mixture was stirred at −78 °C for 30 min under argon, and then a solution containing 1.10 g (3.21 mmol) of **4** in 10 mL of anhydrous THF was added. The reaction mixture was stirred at −78 °C for 30 min under argon, then diluted with 50 mL of saturated aqueous NH_4Cl, and extracted with two 50 mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 cm × 2 cm). Elution with 3:1 hexanes/ethyl acetate solvent gave the expected product **5** as a yellow oil: yield 1.01 g (65%); silica gel TLC R_f = 0.89 (1:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.57 (d, 3H, J = 6.8 Hz), 0.85 (d, 3H, J = 7.2 Hz), 2.03−2.09 (m, 1H), 2.34 (s, 3H), 3.12−3.19 (m, 3H), 3.61 (s, 3H), 3.63 (s, 3H), 4.27−4.30 (m, 1H), 7.23 (d, 2H, J = 8.4 Hz), 7.41 (d, 1H, J = 8.0 Hz), 7.44 (s, 1H), 7.60 (d, 1H, J = 8.4 Hz), 7.67 (d, 2H, J = 8.0 Hz), 8.23 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 16.6, 19.0, 21.7, 28.9, 31.6, 52.4, 52.5, 55.5, 60.6, 107.4, 117.9, 118.5, 119.7, 121.1, 125.9, 126.8, 128.1, 130.3, 133.8, 134.8, 134.9,

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145.6, 161.9, 164.3; mass spectrum (APCI) m/z 493.1910 $(M + H)^+$ ($C_{26}H_{29}N_4SO_4$ requires m/z 493.1909).

*Methyl (S)-2-[(4,5-Dimethoxy-2-nitrobenzyloxy)-carbonylamino]-3-(6-cyano-1-tosyl-1H-indol-3-yl)-propionate (**7**).* To a stirred solution containing 468 mg (0.95 mmol) of **5** in 10 mL of THF at 0 °C was added 8 mL of 2 N aqueous HCl. The reaction mixture was stirred at room temperature for 3 h. The reaction mixture was then poured slowly into 30 mL of saturated aqueous $NaHCO_3$ and then extracted with two 30 mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. Methyl (*S*)-2-amino-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**6**) was obtained as a yellow solid and used directly in the next step without further purification. To a stirred solution containing 180 mg (0.45 mmol) of **6** in 2 mL of a 1:1 dioxane/water solvent was added 150 mg (1.08 mmol) of K_2CO_3 followed by 104 mg (0.48 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 14 h under argon, then diluted with 50 mL of brine, and extracted with two 50 mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 cm × 2 cm). Elution with 1:1 hexanes/ethyl acetate solvent gave the expected product **7** as a yellow oil: yield 256 mg (79% for two steps); silica gel TLC R_f = 0.38 (1:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.35 (s, 3H), 3.17−3.31 (m, 2H), 3.66 (s, 3H), 3.93 (s, 3H), 3.94 (s, 3H), 4.66−4.71 (m, 1H), 5.47−5.57 (m, 3H), 6.95 (s, 1H), 7.26 (d, 2H, J = 8.4 Hz), 7.44 (d, 1H, J = 8.0 Hz), 7.54 (s, 1H), 7.57 (s, 1H), 7.69−7.73 (m, 3H), 8.24 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.7, 27.7, 52.8, 54.0, 56.5, 56.6, 64.2, 108.0, 108.3, 110.4, 116.9, 118.1, 119.4, 120.4, 126.4, 126.89, 126.93, 127.4, 127.9, 130.3, 133.9, 134.0, 134.6, 146.0, 148.4, 153.7, 155.3, 171.5; mass spectrum (APCI) m/z 637.1590 $(M + H)^+$ ($C_{30}H_{29}N_4O_{10}S$ requires m/z 637.1604).

*Cyanomethyl (S)-2-[(4,5-Dimethoxy-2-nitrobenzyloxy)-carbonylamino]-3-(6-cyano-1-tosyl-1H-indol-3-yl)-propionate (**9**).* To a stirred solution containing 55.0 mg (0.08 mmol) of **7** in 1.5 mL of an anhydrous 2:1 THF/methanol solvent was added 56.5 mg (0.16 mmol) of Cs_2CO_3. The reaction mixture was stirred at room temperature for 3 h under argon, then diluted with 20 mL of brine, and extracted with two 50 mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 cm × 2 cm). Elution with ethyl acetate gave **8** as a yellow oil: yield 27 mg (64%); silica gel TLC R_f = 0.3 (1:1 hexanes/ethyl acetate). To a stirred solution containing 27 mg (0.05 mmol) of **8** in 1 mL of a 1:3:1 water/THF/methanol solvent was added 150 μL (3.59 mg, 0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h and then concentrated under diminished pressure. The residue was redissolved in 1 mL of anhydrous DMF, and 24.7 μL (18.1 mg, 0.15 mmol) of Et_3N was added followed by 10.0 μL (11.3 mg, 0.15 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C for 16 h. The residue was purified by chromatography on a silica gel column (10 cm × 2 cm). Elution with a 1:2 hexanes/ethyl acetate solvent gave the desired product **9** as a light yellow solid: yield 19 mg (44% for three steps); silica gel TLC R_f = 0.25 (1:1 hexanes/ethyl acetate); ^{1}H NMR (DMSO-d_6) δ 3.12−3.27 (m, 2H), 3.84 (s, 3H), 3.87 (s, 3H), 4.43−4.48 (m, 1H), 5.00 (s, 2H), 5.33 (ABq, 2H, J = 14.8 Hz), 7.10 (s, 1H), 7.31 (d, 1H, J = 8.4 Hz), 7.52 (s, 1H), 7.69−7.73 (m, 2H), 7.85 (s, 1H), 8.22 (d, 1H, J = 7.6 Hz), 11.5 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 26.1, 49.4, 54.5, 55.98, 56.03, 62.6, 102.3, 108.0, 110.2, 110.3, 115.5, 116.3, 119.2, 120.6, 121.1, 127.5, 128.6, 130.0, 134.8, 139.0, 147.6, 153.3, 155.5, 170.9; mass spectrum (APCI) m/z 508.1488 $(M + H)^+$ ($C_{24}H_{22}N_5O_8$ requires m/z 508.1468).

*(S)-2-[(4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino]-3-(6-cyano-1-tosyl-1H-indol-3-yl)propionic Acid pdCpA Ester (**10**).* To a stirred solution containing 5.20 mg (4.00 μmol) of pdCpA tetrabutylammonium salt in 100 μL of a 9:1 anhydrous DMF/triethylamine solvent was added 10.7 mg (21.0 μmol) of cyanomethyl (*S*)-2-[(4,5-dimethoxy-2-nitrobenzyloxy)-carbonylamino]-3-(6-cyano-1-tosyl-1*H*-indol-3-yl)propionate (**9**). The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 mm × 10 mm) using a 99:1 to 1:99 linear gradient of 50 mM aqueous ammonium acetate (pH 4.5)/acetonitrile. The retention time of the desired product was 24.2 min. The fractions containing the product were lyophilized to afford **10** as a colorless solid: yield 2.9 mg (71%); mass spectrum (ESI) m/z 1085.2208 $(M - H)^-$ ($C_{41}H_{43}N_{12}O_{20}P_2$ requires m/z 1085.2192).

