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Sensitive, Site-Specific, and Stable Vibrational Probe of Local Protein Environments: 4-Azidomethyl-L-Phenylalanine

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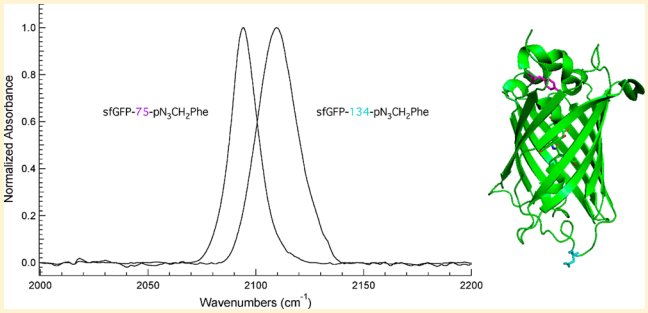
Supporting Information

ABSTRACT: We have synthesized the unnatural amino acid (UAA), 4-azidomethyl-L-phenylalanine (pN_3CH_2Phe), to serve as an effective vibrational reporter of local protein environments. The position, extinction coefficient, and sensitivity to local environment of the azide asymmetric stretch vibration of pN_3CH_2Phe are compared to the vibrational reporters: 4-cyano-L-phenylalanine (pCNPhe) and 4-azido-L-phenylalanine (pN_3Phe). This UAA was genetically incorporated in a site-specific manner utilizing an engineered, orthogonal aminoacyl-tRNA synthetase in response to an amber codon with high efficiency and fidelity into two distinct sites in superfolder green fluorescent protein (sfGFP). This allowed for the dependence of the azide asymmetric stretch vibration of pN_3CH_2Phe to different protein environments to be measured. The photostability of pN_3CH_2Phe was also measured relative to the photoreactive UAA, pN_3Phe.



INTRODUCTION

The ability to probe local protein environments has been significantly increased by the use of unnatural amino acids containing vibrational reporters that can be genetically incorporated into proteins with site-specificity.[1,2] For instance, the unnatural amino acids, 4-cyano-L-phenylalanine (pCNPhe) and 4-azido-L-phenylalanine (pN_3Phe), which contain the nitrile and azide vibrational reporters, respectively, have been successfully incorporated with high efficiency and fidelity into proteins in a site-specific manner utilizing an engineered, orthogonal aminoacyl-tRNA synthetase to study local protein environments.[1−8] The nitrile and azide groups are effective vibrational reporters in part due to the position of the nitrile symmetric stretch vibration and the azide asymmetric stretch vibration, which occur in a relatively clear region of the infrared; the oscillator strengths of these vibrations; and the sensitivity of these vibrations to local environment.[2,9,10] These two groups also represent two-atom (nitrile) and three-atom (azide) probes, which have the potential to be minimally invasive.[2]

Previous work with the modified nucleoside, 2′-azido-5-cyano-2′-deoxyuridine (N_3CNdU), that contains both the nitrile and azide vibrational reporters allowed these two probes to be directly compared.[10] The azide asymmetric stretch vibration of N_3CNdU was found to have an extinction coefficient that was approximately twice as large as the nitrile symmetric stretch vibration and the azide asymmetric stretch vibration demonstrated a greater sensitivity to local environment as shown by a larger frequency shift between solvents selected to mimic environments of biological significance.[10] However, previous work has also demonstrated that the azide modified unnatural amino acid (UAA), pN_3Phe, is photoreactive,[11−13] while the nitrile modified UAA, pCNPhe, is not, which is a significant factor for the increased use of pCNPhe as a vibrational reporter compared to pN_3Phe, as well as the ability of pCNPhe to serve as a fluorescence probe by forming a FRET pair with tryptophan.[4,14−17]

Here, we have proposed a new vibrational reporter UAA that has the aforementioned advantages of both the nitrile and azide vibrational reporters, while being able to be genetically incorporated into proteins site-specifically using an engineered, orthogonal aminoacyl-tRNA synthetase. Specifically, we have synthesized 4-azidomethyl-L-phenylalanine (pN_3CH_2Phe, Figure 1B, Scheme 1), which contains the azide vibrational

