JOC*Note*

Unnatural Amino Acid Mutagenesis of Green Fluorescent Protein

Lei Wang, Jianming Xie, Ashok A. Deniz,† and Peter G. Schultz*

Department of Chemistry and Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

schultz@scripps.edu

Received October 15, 2002

Abstract: Unnatural amino acid mutagenesis has been used to selectively substitute tyrosine 66 of green fluorescent protein (GFP) with five novel amino acids: *p*-amino-L-phenylalanine, *p*-methoxy-L-phenylalanine, *p*-iodo-L-phenylalanine, *p*-bromo-L-phenylalanine, and L-3-(2-naphthyl)alanine. The absorbance and emission maxima of the resulting mutant GFPs span the range from 375 to 435 nm and 428 to 498 nm, respectively. The spectral properties of the mutant GFPs, including the absorbance and fluorescence maxima and quantum yields, correlate with the structural and electronic properties of the substituents on the amino acids.

We recently developed a general methodology that allows for the site-specific incorporation of unnatural amino acids into proteins directly in living cells.[1,2] A new set of components was added to the biosynthetic machinery including a tRNA-codon pair, an aminoacyl-tRNA synthetase, and a desired unnatural amino acid. These new components are orthogonal to the endogenous counterparts; that is, they do not cross interact with the existing tRNA-codon pairs, synthetases, and natural amino acids. The orthogonal synthetase aminoacylates the orthogonal tRNA with the unnatural amino acid, and the acylated tRNA inserts the unnatural amino acid in response to the amber nonsense codon, TAG. When a codon in a gene of interest is mutated to the amber nonsense codon and the mutated gene is coexpressed with the orthogonal tRNA and synthetase in the presence of the unnatural amino acid, the unnatural amino acid is selectively incorporated with high translation fidelity and efficiency. This methodology effectively expands the genetic code of *Escherichia coli* to include a wide variety of amino acids with novel chemical, structural, and physical properties.[2−4]

To begin to explore the effects of such novel amino acids on protein structure and function, we chose first to examine green fluorescent protein (GFP). GFP is a unique light-emitting protein whose chromophore is formed post-translationally from the sequence Ser65-Tyr66-Gly67 by an oxidative reaction. GFP has become an invaluable tool as a tag for monitoring gene expression, protein localization, and protein dynamics in vitro and in vivo.[5] Extensive studies have been carried out to alter the spectral properties of GFP using conventional amino acid mutagenesis. Here, we report the synthesis and characterization of five GFP mutants in which unnatural amino acids with different functional groups were substituted for Tyr66 of GFP.

Tyr66 in GFP was chosen for mutation since previous studies using conventional mutagenesis showed that an aromatic amino acid is necessary at this position for the generation of fluorescence. Replacement of Tyr66 with Trp, His, and Phe results in GFP mutants with shifted absorbance and emission bands.[5] To begin to systematically study the effects of substituents on the aromatic ring while minimizing structural perturbations to the protein, we substituted four Tyr analogues bearing different substituents at the para position of the phenyl ring (Scheme 1). For in vivo unnatural amino acid mutagenesis, an orthogonal amber suppressor tRNA ($mutRNA^{Tyr}_{CUA}$),[6] together with an orthogonal aminoacyl-tRNA synthetase with specificity for each of the unnatural amino acids,[1,3,4] were used to incorporate the desired analogue into position 66 of GFP. The gene encoding αGFP (also called cycle 3 GFP) was used in this study; αGFP has similar spectral properties to wild type (wt) GFP but has improved folding and reduced aggregation when expressed in *E. coli*.[7,8] The codon for Tyr66 was first mutated into the amber nonsense codon, and a His_6 tag was added to the C-terminus to facilitate protein purification.