Measurement of Photophysical Properties of Tryptophan Derivatives A−C. The UV/vis absorption spectra (220−400 nm) were recorded using a Cary 60 UV/vis spectrophotometer. Fluorescence quantum yields (Table 1) of fluorescent compounds were determined using the gradient method.[24] Tryptophan derivatives **A**−**C** were dissolved in methanol. Solutions of each compound were made such that the UV absorptions at the maximal wavelength were 0.02, 0.04, 0.06, 0.08, and 0.1. Anthracene (Φ_F = 0.27; λ_{ex} = 340 nm) was used as a reference standard to calculate the fluorescence quantum yields of the tryptophan analogues according to the formula $\Phi_x = \Phi_s \times (\mathrm{Grad}_x \times n_x^2)/(\mathrm{Grad}_s \times n_s^2)$, where Grad is the gradient of the plot of integrated intensity versus absorbance, n is the refractive index of the solvent, s is the standard of known Φ_F, and x is the tested sample.[24]

Ligation of Suppressor $tRNA_{CUA}$-C_{OH} with Tryptophan Analogues and Deprotection of the *N*-NVOC Group. Yeast suppressor $tRNA^{Phe}_{CUA}$ was prepared as reported previously.[25] Briefly, the activation of abbreviated suppressor $tRNA_{CUA}$-C_{OH} was conducted in 200 μL (total volume) of 100 mM Hepes buffer (pH 7.5) containing 2.0 mM ATP, 15 mM $MgCl_2$, 200 μg of suppressor tRNA-C_{OH}, 4.0 absorbance units (A_{260}) of *N*-NVOC-protected aminoacyl-pdCpA (5−10-fold molar excess), 15% DMSO, and 400 units of T4 RNA ligase. After incubation at 37 °C for 1 h, the reaction was quenched by the addition of 20 μL of 3 M NaOAc (pH 5.2) followed by 600 μL of ethanol. The reaction mixture was incubated at −20 °C for 30 min and then centrifuged at 15000*g* and 4 °C for 30 min. The supernatant was carefully decanted, and the tRNA pellet was washed with 200 μL of 70% ethanol and then dissolved in 60 μL of RNase free H_2O. The efficiency of ligation was estimated by 8% denaturing polyacrylamide gel electrophoresis (PAGE) (pH 5.2).[26] The NVOC protecting group was removed by exposure to high-intensity UV light at 4 °C for 3 min.[27]

Preparation of Plasmid Constructs. The modified DHFR plasmids were obtained by site-directed mutagenesis, as described previously, using the wild-type DHFR plasmid.[28] The DNA primer GTAGATCGCGTTATCGGCATGCGGGAACGCCATGCCGTGGAACCTG was used for the incorporation of the four-base codon CGGG at position 17.[28] Two other mutant DHFR constructs were designed using the DNA primer GCATGGAAAACGCCATGCCGTAGAACCTGCCTGCCGATCTCGC to insert a TAG (amber suppressor) codon

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at position 22, whereas GGTACGGACGATCGCGTAACGTAGGTGAAGTCGGTGGATGAAGC was used to insert TAG at position 74.

The cDNA for hnRNP LL protein (542 amino acids) was obtained from GenBank (accession number NP_612403). The construct RRM2 was designed with a few extra native amino acids flanking the second RRM (RNA recognition motif) at both termini (G157−G272) and codons optimized for expression in *E. coli*. A 24-nucleotide (TGGTCTCACCCGCAGTTCGAAAAA) sequence was attached at the 3′-end of the construct for encoding an additional eight-amino acid (WSHPQFEK) peptide (Strep tag) for purification. RRM2 and DNA primers, CAACGACTCTTTCGACTACACCAAA and TCGTTACGGATAACGTTC, were used in a polymerase chain reaction (PCR) mutagenesis (Q_5 mutagenesis kit) for replacing W257 with phenylalanine, affording RRM2F. RRM2F-201TAG was prepared by subjecting RRM2F to an additional round of mutagenesis, with DNA primers TATCGTTATCTAGAAACGTAACGGTAT and CGCTGAACTTTACCAACC, for the replacement of F201 with a TAG codon.

The expression plasmid for the N-terminal hexa-His-tagged mutant Klenow fragment D355A[29] (denoted Klenow1 for the sake of simplicity) was constructed as follows. The gene for the Klenow fragment (amino acids 324−928 of DNA polymerase I) was cloned from *E. coli* strain B genomic DNA (Sigma) with forward primer 5′-GGCAGCCATATGATTTCTTATGACAACTACGTCACCATCC-3′ (includes the V324M mutation) and reverse primer 5′-GAATTCGGATCCTCAGTGCGCCTGATCCCAGTTTTCG-3′ using deep vent DNA polymerase. The PCR product and pET-28a(+) plasmid were doubly digested with restriction enzymes *Nde*I and *Bam*HI according to the manufacturer's conditions, and the plasmid was dephosphorylated using calf intestinal alkaline phosphatase. Following gel purification and extraction, the vector and insert were ligated using T4 DNA ligase at a 1:5 vector:insert ratio. Colonies resulting from transforming chemically competent DH5α cells with the ligation mixture were screened. The sequence of the final construct was verified by Sanger sequencing at the Genomics Core Facility at the Pennsylvania State University. Site-directed mutagenesis was then used to modify the plasmid DNA such that the expressed protein had a D355A mutation (i.e., Klenow1). Klenow1−484TAG was then prepared by subjecting Klenow1 to an additional round of mutagenesis (using the NEB Q_5 mutagenesis kit), with DNA primers CAAAAATCAATAGACCTTTAACCAGATTGC and CCTTTACCAGCAATCTCTTC, for the replacement of the codon in position 484 with a TAG codon.

***In Vitro* Translation of Protein.** *In vitro* translation of DHFR containing tryptophan analogues at positions 22 and 74 was done by using a method described previously.[30] The *in vitro* expression mixture (total volume of 500 μL) contained 50 μg of modified DHFR (TAG at positions 22 and 74 and/or CGGG at position 17) plasmid DNA, 200 μL of premix [35 mM Tris-acetate (pH 7.0), 190 mM potassium glutamate, 30 mM ammonium acetate, 2.0 mM dithiothreitol, 11 mM magnesium acetate, 20 mM phosphoenolpyruvate, 0.8 mg/mL *E. coli* tRNA, 0.8 mM IPTG, 20 mM ATP and GTP, 5 mM CTP and UTP, and 4 mM cAMP], each of the 20 proteinogenic amino acids (100 μM), 150 μCi of [^{35}S]-L-methionine, 10 μg/μL rifampicin, 150 μL of S-30 extract from *E. coli* strain BL21(DE3), 150 μg of deprotected misacylated $tRNA_{CUA}$, and/or 150 μg of deprotected coumarinyl-$tRNA_{CCCG}$.[30] The reaction mixture was incubated at 30 °C for 45 min for high yield.[31] Plasmid DNA containing the gene for wild-type DHFR was used as the positive control, and an abbreviated tRNA (tRNA-C_{OH}) lacking any amino acid was used as the negative control. Similarly, RRM2F-201TAG and Klenow1−484TAG were used in combination with tRNA activated with 6-CNTrp (**A**) in the *in vitro* protein translation reactions to produce RRM2F-201-**A** and Klenow1−484-**A**, respectively. Small aliquots of protein samples were treated with loading buffer and heated at 90 °C for 2 min. The samples were analyzed by 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) at 100 V for 2 h.