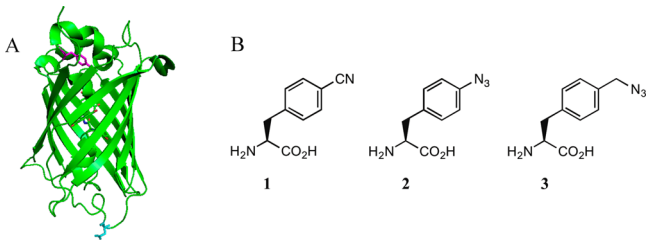


Figure 1. (A) Structure of wt-sfGFP (PDB ID 2B3P) with site 75 (magenta) and site 134 (cyan) highlighted. (B) Structure of 4-cyano-L-phenylalanine (pCNPhe, **1**), 4-azido-L-phenylalanine (pN_3Phe, **2**), and 4-azidomethyl-L-phenylalanine (pN_3CH_2Phe, **3**).

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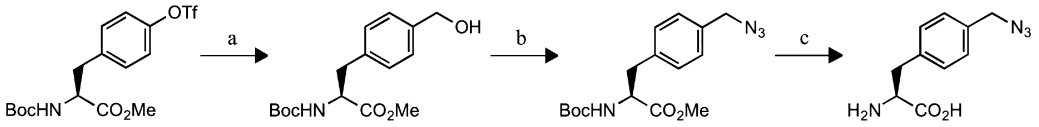
Scheme 1[a].



[a]a Conditions: (a) (i) $Pd(OAc)_2$, dppp, Et_3N, DMF, CO, Oct_3SiH, 70 °C; (ii) $NaBH_4$, CH_3OH, 0 °C to RT; (b) (i) MsCl, Et_3N, DMF, 0 °C to RT; (ii) MeOH, RT; (iii) NaN_3, RT; (c) (i) $LiOH{\cdot}H_2O$, THF/H_2O, RT; (ii) HCl, $C_4H_8O_2$, RT.

reporter that is expected to have a larger oscillator strength compared to the nitrile vibrational reporter of pCNPhe and have a greater sensitivity to local environment compared to the nitrile probe. The addition of the methylene group between the azide and phenyl groups of pN_3CH_2Phe is expected to greatly diminish the photoreactivity of pN_3CH_2Phe relative to pN_3Phe, thus allowing pN_3CH_2Phe to serve as a stable, sensitive azide modified UAA vibrational reporter of local protein environments. The ability of pN_3CH_2Phe to serve as an effective probe of local protein environment was investigated by measuring the position of the azide asymmetric stretch vibration of pN_3CH_2Phe in various solvents and by incorporating this UAA site-specifically into two distinct sites of the 247-residue monomeric protein, superfolder green fluorescent protein[18] (sfGFP, Figure 1A) with high efficiency and fidelity. The photostability of this UAA compared with pN_3Phe incorporated into sfGFP was also examined.

EXPERIMENTAL SECTION

General Information. Chemical reagents were purchased from Sigma-Aldrich, Strem Chemicals, Gelest, and Praxair and used without further purification. Deuterated chloroform (98.8% D enrichment) and deuterium oxide (99.9% D enrichment) were purchased from Cambridge Isotope Laboratories. DH10B cells and *pBadA* were purchased from Invitrogen. All aqueous solutions were prepared with 18 MΩ cm water.

Reactions were stirred with a magnetic stir bar and conducted under a dry argon atmosphere unless otherwise noted. Reactions performed above or below ambient temperature were carried out in an oil bath or an ice bath, respectively. Analytical thin layer chromatography (TLC) was performed on 0.2 mm silica plastic coated sheets (Selecto Scientific) with F_{254} indicator. Flash column chromatography was performed on 230−400 mesh silica gel.

^1H (499.7 MHz) NMR spectra were obtained at 499.7 MHz with a Varian INOVA 500 multinuclear Fourier transform NMR spectrometer. Chemical shifts are reported in parts per million (ppm) and coupling constants (J) are reported in hertz (Hz). ^1H spectra in $CDCl_3$ were referenced to the solvent peak at 7.26 ppm and ^1H spectra in D_2O were referenced to the solvent peak at 4.65 ppm.