Mutant GFP proteins containing different unnatural amino acids at position 66 were expressed in *E. coli* in glycerol minimal media at 30 °C. The mutant proteins were purified with Ni^{2+} affinity chromatography. The yields are in the range of 10−22 mg/L of minimal media. For comparison, an orthogonal synthetase with specificity for Tyr (*Methanococcus jannaschii* TyrRS)[6,9] was also used to produce wt αGFP with a yield of 24 mg/L of minimal media. All mutant GFPs were fluorescent except the one containing L-3-(2-naphthyl)alanine. This unnatural amino acid may be too bulky to fit well into the cavity. Indeed, when Tyr66 is mutated to Trp, additional mutations are required to compensate for the larger size of Trp compared with Tyr in order to realize a significant fluorescence efficiency.[10] The absorbance and fluorescence spectra of the other mutant GFPs are shown in Figure 1. The absorbance and emission maxima, extinction coefficients, and quantum yields are summarized in Table 1. As expected, the spectral properties of the αGFP produced using the orthogonal tRNA/synthetase to suppress TAG66 codon with Tyr are identical to those of wt αGFP. The extinction coefficients and quantum yield are also consistent with measurements reported previously.[8]

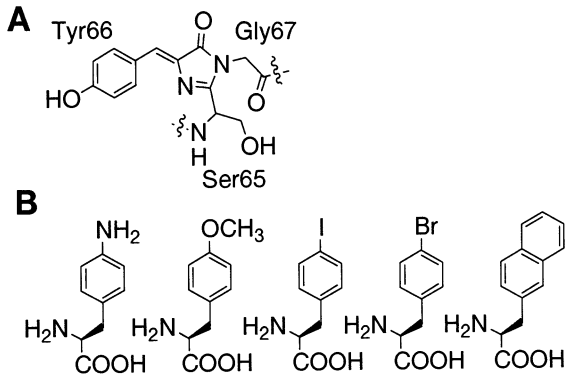
Wild type αGFP has two absorbance maxima, a major one at 397 nm and a minor one at 475 nm. These two

† Department of Molecular Biology.

(1) Wang, L.; Brock, A.; Herberich, B.; Schultz, P. G. *Science* **2001**, *292*, 498.
(2) Wang, L.; Schultz, P. G. *Chem. Commun.* **2002**, 1.
(3) Wang, L.; Brock, A.; Schultz, P. G. *J. Am. Chem. Soc.* **2002**, *124*, 1836.
(4) Santoro, S. W.; Wang, L.; Herberich, B.; King, D. S.; Schultz, P. G. *Nat. Biotechnol.* **2002**, *20*, 1044.
(5) Tsien, R. Y. *Annu. Rev. Biochem.* **1998**, *67*, 509.
(6) Wang, L.; Schultz, P. G. *Chem. Biol.* **2001**, *8*, 883.
(7) Crameri, A.; Whitehorn, E. A.; Tate, E.; Stemmer, W. P. *Nat. Biotechnol.* **1996**, *14*, 315.
(8) Patterson, G. H.; Knobel, S. M.; Sharif, W. D.; Kain, S. R.; Piston, D. W. *Biophys. J.* **1997**, *73*, 2782.
(9) Wang, L.; Magliery, T. J.; Liu, D. R.; Schultz, P. G. *J. Am. Chem. Soc.* **2000**, *122*, 5010.

10.1021/jo026570u CCC: $25.00
Published on Web 11/16/2002

SCHEME 1[a]



[a] (A) The chromophore of wt GFP formed by Ser65-Tyr66-Gly67. (B) Structures of unnatural amino acids substituted for Tyr66 of GFP: from left to right, *p*-amino-L-phenylalanine (*p*-amino-Phe), *p*-methoxy-L-phenylalanine (*p*-methoxy-Phe), *p*-iodo-L-phenylalanine (*p*-iodo-Phe), *p*-bromo-L-phenylalanine (*p*-bromo-Phe), and L-3-(2-naphthyl)alanine (naphthyl-Ala)