Protein Purification. The DHFR and Klenow polymerase analogues containing an N-terminal hexahistidine fusion peptide were purified by Ni-NTA chromatography.[32] The *in vitro* translation reaction mixture (500 μL) was diluted with 1.5 mL of 50 mM Tris-HCl (pH 8.0) containing 300 mM NaCl and 10 mM imidazole, and mixed gently with 150 μL of a 50% slurry of Ni-NTA resin at 4 °C for 1 h. Then the mixture was packed in a column and washed with 800 μL of 50 mM Tris-HCl (pH 8.0) containing 300 mM NaCl and 20 mM imidazole. Finally, the column containing the protein analogue was eluted three times with 200 μL of 50 mM Tris-HCl (pH 8.0) containing 30 mM NaCl and 150 mM imidazole. Three of the Ni-NTA column eluates were combined and applied to a 90 μL DEAE-Sepharose CL-6B column.[33] The column was washed successively with 300 μL of 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, then 300 μL of 50 mM Tris-HCl (pH 8.0) containing 200 mM NaCl, and finally three 100 μL portions of 50 mM Tris-HCl (pH 8.0) containing 300 mM NaCl. Small aliquots of each fraction were analyzed by 15% SDS−PAGE.

Purification of RRM2F and RRM2F-201-**A** was conducted by following the Strep-Tactin manufacturer's protocol (IBA Lifesciences). The protein was eluted with 100 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 2.5 mM desthiobiotin, and 1 mM EDTA. The proteins were visualized on a 15% Tris-glycine SDS−PAGE gel. The protein concentration was measured using two different methods. Briefly, the absorbance of protein samples was measured at two different wavelengths. The concentration was obtained by following the equation $C = (1.55A_{280}) - (0.76A_{260})$, where C is the concentration of the protein in milligrams per milliliter, A_{280} is the absorbance at 280 nm, and A_{260} is the absorbance at 260 nm. The protein concentration obtained by this method was comparable with BSA protein quantification assay results.

Enzymatic Activities of DHFR Analogues. The enzymatic activities of wild-type and modified DHFRs containing tryptophan derivatives were measured in 1 mL of MTEN buffer [50 mM MES, 25 mM Tris, 25 mM ethanolamine, 100 mM NaCl, 0.1 mM EDTA, and 10 mM β-mercaptoethanol (pH 7.0)]. MTEN buffer (0.97 mL) was mixed with 10 μL of 10 mM β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) and 100 ng of protein.[34] The mixture was incubated at 37 °C for 3 min. Then 20 μL of 5 mM dihydrofolate in MTEN buffer (pH 7.0) was added. The A_{340} value was monitored over a period of 10 min.

Fluorescence Emission Spectra of Protein Analogues and the FRET Experiment. The fluorescence emission spectra of modified proteins containing tryptophan derivatives were measured using a Varian Cary Eclipse Fluorescence Spectrophotometer with the excitation and emission slit widths set at 10 nm. The protein samples (0.2−1.0 μM) were excited with light at 310 nm, and the emission spectra were recorded. The FRET between Klenow1−484-**A** and the DNA oligomers was measured

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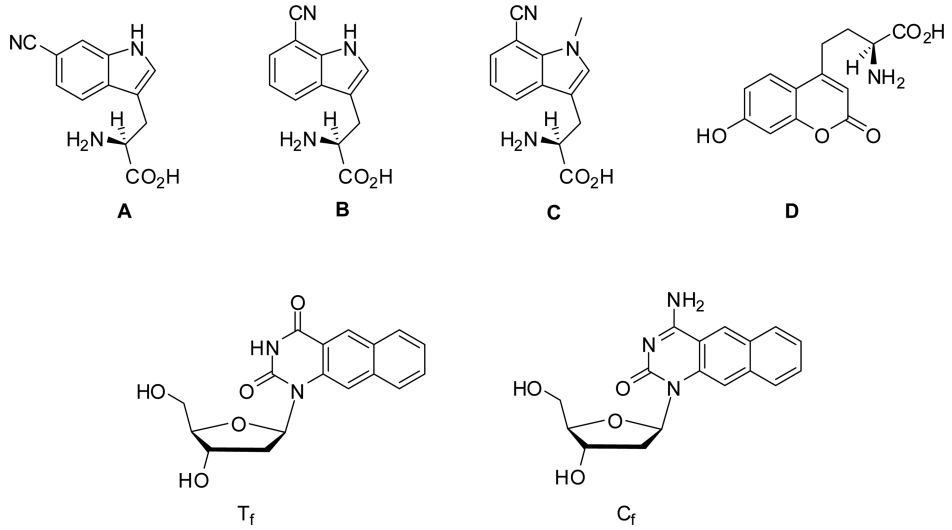
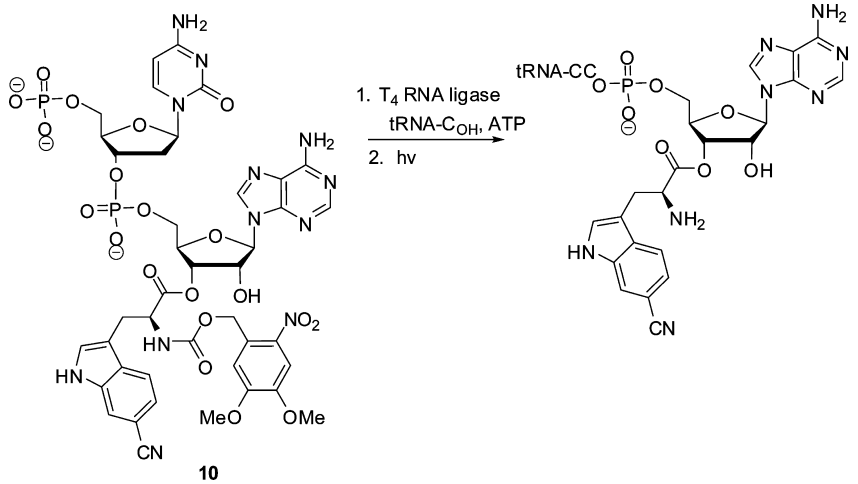


Figure 1. Tryptophan analogues (**A**–**C**) and L-(7-hydroxycoumarin-4-yl)ethylglycine (**D**), which were incorporated into dihydrofolate reductase, and benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione 2′-deoxyriboside (T_f) and 4-aminobenzo[*g*]quinazoline-2-one 2′-deoxyriboside (C_f), which were incorporated into the DNA oligomers DNA1/DNA2 and *BCL2* i-motif, respectively.

Scheme 2. Preparation of Suppressor $tRNA_{CUA}$ Activated with Amino Acid A



experimentally by incubating 300 nM Klenow1−484-**A** in the presence of 300 nM DNA1/DNA2 in 20 mM Tris-HCl (pH 7.8) containing 200 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. The FRET following excitation at 310 nm was recorded after incubation for 60 min at 37 °C. Emission was measured in the range of 340−500 nm. Similarly, the FRET between RRM2 and the i-motif was measured by incubating 5 μM RRM2F-201-**A** in the presence or absence of 50−200 nM *BCL2*-i22C_f in 50 μL of 50 mM Tris-HCl (pH 6.6) containing 100 mM NaCl and 1 mM EDTA. The proteins were incubated with the DNA for 15 min and then excited at 310 nm. Emission was measured immediately in the range of 325−550 nm.