Synthesis of *N*-(*tert*-Butoxycarbonyl)-4-hydroxymethyl-L-phenylalanine Methyl Ester (5). **5** was synthesized based upon previous literature procedures with minor modifications.[19] A mixture of *N*-(*tert*-butoxycarbonyl)-4-((trifluoromethyl)sulfonyl)-L-phenylalanine methyl ester (**4**) (3.36 g, 7.86 mmol) synthesized as previously described,[20−22] palladium(II) acetate (159 mg, 0.24 mmol), 1,3-bis-(diphenylphosphino)propane (97 mg, 0.24 mmol) and triethylamine (2.74 mL, 19.7 mmol) in anhydrous DMF (39.3 mL) was purged with carbon monoxide for 10 min. Trioctylsilane (7.1 mL, 15.7 mmol) was then added in one portion and the reaction mixture was stirred under CO gas for 16 h at 70 °C. The mixture was subsequently flushed with N_2 gas and cooled to 0 °C. Sodium borohydride (446 mg, 11.8 mmol) was added in one portion followed by the dropwise addition of anhydrous methanol (7.86 mL) at 0 °C. The reaction mixture was then stirred at room temperature for 1 h and subsequently treated with 10% aqueous acetic acid for 10 min. The mixture was extracted three times with ethyl acetate, and the combined organic layers were washed twice with water, dried over magnesium sulfate, filtered through a Celite cake, and concentrated in vacuo to an oil. The oil was purified by flash chromatography (4:6 ethyl acetate:petroleum ether) to give 1.01 g (42%) of **5** as a pale yellow oil that crystallized upon standing. ^1H NMR ($CDCl_3$): 1.41 (s, 9H, $C(CH_3)_3$), 3.04 (dd, 1H, $C_ß-H_1$, J = 5.8 Hz, J = 13.7 Hz), 3.12 (dd, 1H, $C_ß-H_2$, J = 5.6 Hz, J = 13.9 Hz), 3.72 (s, 3H, OCH_3), 4.57 (m, 1H, $C_α-H$), 4.66 (s, 2H, CH_2OH), 4.97 (d, 1H, NH, J = 7.8 Hz), 7.11 (d, 2H, ArH, J = 8.4 Hz), 7.29 (d, 2H, ArH, J = 8.3 Hz).

Synthesis of *N*-(*tert*-Butoxycarbonyl)-4-azidomethyl-L-phenylalanine Methyl Ester (6). A mixture of **5** (850 mg, 2.75 mmol) and triethylamine (498 μL, 3.57 mmol) in anhydrous DMF (2.2 mL) was cooled to 0 °C. Methanesulfonyl chloride (276 μL, 3.57 mmol) was added dropwise at 0 °C and the reaction mixture was stirred for 1 h at room temperature. Anhydrous methanol (111 μL, 2.75 mmol) was then added in one portion and the reaction mixture was stirred for 20 min at room temperature. Sodium azide (268 mg, 4.12 mmol) was subsequently added and the reaction mixture was stirred for an additional 16 h at room temperature. The mixture was diluted with water and extracted three times with ethyl acetate. The combined organic layers were washed with water, 1 M ammonium chloride, water, brine (2×), dried over magnesium sulfate, filtered through a Celite cake, and concentrated in vacuo to an oil that crystallized upon standing to give **6** as a white solid (833 mg, 91%). ^1H NMR ($CDCl_3$): 1.42 (s, 9H, $C(CH_3)_3$), 3.05 (dd, 1H, $C_ß-H_1$, J = 6.4 Hz, J = 13.7 Hz), 3.13 (dd, 1H, $C_ß-H_2$, J = 6.2 Hz, J = 13.5 Hz), 3.71 (s, 3H, OCH_3), 4.32 (s, 2H, CH_2N_3), 4.59 (m, 1H, $C_α-H$), 4.98 (d, 1H, NH, J = 7.3 Hz), 7.15 (d, 2H, ArH, J = 7.8 Hz), 7.25 (d, 2H, ArH, J = 7.8 Hz).