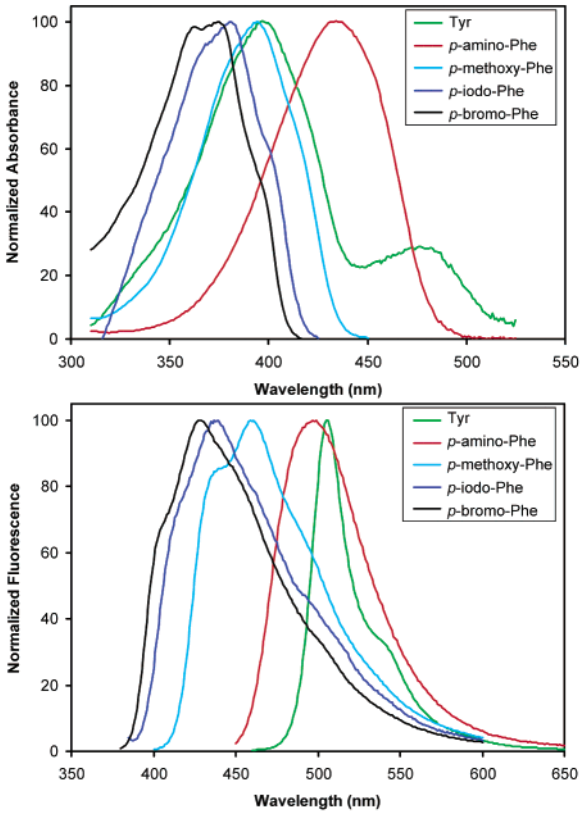


FIGURE 1. Absorption (top) and fluorescence (bottom, corrected) spectra for αGFP and mutants containing different unnatural amino acids at residue 66 prepared using in vivo unnatural amino acid mutagenesis.

peaks are believed to correspond to the GFP populations with a neutral chromophore (phenol of Tyr66) and an anionic chromophore (phenolate anion of Tyr66), respectively.[5] When Tyr66 is replaced with *p*-methoxy-Phe, only one absorbance maximum at 394 nm is observed, which is very close to the 397 nm maximum of wt αGFP. This result corroborates the above assignment of the two absorbance maxima for wt αGFP, since the methoxy group cannot be present in anionic form. The wavelength of the fluorescence emission maximum (λ_{max}^{em}) of wt αGFP is approximately 506 nm when excited at 397 or 475 nm. This emission corresponds to the deprotonated form of the chromophore in the excited state. Excited-state proton transfer is responsible for the disappearance of the emission peak corresponding to the protonated chromophore (at 460 nm as revealed by picosecond spectroscopy.)[12] In this experiment, when *p*-methoxy-Phe is inserted in the chromophore, a single emission maximum at 460 nm is observed. Substitution of the hydroxyl group of Tyr with a methoxy group removes the possibility of proton transfer and confirms the previous assignment for wt GFP absorbance and emission directly.

When other unnatural amino acids with different subsituents at the para position of the phenyl ring were incorporated, single major maxima were observed for absorbance and emission spectra. This is likely due to the fact that the amino acids do not have multiple charge states under the assay conditions. The emission maxima of the mutant GFPs span the region from blue, cyan, to green. The wavelengths for both absorbance (λ_{max}^{abs}) and emission peaks increase in the order of *p*-bromo (λ_{max}^{abs} = 375 nm, λ_{max}^{em} = 428 nm), *p*-iodo (λ_{max}^{abs} = 381 nm, λ_{max}^{em} = 438 nm), *p*-methoxy (λ_{max}^{abs} = 394 nm, λ_{max}^{em} = 460 nm), hydroxyl (λ_{max}^{abs} = 397 nm, λ_{max}^{em} = 460 nm), *p*-amino (λ_{max}^{abs} = 435 nm, λ_{max}^{em} = 498 nm), and deprotonated hydroxyl (λ_{max}^{abs} = 475 nm, λ_{max}^{em} = 506 nm). This trend is consistent with the electron-donating ability of these substituents. For comparison, the spectral properties of a series of monosubstituted benzenes are listed in Table 2.[11] Because the para substituents tested here are less electron-donating than the oxygen anion, the fluorescence emission peaks of all GFP mutants are blue shifted relative to wt αGFP.