RESULTS

Synthesis of Fluorescent Aminoacyl pdCpAs Esters. The synthesis of the aminoacylated pdCpA derivative of amino acid **A** (Figure 1) was accomplished starting from commercially available 6-cyanoindole, which was formylated to yield **1** (Scheme 1).[35] The latter was N-tosylated with *p*-TsCl and NaH, affording **2** in 68% overall yield.[14] Reduction of the aldehyde moiety with $NaBH_4$ afforded alcohol **3** in 80% yield. Chlorination of **3** with thionyl chloride then provided **4** as a yellowish solid. Regioselective lithiation (*n*-butyllithium, THF, −78 °C) of the Schöllkopf reagent produced the lithium enolate that afforded adduct **5** with high diastereoselectivity.[14,15] Mild hydrolysis (2 N HCl) provided α-substituted amino acid methyl ester **6** that was protected as the NVOC carbamate to yield **7** in 79% overall yield. N-Detosylation of **7** using cesium carbonate in 2:1 THF/MeOH solvent afforded ester **8**. Hydrolysis of **8** afforded the free acid, which was activated as cyanomethyl ester **9** in 44% overall yield (Scheme 1). To evaluate the properties of **A** as a constituent of proteins, this amino acid was used to activate a suppressor tRNA (Scheme 2). Treatment of cyanomethyl ester **9** with the tris(tetrabutylammonium) salt of pdCpA (TBA-pdCpA)[36] in anhydrous DMF afforded pdCpA ester **10** in 71% yield. The aminoacylated dinucleotide was ligated to an abbreviated $tRNA_{CUA}$-C_{OH} transcript in the presence of T4 RNA ligase and ATP to afford the NVOC-aminoacyl-tRNA. The activated tRNA was deprotected by UV irradiation to afford free aminoacyl-tRNA (Scheme 2).[14]

The synthesis of the aminoacylated pdCpA derivative of amino acid **B** (Figure 1) employed a similar route (Scheme S1 of the

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Supporting Information). Initially, commercially available 7-cyanoindole was formylated to yield **11**, the latter of which was N-tosylated with *p*-TsCl and NaH, affording **12** in 66% overall yield. Treatment of aldehyde **12** with $NaBH_4$ afforded alcohol **13** in 77% yield. Chlorination of **13** with thionyl chloride then provided **14**. Regioselective lithiation of the Schöllkopf reagent produced the lithium enolate, which afforded adduct **15** with high diastereoselectivity. Mild hydrolysis (2 N HCl) provided α-substituted amino acid methyl ester **16**, which was protected as the NVOC carbamate to yield **17** in 73% yield. N-Detosylation of **17** using cesium carbonate in 2:1 THF/MeOH solvent afforded ester **18**. Hydrolysis of **18** afforded the free acid, which was activated as cyanomethyl ester **19** in 55% overall yield. Treatment of the cyanomethyl ester with a solution of TBA-pdCpA[36] in anhydrous DMF gave the corresponding amino-acylated pdCpA containing amino acid **B** in 65% yield. Following the route discussed in Scheme 2, **B** was ligated to an abbreviated $tRNA_{CUA}$-C_{OH} transcript in the presence of T4 RNA ligase and ATP to afford NVOC-aminoacyl-tRNA; the activated tRNA was then deprotected by UV irradiation to afford the free aminoacyl-tRNA.

The synthesis of the aminoacylated pdCpA derivative of amino acid **C** (Figure 1) was accomplished starting from intermediate **15** (Scheme S2 of the Supporting Information). N-Detosylation of **15** was performed using cesium carbonate in 2:1 THF/MeOH solvent to yield **21** in 68% yield. N-Methylation of **21** with methyl iodide (MeI) and sodium hydride afforded **22** in 74% yield.[13] Mild hydrolysis (2 N HCl) afforded the α-substituted amino acid methyl ester **23**. Amine **23** was protected as the NVOC carbamate to yield **24** in 65% overall yield. Methyl ester **24** was then hydrolyzed to afford the free acid, which was subsequently treated with chloroacetonitrile to afford the requisite cyanomethyl ester **25** in 52% yield. Treatment of the cyanomethyl ester with a solution of TBA-pdCpA[36] in anhydrous DMF gave the corresponding aminoacylated pdCpA derivative **26** in 69% yield (Scheme S2 of the Supporting Information). Following the same strategy discussed in Scheme 2, the aminoacylated dinucleotide was ligated to an abbreviated $tRNA_{CUA}$-C_{OH} transcript in the presence of T4 RNA ligase and ATP to afford the NVOC-aminoacyl-tRNA. The latter was then deprotected by UV irradiation to afford the free aminoacyl-tRNA.

The synthesis of N-acetylated methyl esters of Trp analogues is illustrated in Scheme S3 of the Supporting Information. Compounds **28** and **30** were prepared from intermediates **6** and **16**, respectively, which were acetylated using Ac_2O and then detosylated using cesium carbonate.[14] Compound **31** was synthesized by acetylating intermediate **23** (Scheme S3 of the Supporting Information).

Activation of Suppressor $tRNA_{CUA}$ and Synthesis of Modified Proteins. The individual *N*-NVOC-protected amino-acylated pdCpA derivatives were ligated to a suppressor $tRNA_{CUA}$, lacking its 3′-terminal cytidine and adenosine residues ($tRNA_{CUA}$-C_{OH}),[25] by the use of T4 RNA ligase. The NVOC protecting groups were then removed by exposure to high-intensity UV light at 4 °C. The aminoacyl-tRNAs so obtained were employed in an *in vitro* cell free protein synthesizing system, which was programmed with DHFR DNA plasmids containing TAG codons at the positions corresponding to residue Trp22 or Trp74 of DHFR. Modified DHFR translation was conducted in an *in vitro* coupled transcription−translation system in the presence of the tryptophanyl-$tRNA_{CUA}$ derivatives. As shown in Table 2, each of the three tryptophanyl-tRNAs afforded good suppression of the UAG codons at positions 22 and 74 of DHFR, with relative yields ranging from 15 to 41% compared to that of the cell free synthesis of wild-type DHFR. Each modified DHFR contained a hexahistidine motif at the N-terminus of DHFR, which permitted convenient purification of the proteins on a Ni-NTA column.[37] Final purification was then accomplished on a DEAE-Sepharose CL-6B column.

Table 2. Enzymatic Activities of DHFRs Singly Modified at Positions 22 and 74[a]

	position 22 (Trp)		position 74 (Trp)	
DHFR	yield (%)	k (s^{-1})	yield (%)	k (s^{-1})
wild type	100	12	100	12
A	15 ± 2	1.7 ± 0.1	37 ± 3	11.8 ± 0.5
B	15 ± 2	1.1 ± 0.1	35 ± 3	13.3 ± 0.5
C	19 ± 2	1.3 ± 0.1	41 ± 3	15.2 ± 0.6

[a]Standard deviation based on data from three experiments.

The enzymatic activities of the modified DHFRs were measured by their ability to consume NADPH (Table 2) under steady-state conditions. Replacement of Trp22, which is in the catalytically relevant Met20 loop subdomain of DHFR,[38] with tryptophan analogues **A**−**C** resulted in a substantial reduction in enzyme activity (the turnover rate constants were found to be 9−14% of that obtained for wild-type DHFR[39] under the same assay conditions). The reduction in enzyme activity due to the incorporation of these amino acids at position 22 may have occurred because they were located near the substrate binding site (Met20 loop).[38,40] Thus, substituted Trp derivatives **A**−**C** may have disturbed substrate binding and reduced the DHFR activities. We then replaced another tryptophan residue (Trp74), which is not located in the active site, with the same three tryptophan analogues. All of the modified DHFRs had approximately the same NADPH consuming activity as the wild type (Table 2). The results demonstrated that these three tryptophan derivatives, having favorable properties as fluorescence donors, are suitable for studying protein conformational changes with minimal structural perturbation.

We evaluated the UV and fluorescence spectral properties of the tryptophan analogues as potentially useful fluorescence probes. The study was conducted using the respective N-acetylated methyl esters to mimic their anticipated behavior when present in proteins. The synthesis of the N-acetylated methyl esters of the Trp analogues (**A**−**C**) is illustrated in Scheme S3 of the Supporting Information. As shown in Table 1, analogue **A** has a molar absorption coefficient (10300) and a quantum yield (0.53) higher than those of tryptophan (6900 and 0.18, respectively). It has stronger fluorescence and should minimize the background from the tryptophans in DHFR. Analogues **B** and **C** are also brighter than tryptophan (Table 1). Their extended red-shifted absorbances allow selective excitation of these fluorophores; the emission maxima for both **B** and **C** are substantially red-shifted from that of tryptophan, such that the emission signals from these Trp analogues can be easily isolated from those of the native tryptophans in DHFR. These interesting photophysical characteristics make **A**−**C** potentially attractive fluorescent probes. The fluorescence emission spectra of the N-acetylated methyl esters of Trp analogues (5 μM) in MeOH are shown in Figure 2. As illustrated below, inclusion of these probes into a number of positions in three different proteins had no significant effects on their photophysical properties.