Synthesis of 4-Azidomethyl-L-phenylalanine Hydrochloride (3·HCl). Lithium hydroxide monohydrate (124 mg, 2.95 mmol) was added to a solution of **6** (760 mg, 2.27 mmol) in THF/H_2O (3:1, 26.2 mL) and stirred for 16 h at room temperature. The pH of the reaction mixture was adjusted to ~2.5 with 0.5 M sodium bisulfate and the mixture was extracted three times with ethyl acetate. The combined organic layers were washed with water and brine, dried over magnesium sulfate, filtered through a Celite cake, and concentrated in vacuo to yield *N*-(*tert*-butoxycarbonyl)-4-azidomethyl-L-phenylalanine as a pale yellow oil. *N*-(*tert*-butoxycarbonyl)-4-

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azidomethyl-L-phenylalanine was subsequently dissolved in 2.5 M HCl in 1,4-dioxane (5.7 mL) and the reaction mixture was stirred at room temperature for 4 h. The reaction mixture was concentrated and pentane was added to precipitate **3·HCl**. The white solid product was isolated by filtration to give 510 mg (87%). ^{1}H NMR (D_2O): δ 3.11 (dd, 1H, $C_ß$-H_1, J = 7.9 Hz, J = 14.7 Hz), 3.24 (dd, 1H, $C_ß$-H_2, J = 5.9 Hz, J = 14.7 Hz), 4.17 (dd, 1H, $C_α$−H, J = 5.4 Hz, J = 7.8 Hz,), 4.32 (s, 2H, CH_2N_3), 7.25 (d, 2H, ArH, J = 8.3 Hz), 7.31 (d, 2H, ArH, J = 7.9 Hz); MS: 221.0 (M+1).

Expression and Purification of sfGFP Constructs. The codon-optimized gene containing a C-terminal 6-His affinity tag for wild-type sfGFP (wt-sfGFP)[18,23,24] was inserted into *pBadA* generating *pBad-sfGFP*. The codons for Y75 and D134 were individually replaced by site-directed mutagenesis with the amber stop codon (TAG) generating *pBad-sfGFP-75TAG* and *pBad-sfGFP-134TAG*, respectively. The aminoacyl-tRNA synthetase for the incorporation of pN_3Phe and pN_3CH_2Phe was inserted into *pDule* generating *pDule-pN$_3$/pN$_3$CH$_2$Phe*.[5,17] Each of these plasmids, except *pBad-sfGFP-75TAG*, was obtained from Dr. Ryan A. Mehl (Oregon State University).

pBad-sfGFP was transformed into DH10B *Escherichia coli* cells, while *pBad-sfGFP-75TAG* and *pBad-sfGFP-134TAG* were individually cotransformed with *pDule-pN$_3$/pN$_3$CH$_2$Phe* into DH10B *E. coli* cells. The transformed cells were used to inoculate 5 mL of noninducing media that was grown to saturation while shaking (250 rpm) at 37 °C. A portion (2.5 mL) of the cultured cells was used to inoculate 250 mL of autoinduction media containing pN_3Phe or pN_3CH_2Phe at 1 mM except for negative control experiments where the UAAs were excluded from the autoinduction media. The cells from the autoinduction media were collected by centrifugation after shaking at 37 °C for 24−30 h and the expressed protein was purified using TALON cobalt ion-exchange chromatography (Clontech) similar to previous procedures.[5,23,24]

Site-specific incorporation of pN_3Phe or pN_3CH_2Phe into site 75 or 134 with high efficiency and fidelity was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass analysis. Typical yields for the sfGFP constructs containing pN_3Phe or pN_3CH_2Phe were 240 mg and 130 mg of purified protein per liter of autoinduction media, respectively. The protein expression yields were calculated using the extinction coefficient of sfGFP at 488 nm.[18]

Equilibrium FTIR Measurements. Equilibrium FTIR absorbance spectra were recorded on a Bruker Vertex 70 FTIR spectrometer equipped with a globar source, KBr beamsplitter, and a liquid nitrogen cooled mercury cadmium telluride (MCT) detector. The spectra were recorded using a temperature-controlled transmission cell consisting of calcium fluoride windows with a path length of ∼100 μm or using a Harrick BioATRcell II accessory. The temperature was measured using an embedded thermocouple in the cells. The spectra were the result of 1024 scans recorded at a resolution of 1.0 cm^{-1}. The spectra were analyzed in Igor Pro (Wavemetrics).