The extinction coefficients of the mutant GFPs containing *p*-amino-Phe and *p*-methoxy-Phe at position 66 are higher than that of wt αGFP (31 000, 27 000, and 25 000 M^{-1} cm^{-1}, respectively), but their fluorescence quantum yields are lower (0.43, 0.37, and 0.76, respectively). The experimental sensitivities, the product of quantum yield and extinction coefficient, for these two mutants are close to that of wt αGFP. The quantum yields of the mutant GFPs containing *p*-iodo-Phe and *p*-bromo-Phe are low due to spin−orbit coupling between the fluorophore and the halogen substituent.[13]

Mutant GFPs with phenolate anions in the chromophore, such as EGFP and Emerald, have been widely used due to their single excitation maxima, and emission maxima at wavelengths close to that of fluorescein, a commonly used small-molecule fluorophore.[14] The *p*-amino-Phe mutant GFP has the same advantages and should be a useful alternative. In addition, the Stokes' shift of this mutant (63 nm) is larger than that of its

(10) Heim, R.; Tsien, R. Y. *Curr. Biol.* **1996**, *6*, 178.
(11) Bowen, E. J. *Luminescence in Chemistry*; D. Van Nostrand Co., Ltd.: New York, 1968.
(12) Chattoraj, M.; King, B. A.; Bublitz, G. U.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8362.
(13) (a) Kasha, M. *J. Chem. Phys.* **1952**, *20*, 71. (b) McGlynn, S. P.; Azumi, T.; Kinoshita, M. *Molecular Spectroscopy of the Triplet State*; Prentice-Hall, Inc.: Engelwood Cliffs, New Jersey, 1969. (c) Bonnett, R.; Harriman, A.; Kozyrev, A. N. *J. Chem. Soc. Faraday Trans.* **1992**, *88*, 763.
(14) Cubitt, A. B.; Woollenweber, L. A.; Heim, R. *Methods Cell Biol.* **1999**, *58*, 19.

TABLE 1. Spectral Properties of GFP Mutants

amino acid at position 66	absorbance maximum (nm)		extinction coefficient (M^{-1} cm^{-1})		emission maximum (nm)	quantum yield
	major	minor	major	minor		
Tyr	397	475	25 000 ± 2000	6000 ± 500	506	0.76 ± 0.02
p-amino-Phe	435		31 000 ± 4000		498	0.43 ± 0.01
p-methoxy-Phe	394		27 000 ± 1000		460	0.37 ± 0.01
p-iodo-Phe	381		16 000 ± 1000		438	0.013 ± 0.001
p-bromo-Phe	375		20 000 ± 1000		428	0.014 ± 0.001

TABLE 2. Fluorescence Properties of Monosubstituted Benzenes[11]

compd	substituent	excitation maximum (nm)	emission maximum (nm)
benzene	H	260	291
phenoxide ion	O^-	289	345
aniline	NH_2	280	345
phenol	OH	272	320
anisole	OCH_3	269	302
iodobenzene	I	–	none
bromobenzene	Br	–	none

counterparts (21 nm for EGFP and Emerald). Previously, substitution of His for Tyr66 resulted in a blue fluorescent protein (BFP).[15] Unfortunately, the relatively low quantum yield (0.24) and rapid photobleaching limit its usage. The *p*-methoxy-Phe mutant GFP has a higher experimental sensitivity and good photostability. Therefore, this mutant should be useful for double-labeling experiments together with a UV-excitable GFP.[16] The current preferred donor−acceptor partners for fluorescence resonance energy transfer (FRET) are cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The BFP−EGFP pair is disfavored because of the aforementioned problems with BFP. The *p*-methoxy-Phe mutant GFP may be a useful substitute for BFP here as well.

In summary, we have incorporated five amino acid analogues of Tyr into position 66 of αGFP. The resultant mutant GFPs show spectral characteristics consistent with the structural and electronic properties of the substituents. These mutant GFPs may further expand the applications of this class of bioluminescent proteins. It may be possible to incorporate other unnatural amino acids at position 66 to generate GFP mutants with faster maturation rates and higher intensities, or at other positions, such as position 203, for GFP mutants with longer emission wavelengths (>530 nm).