The fluorescence emission spectra of the DHFRs containing the cyanotryptophans at position 74 are shown in Figure 3. We

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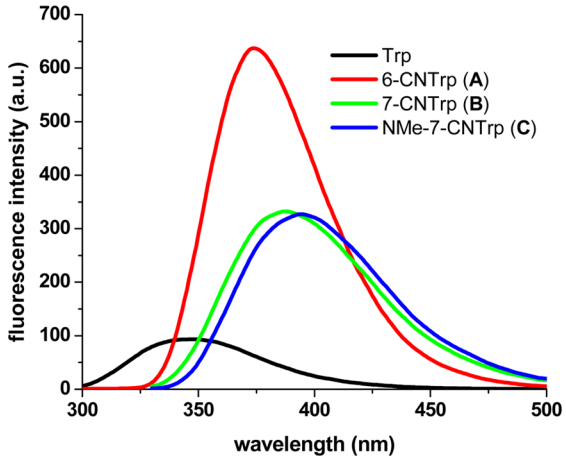

Figure 2. Fluorescence emission spectra of the N-acetylated methyl esters of Trp analogues **A**–**C** (0.005 mM) in MeOH: Trp (black), 6-CNTrp (**A**, red), 7-CNTrp (**B**, green), and *N*Me-7CNTrp (**C**, blue). The emission spectra were recorded following excitation at their maximal absorption wavelengths using the excitation and emission slit widths set at 5 nm.

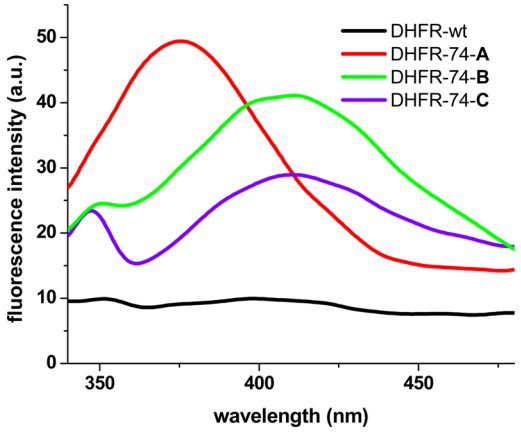

Figure 3. Fluorescence emission spectra of modified DHFRs containing amino acid **A** at position 74 (red), **B** at position 74 (green), and **C** at position 74 (violet) and wild-type DHFR (black). The spectra were recorded at pH 8.0 with a λ_{ex} of 310 nm.

chose position 74 for fluorescence experiments as this position tolerated the introduction of all of the amino acids without a significant loss of enzyme function. The modified DHFRs were subjected to irradiation at 310 nm to minimize the fluorescence from Trp residues. The high quantum yield of **A** allowed us to observe strong fluorescence emission at 370 nm after excitation at 310 nm, even though this was 20 nm from its maximal absorption wavelength. As expected, **B** and **C** exhibited substantially red-shifted emission spectra centered around 410 nm (Figure 3, green and violet traces). Thus, it was possible to excite and monitor **A**–**C** selectively in the modified DHFR, which also contained four native tryptophans.

To explore the capability of these tryptophan derivatives as FRET donors, we selected 6-CNTrp (**A**). The emission wavelength (λ_{em}) of fluorescence donor **A** was at 370 nm (Table 1). As the fluorescence acceptor, we selected L-(7-hydroxycoumarin-4-yl) ethylglycine (HCO, **D**)[22] whose excitation and emission maxima were found to be 345 and 440 nm, respectively. Because **D** is small and has a relatively large Stokes shift, a high quantum yield (Φ_F = 0.63 in water), and a blue wavelength fluorescence emission, it has been used as a fluorescence acceptor for FRET studies.[22] For this FRET pair, we calculated the Förster distance (R_0) as 28.3 Å by following the standard protocol.[41] As noted, position 74 tolerated the introduction of all of the cyanotryptophans and was chosen as the position for donor **A**. For the acceptor position, we chose position 17; in a previous study, we demonstrated that this DHFR position tolerated small fluorescent amino acids well.[22] Its distance to W74 is 25 Å (calculated from the crystal structure of Protein Data Bank entry 1RA1), which is suitable for studying FRET between this pair. By decoding a four-base codon CGGG with the coumarinyl-tRNA$_{CCCG}$ and the nonsense codon UAG with cyanotryptophanyl-tRNA$_{CUA}$, we incorporated the acceptor (**D**) and donor (**A**) amino acids into DHFR at positions 17 and 74, respectively.[22] The incorporation yield was 13% relative to wild type, and the rate of NADPH consumption decreased to 9.4 ± 0.5 s^{-1} compared to a rate of 12 s^{-1} for the wild type. After purification on Ni-NTA and DEAE-Sepharose CL-6B columns, the doubly modified DHFR was excited at 310 nm to decrease the level of interference from the fluorescence emission of tryptophan (λ_{max} = 280 nm). As shown in Figure 4, the modified

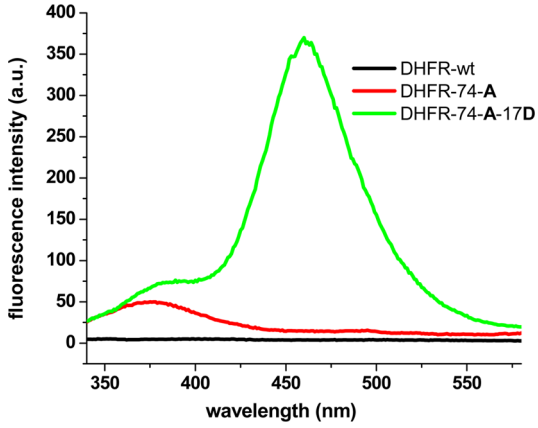

Figure 4. Fluorescence emission spectra of DHFRs containing amino acid **A** at position 74 (red), **D** at position 17 and **A** at position 74 (green), and wild-type DHFR (black). The spectra were recorded at pH 8.0 with a λ_{ex} of 310 nm.

DHFR containing fluorescence acceptor **D** at position 17 and donor **A** at position 74 exhibited an efficient FRET signal at 460 nm. This result demonstrated that donor **A** formed a FRET pair with acceptor **D** at position 17 of DHFR.

Using the *in vitro* suppression method, the 6-cyanotryptophanyl-tRNA afforded good suppression of the UAG codon at position 484 of Klenow1 polymerase mRNA (Figure 5A) with a relative yield of 80%, compared to Klenow1 polymerase. The modified Klenow protein, containing a hexahistidine fusion peptide at the N-terminus, was purified by successive chromatographies on Ni-NTA and DEAE-Sepharose CL-6B columns. Because the absorption spectrum of 6-CNTrp overlaps somewhat with that of Trp (Table 1 and Figure 2), the influence of naturally occurring Trp residues in the protein was taken into consideration. As discussed, Trp analogue **A** has a stronger fluorescence signal than that of tryptophan, and because the absorption of Trp decreases significantly above 300 nm, we chose to excite the modified protein at 310 nm. A strong emission was observed, centered at ~370 nm (Figure 5B). To study FRET between this protein and its DNA substrates, we selected benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione 2′-deoxyriboside (T_f)

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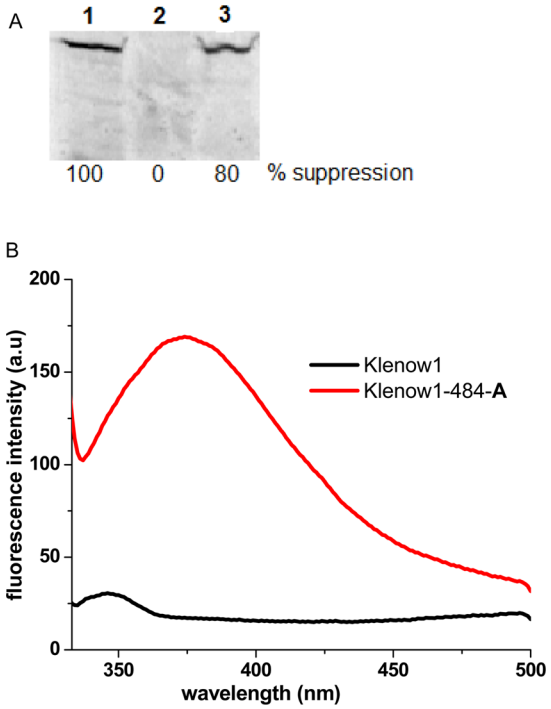

Figure 5. (A) Autoradiogram of a 15% SDS−polyacrylamide gel (100 V, 2 h) illustrating the incorporation of 6-CNTrp (**A**) into position 484 of Klenow1: lane 1, Klenow1 expression; lane 2, Klenow1−484TAG DNA in the presence of abbreviated suppressor $tRNA_{CUA}$-C_{OH}; lane 3, Klenow1 containing 6-CNTrp (**A**) at position 484. Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 instrument equipped with ImageQuant version 5.2 from Molecular Dynamics. (B) Fluorescence emission spectra of Klenow1 containing amino acid **A** at position 484 (red) and Klenow1 (black). The spectra were recorded at pH 7.8 with a λ_{ex} of 310 nm.

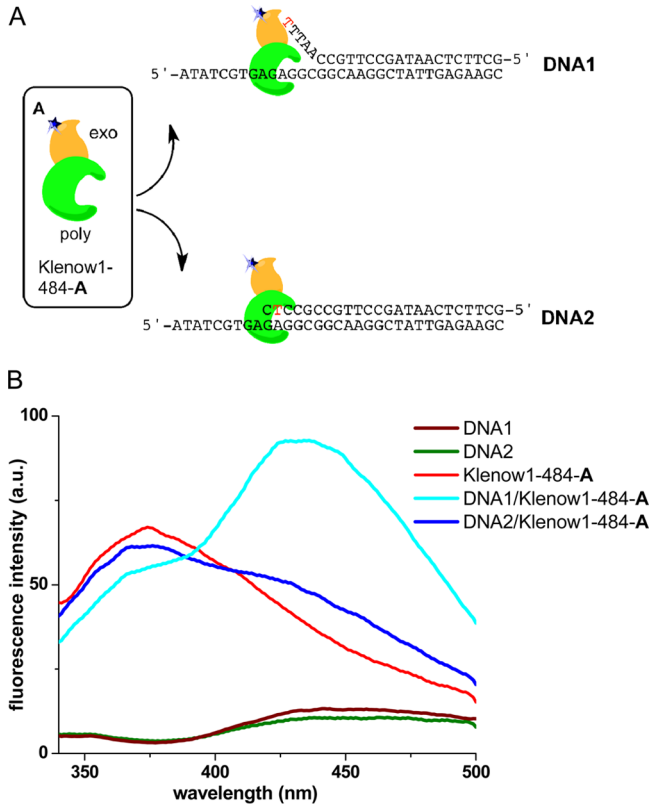

Figure 6. (A) DNA constructs used in the FRET assay. The fluorescent nucleoside (T_f) is colored red. DNA1 with a mismatched 3′-end of the primer strand, positioned in the exonuclease domain (smaller N-terminal domain, yellow). DNA2 containing a perfectly base-paired 3′-end of the primer strand, positioned in the polymerase domain (larger C-terminal domain, green). (B) FRET between DNA1/DNA2 and Klenow1−484-**A**. Klenow1−484-**A** (300 nM) was allowed to interact with 300 nM DNA1 or DNA2, and FRET was obtained after incubation for 60 min.

as the fluorescence acceptor to be incorporated into DNA (Figure 1). T_f displays a strong fluorescence emission following excitation at either 260 or 360 nm. The benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione has been used as a fluorescent substitute for thymine in nucleic acid complexes, because it has high quantum yield (Φ_F = 0.81 in water) and a blue wavelength fluorescence emission.[42] Once the FRET pair was selected, we calculated the Förster distance (R_0) of this pair as 25.2 Å.[41] The main goal was to observe a change in the FRET signal when the fluorescent base was positioned at the exonuclease active site versus when it was at the polymerase active site, as the two active sites of the Klenow fragment are separated by ∼30 Å.[29] Experimentally, this was achieved by forming the complex between the Klenow fragment and a DNA oligomer [DNA1 (Figure 6A)] having a mismatched 3′-end of the primer strand (such that the primer terminus should be localized in the exonuclease active site) or a DNA oligomer [DNA2 (Figure 6A)] containing a perfectly base-paired 3′-end of the primer strand (such that the primer terminus should be localized in the polymerase active site). Both Klenow1 and the modified enzyme containing 6-CNTrp at position 484 were found to bind to DNA1 with comparable efficiencies in an electrophoretic mobility shift assay (Figure S1 of the Supporting Information). To determine whether 6-CNTrp (**A**) could be used successfully as a probe for FRET measurements, 300 nM Klenow1−484-**A** was allowed to interact with 300 nM DNA1 or DNA2, and FRET was measured following excitation at 310 nm after incubation at 37 °C for 60 min. Figure 6B shows the FRET signal when the protein−DNA1 complex was formed. The increase in fluorescence upon formation of the protein−nucleic acid complex was significantly lower when the protein−DNA2 complex was formed (Figure 6B). In the protein−DNA1 complex, the 3′-end of the primer strand is in a single-stranded conformation; hence, it binds to the exonuclease domain. In this conformation, it is located close to 6-CNTrp at position 484 of Klenow fragment; hence, an increased FRET signal magnitude was observed (Figure 6B). In comparison, for the protein−DNA2 complex, the 3′-end of the primer strand is in a double-stranded conformation, which binds to the polymerase domain. In this conformation, the fluorescent acceptor is located far from the 6-CNTrp residue at position 484 of Klenow fragment; hence, a small FRET signal was observed (Figure 6B).