Experimental Section

In Vivo Unnatural Amino Acid Mutagenesis and Mutant Protein Preparation. The αGFP gene was cloned from plasmid $pGFP_{UV}$ (Clontech, Palo Alto, CA) using primer LW216 (5′-ATGAGTAAAGGAGAAGAACTTTTCAC-3′) and LW217 (5′-TTTGTAGAGCTCATCCATGCC-3′). An amber nonsense codon was introduced at position 66 using polymerase chain reaction (PCR) with primer LW209 (5′-CACTTGTCACTACTTTCTCTTAGGGTGTTCAATGCTTTTCCCG-3′) and LW208 (5′-GAGAAAGTAGTGACAAGTGTTG-3′). The mutated GFP gene was inserted into plasmid pLEI to afford plasmid pLEIG. A His_6 tag in pLEI was fused to the C-terminus of the mutated GFP gene in frame. The whole GFP gene is under the control of a bacteriophage *T5* promoter and t_0 terminator. Plasmid pLEIG also expresses the $mutRNA^{Tyr}_{CUA}$ gene under the control of the *lpp* promoter and *rrnC* terminator. The genes for the orthogonal aminoacyl-tRNA synthetases with specificities toward different unnatural amino acids were encoded in a pBK plasmid under the control of the constitutive *E. coli* GlnRS promoter and terminator. *E. coli* DH10B cells cotransformed with pLEIG and a pBK plasmid were grown in minimal media containing 1% glycerol and 0.3 mM leucine (GMML media) with 25 μg/mL kanamycin, 34 μg/mL of chloramphenicol, and 1.0 mM corresponding unnatural amino acid at 30 °C. When cells reach an OD_{600} of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) (1mM) was added to induce protein expression. After 6 h, cells were pelleted and the protein was purified by Ni^{2+} affinity chromatography according to the manufacturer's protocol under native conditions (Qiagen, Valencia, CA). Purified proteins were exchanged into assay buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH = 8) with a PD-10 column (Amersham Pharmacia, Piscataway, NJ). Protein concentrations were determined by Bradford assay (BCA kit, Biorad, Hercules, CA). Aliquots of protein were analyzed with SDS−PAGE to determine protein purity. Fractions with purities greater than 95% were used for spectral measurement.

UV−vis and Fluorescence Spectroscopy. Absorbance measurements were performed with a Hewlett-Packard 8453 UV-visible spectrophotometer. Protein samples were diluted so that their absorbances were within the range of 0.5 to 1.0. A FluoroMax-2 spectrofluorometer (Instruments S. A., Inc., Edison, NJ) equipped with a 150 W continuous xenon lamp was used to measure fluorescence emission spectra and quantum yields with both excitation and emission band-pass of 2 nm; a PMT voltage of 950 V; and at a scan rate of 1 nm/s. For the quantum yield measurement of wt GFP, appropriate amount of wt GFP in assay buffer and fluorescein in 0.1 M NaOH were adjusted so that both samples have the matched absorbances at 480 nm. The fluorescence emission spectra were recorded with excitation at 480 nm, and corrected using the correction factors supplied with the instrument. The 490−650 nm region of the fluorescence emission spectra were integrated. The quantum yield of wt GFP was calculated by comparison of the integrated emission intensity from these two spectra and by using the known fluorescence quantum yield of fluorescein (0.90).[17] Quantum yields for other GFP mutants were determined in a similar manner: for the *p*-amino-Phe mutant, excitation at 440 nm, integration from 450 to 650 nm, reference fluorescein; for the *p*-methoxy-Phe mutant, excitation at 394 nm, integration from 400 to 650 nm, reference quinine sulfate in 0.5 M H_2SO_4 (quantum yield = 0.55);[17] for the *p*-iodo-Phe mutant, excitation at 380 nm, integration from 385 to 650 nm, reference quinine sulfate; for the *p*-bromo-Phe mutant, excitation at 375 nm, integration from 380 to 650 nm, reference quinine sulfate. Both fluorescein and quinine sulfate were purchased from Molecular Probes (Eugene, OR).

Acknowledgment. We acknowledge support of this work by the Department of Energy (DE-FG03-00ER45812), National Institutes of Health (GM62159), and the Skaggs Research Institute.

JO026570U

(15) Heim, R.; Prasher, D. C.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12501.

(16) Rizzuto, R.; Brini, M.; De Giorgi, F.; Rossi, R.; Heim, R.; Tsien, R. Y.; Pozzan, T. *Curr. Biol.* **1996**, *6*, 183.

(17) Demas, J. N.; Crosby, G. A. *J. Phys. Chem.* **1971**, *75*, 991.