Transcription factor hnRNP LL has been shown to bind to the *BCL2* i-motif DNA and to unfold the i-motif to initiate transcription.[43] hnRNP LL has four RRM (RNA recognition motif) domains, each potentially capable of binding to the i-motif DNA.[43] A construct (Figure 7A) was designed with the native amino acid sequence of the second RRM of hnRNP LL. We demonstrated that RRM2 is capable of binding to the *BCL2* i-motif DNA by the use of an electrophoretic mobility shift assay (EMSA) (Figure S2 of the Supporting Information). A modification in the RRM2F construct, namely W257F, effected the replacement of the only innate Trp with a Phe during protein synthesis. *In vitro* suppression of a UAG codon at position 201,

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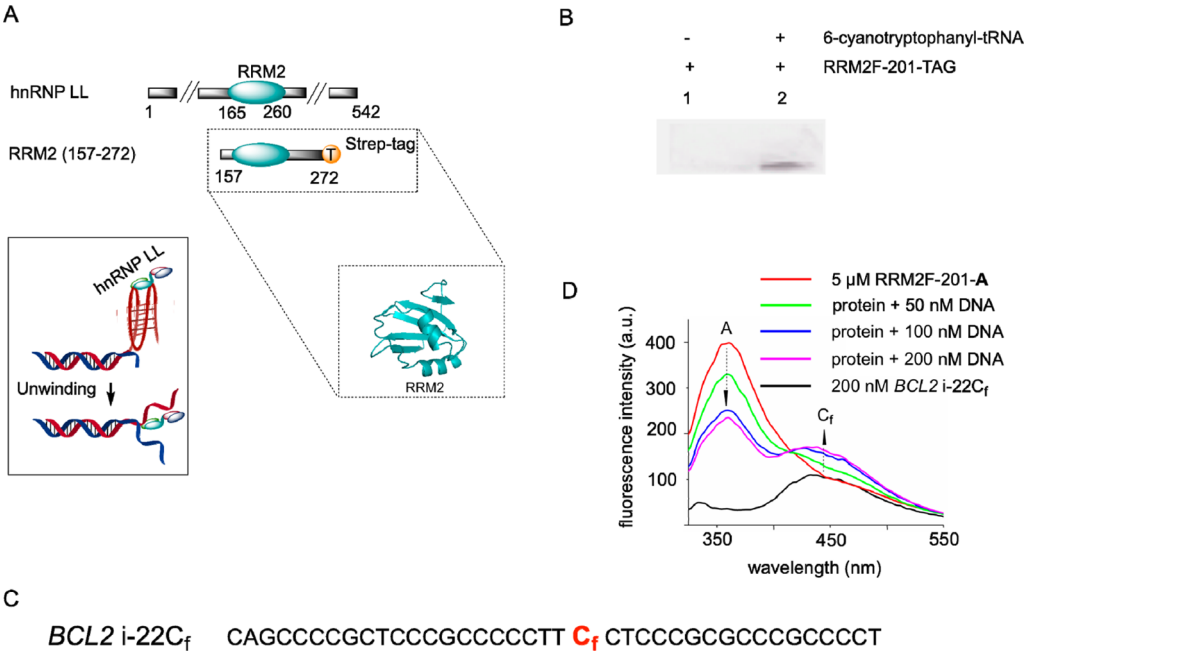

Figure 7. RRM2F-201-**A** and i-motif DNA binding interaction. (A) Location of RRM2 in hnRNP LL. RRM2 is separated from RRM1 and RRM3 (which are upstream and downstream, respectively) by short sequences of amino acids. RRM2F was constructed with an additional 15−20 native amino acids on both the C- and N-termini of the construct, flanking the sequence optimized for expression in *E. coli*. The genes were equipped with a Strep tag at the C-terminus for Strep-Tactin mediated protein purification. The i-motif DNA relaxation upon binding with hnRNP LL is schematically shown in the inset. (B) Incorporation of 6-CNTrp (**A**) at position 201 of RRM2F. The suppression efficiency was 70%, relative to that of the cell free expression of RRM2, and ∼95% relative to that of the expression of RRM2F. (C) Sequence of the *BCL2* i-motif DNA. The nucleoside in red was substituted with a non-natural nucleoside (C_f) (Figure 1). (D) FRET between *BCL2*i-22C and RRM2F-201-**A**. RRM2F-201-**A** (5 μM) was allowed to interact with 50−200 nM *BCL2*i-22C, and FRET was monitored after incubation for 15 min.

using a $tRNA_{CUA}$ activated with 6-CNTrp (**A**), afforded RRM2F-201-**A** (Figure 7B) with a relative yield of ∼95%, compared to that of "wild-type" RRM2F protein expression. Similarly, a substrate i-motif DNA, *BCL2* i-22C_f, was designed by replacing a cytidine nucleotide (C22) of the i-motif DNA with 4-aminobenzo[*g*]quinazoline-2-one 2′-deoxyriboside (C_f) (Figure 7C). To investigate whether the binding of RRM2 to the *BCL2* i-motif DNA could be measured by fluorimetry, we studied the possible FRET between RRM2F-201-**A** and *BCL2* i-22C_f (Figure 7D). 4-Aminobenzo[*g*]quinazoline-2-one (C_f; λ_{max} = 370 nm) has been used as a fluorescence probe as a substitute for cytosine in nucleic acid complexes, because it has a high quantum yield (Φ_F = 0.62 in water) and a blue wavelength fluorescence emission at 456 nm.[44] We calculated the Förster distance (R_0) for this pair as 24.1 Å. Protein RRM2F-201-**A** was incubated with the DNA (*BCL2*i-22C_f) for 15 min and then excited at 310 nm. Emission was measured immediately from 325 to 550 nm. Upon excitation of RRM2F-201-**A** at 310 nm, a strong fluorescence signal at 370 nm was obtained (red trace in Figure 7D). The addition of increasing concentrations of the labeled i-motif DNA to 5 μM RRM2F-201-**A** resulted in a steady increase in the magnitude of the C_f peak at 445 nm. Because C_f has one of its excitation maxima at 370 nm, the appearance of the C_f emission peak at ∼445 nm supports a binding interaction between RRM2 and the i-motif DNA (Figure 7D).

DISCUSSION

Chemically modified tryptophan analogues with suitable photophysical properties may be useful structure-conserving substitutes for tryptophan in protein fluorescence experiments. Among the most thoroughly investigated analogues have been the azatryptophans,[45] which show fluorescence behavior distinct from that of tryptophan. In a recent study, we incorporated small fluorescent azaindoles into the side chains of amino acids that were introduced into *E. coli* dihydrofolate reductase (*ec*DHFR) to monitor changes in the crowded protein interior space.[13] These probes had the virtue of being quite structurally similar to the native tryptophan. As anticipated, they exhibited extended red-shifted absorbance, permitting selective excitation in the presence of Trp. The modified proteins could be employed for FRET measurements, allowing sensitive monitoring of changes in protein conformation. Unfortunately, their quantum yields proved to be much lower than that of tryptophan itself.[13] Nonetheless, the generally favorable results achieved in that study encouraged us to prepare and study additional fluorescent amino acids.

Prompted by the need to develop smaller and brighter fluorophores that can be readily accommodated within a protein structure without perturbation of protein conformation and function, we have investigated three different cyanotryptophan (CNTrp) analogues as potential probes for fluorescence analysis of protein structure and function. Recently, *p*-cyanophenylalanine (CNPhe) has emerged as a useful spectroscopic probe for studying protein structure and dynamics.[46] Introduction of the cyano group increased the fluorescence quantum yield of CNPhe in water to ∼5 times that of phenylalanine (Phe).[46] The polarity of the cyano group is intermediate between that of a methylene and that of an amide group. This intermediate polarity allows CNPhe to be accepted in both hydrophobic and hydrophilic environments in a protein.[47] These interesting properties encouraged us to explore the fluorescence properties of cyanotryptophans, as tryptophan (Trp) is often located at sites that are important for protein function or stability.[12] Thus far,

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only 5-cyanotryptophan has been used as an infrared probe of the local hydration status of proteins or peptides, but its fluorescence quantum yield is approximately 1 order of magnitude smaller than that of Trp, rendering it less useful as a fluorescence probe.[48]

X-ray crystallographic analysis of *ec*DHFR suggested that Trp22 is involved in substrate binding through H-bonds between the residue and an ordered water molecule.[40] Therefore, replacement of this residue may distort substrate binding and alter the function of DHFR. In this study, the significant reduction in DHFR enzyme activity upon incorporation of the non-native Trp analogues in position 22 corroborated this finding. However, all three tryptophan derivatives were well tolerated in terms of DHFR activity when they replaced the native tryptophan residue at position 74. The latter results suggest that these new fluorescent tryptophan analogues have intrinsic properties that may allow them to substitute for Trp in many cases, thus facilitating the study of subtle conformational changes in protein structure.

A few studies have incorporated donor and acceptor amino acids into a single protein, through decoding a four-base CGGG and a nonsense UAG codon in the presence of an aminoacyl-$tRNA_{CCCG}$ and an aminoacyl-$tRNA_{CUA}$, respectively.[49−51] Studies of this type have allowed the measurement of protein backbone cleavage and relatively large changes in protein structure.[49,50] In previous studies, we have incorporated a series of azatryptophans into *E. coli* dihydrofolate reductase as fluorescence donors and acridon-2-ylalanine (Acd)[13,52] as an acceptor, to study DHFR conformational changes.[13] Here, we describe the preparation of modified DHFR containing 6-CNTrp (**A**) as a fluorescence donor and L-(7-hydroxycoumarin-4-yl)ethylglycine[22] (HCO, **D**) as an acceptor, to permit the study of DHFR conformational changes (Figure 1). The doubly modified DHFR containing **D** at position 17 and **A** at position 74 retained 78% of wild-type activity, as reflected in NADPH consumption assay, and exhibited highly efficient FRET upon being excited at 310 nm (Figure 4).

The smaller size, high quantum yield, and good efficiency of incorporation of amino acid **A** into DHFR encouraged us to incorporate **A** into two DNA binding proteins, including the Klenow fragment of DNA polymerase I and an i-motif DNA binding domain of transcription factor hnRNP LL. In fact, it was possible in both cases to study DNA−protein interaction using FRET. The study of RNA binding proteins, which often display Trp residues at the RNA binding site, has been reported by the Tor group using the FRET technique.[53] A fluorescent ribonucleoside analogue, containing 5-aminoquinazoline-2,4-(1*H*,3*H*)-dione, acted as a Förster resonance energy transfer acceptor for native tryptophan (R_0 = 22 Å) and displayed emission in the visible region (440 nm).[53] The present results suggest that such methods may also prove to be useful for the study of DNA binding proteins. However, the low abundance of tryptophan in small proteins as well as its complicated photophysics[45] limit the practical implementation of this technique. This could be obviated by the introduction of fluorescent non-natural amino acids, such as 6-CNTrp (which is structurally similar to Trp), in proteins by employing appropriately activated suppressor tRNAs.

The Klenow fragment is the large proteolytic fragment of pol I that retains the polymerase and the 3′−5′ exonuclease activities. The X-ray crystal structure of the Klenow fragment shows that the polypeptide chain is folded into two domains.[29] The larger C-terminal domain comprises the active site for the polymerase reaction, whereas the 3′−5′ exonuclease active site is contained in the smaller N-terminal domain.[29] Thus, the Klenow fragment has two active sites; depending on whether or not the 3′-end of the primer strand is fully base-paired to its complementary strand, the DNA localizes in either the polymerase or 3′−5′ exonuclease active site of the Klenow fragment, respectively. To study the interaction of DNA with the Klenow fragment by FRET, **A** was incorporated into the modified Klenow1 (lacking 3′−5′ exonuclease activity) as a fluorescence donor. A modified fluorescent deoxyribonucleoside analogue (T_f) containing benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione[42] was incorporated at a predetermined position of the primer−template as a FRET acceptor. Benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione displays a strong fluorescence emission (quantum yield of 0.81) at an excitation wavelength of either 260 or 360 nm.[42] Therefore, it acted as a FRET acceptor for **A** [emission at 370 nm (Table 1)]. In case of the DNA oligomer DNA2, for the 3′-end of the primer strand to bind to the polymerase active site, it has to be base paired to the template strand. Because the fluorescent base used in our FRET studies is a thymine derivative, it presumably forms a weak AT-like base pair[42] with the complementary adenine on the template strand. It is well-known that 3′- and 5′-ends of a duplex DNA constantly "breathe" by breaking and forming hydrogen bonds with their complementary bases on the opposite strand.[54] Therefore, to confer maximal stability, DNA2 was designed with the fluorescent probe placed in the second position (from the 3′-end) on the primer strand, and with a cytidine as the terminal (3′) nucleotide (Figure 6A). This study demonstrated the ability of the new Trp fluorophore to distinguish between the known geometric differences[29] in the editing and polymerase complexes formed by the Klenow fragment.

These results also demonstrate RRM2 domain-specific association of the i-motif binding protein hnRNP LL and its substrate i-motif DNA. Recent findings revealed that the *BCL2* i-motif can be a potential target for small molecules[55] and, more interestingly, for transcription factors such as hnRNP LL. In a recent report, the interaction between hnRNP LL and an i-motif DNA has been demonstrated (inset of Figure 7A), which results in unwinding of the i-motif DNA.[43] hnRNP LL has four RRM (RNA recognition motif) domains, each potentially capable of binding to i-motif DNA.[43] Here, we studied the interaction between RRM2 (second RRM domain), substituted with 6-CNTrp (**A**), and the i-motif substituted with 4-aminobenzo[*g*]-quinazoline-2-one 2′-deoxyriboside[44] [C_f (Figure 1)]. In the FRET experiment involving RRM2F-201-**A** and *BCL2* i-22C_f, the C_f peak at 445 nm was gradually augmented with the increment of the DNA−protein ratio (Figure 7D). This suggested a binding interaction between protein and DNA, which was further supported by an EMSA experiment (Figure S2 of the Supporting Information).

Thus, we have demonstrated the utility of the FRET technique, involving 6-CNTrp and T_f /C_f, to study the binding interaction between the Klenow fragment of DNA pol I, or one of the hnRNP LL RRM domains, with DNA. These findings allow a more detailed analysis in which the FRET experiments could be performed using a wide variety of experimental conditions (e.g., varying pH, temperature, and salt concentration) with donors at different locations of the Klenow fragment or RRM domains.

CONCLUSIONS

Three novel tryptophan analogues have been prepared, each containing a single CN moiety. These analogues have quantum

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yields significantly greater than those of tryptophan itself, and fluorescence excitation and emission properties that allow them to be differentiated from tryptophan when both are present in a protein. The three new analogues have been incorporated into two different positions of DHFR, and the fluorescence emission spectra resulting from the presence of the analogues were quite distinct from those of the wild type. When included at position 74 of DHFR, analogue **A** underwent efficient FRET with an acceptor introduced into position 17. Analogue **A** was also introduced into a single position of the Klenow fragment of polymerase I and was able to transfer energy to DNA substrates containing an acceptor fluorophore in the form of a modified nucleobase. One of the DNA substrates afforded an editing complex with the labeled protein, while the other formed a polymerase complex; the efficiencies of the transfer of energy in these two complexes were quite different, reflecting the known differences in the structures of these two complexes. Finally, Trp analogue **A** was introduced into one of the RNA recognition motifs of hnRNP LL, which was shown to bind and transfer energy to a DNA i-motif containing a fluorescent nucleobase. None of the analogues displayed a large change in its excitation and emission properties upon being incorporated into one of the proteins studied.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b01085.

Additional procedures, data, and figures (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

GC−MS, gas chromatography−mass spectrometry; APCI, atmospheric-pressure chemical ionization; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; R_f, ratio of fronts; Schöllkopf's reagent, (*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine; NVOC-Cl, 4,5-dimethoxy-2-nitrobenzyl chloroformate; TBA, tetra-*n*-butylammonium; *p*TsCl, *p*-toluenesulfonyl chloride; DIPEA, *N*,*N*-diisopropylethylamine; THF, tetrahydrofuran; DMF, dimethylformamide.

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