RESULTS AND DISCUSSION

Comparison of pCNPhe, pN_3Phe, and pN_3CH_2Phe. The nitrile symmetric stretch vibration of pCNPhe (2234.6 cm^{-1}) and the azide asymmetric stretch vibration of pN_3Phe (2128.6 cm^{-1}) and pN_3CH_2Phe (2110.7 cm^{-1}) in water appear in a relatively clear region of the infrared as shown in Figure 2.

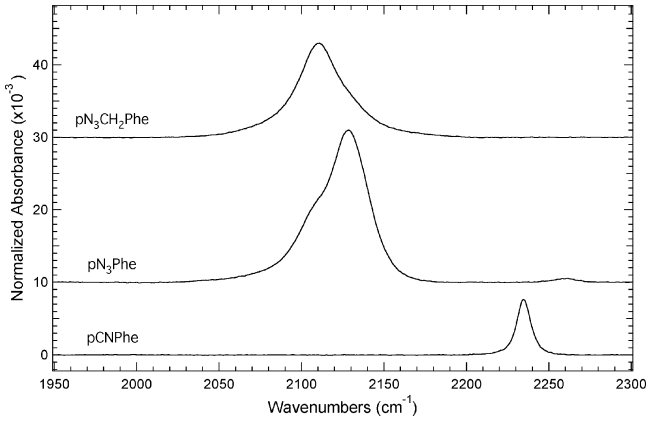

Figure 2. ATR-FTIR absorbance spectra of pCNPhe, pN_3Phe, and pN_3CH_2Phe dissolved in water. The UAAs were dissolved to a concentration of ∼50 mM in either an acidic or basic aqueous solution. The spectra were recorded at 25 °C and were baseline corrected.

These spectra show that the IR absorbance band resulting from the nitrile symmetric stretch vibration of pCNPhe is relatively sharp and symmetric compared to the IR absorbance band of pN_3Phe and pN_3CH_2Phe in this region (1950−2300 cm^{-1}). The IR absorbance band for pN_3Phe has at least two spectral components. The more intense high frequency component is assigned as resulting from the azide asymmetric stretch vibration based upon literature precedent,[6−8] while the low frequency component is likely the result of anharmonic coupling such as accidental Fermi resonance. The IR absorbance band for pN_3CH_2Phe in this region is more symmetric than the absorbance band of pN_3Phe, although it is slightly asymmetric toward higher frequencies, potentially due to anharmonic effects. This band is assigned as arising from the azide asymmetric stretch vibration of pN_3CH_2Phe since this is the expected region for this vibration,[6,7,10,25−29] although 2D IR spectroscopy[30−40] would be required to determine if the observed asymmetry is due to anharmonic coupling.

Figure 2 also shows that the IR absorbance band of pN_3Phe and pN_3CH_2Phe in this region is more intense than the IR absorbance band of pCNPhe. The azide IR absorbance band of pN_3Phe and pN_3CH_2Phe is ∼2.8 and ∼1.7 times more intense than the nitrile IR absorbance band of pCNPhe, respectively. The higher extinction coefficient of the azide asymmetric stretch vibration compared to the nitrile symmetric stretch vibration observed here is consistent with the previous literature comparisons of these two vibrational reporters.[2,10] The observed lower extinction coefficient for the azide asymmetric stretch vibration of pN_3CH_2Phe compared to pN_3Phe is in contrast with the reported higher extinction coefficient for this vibration in the nicotinamide adenine dinucleotide (NAD^+) analogue 3-picolyl azide adenine dinucleotide ($PAAD^+$) compared to azido-NAD^+.[41,42]

Figure 3 illustrates the sensitivity of the azide asymmetric stretch vibration of pN_3Phe and pN_3CH_2Phe to solvent. The solvents dimethyl sulfoxide (DMSO) and water were selected to mimic hydrophobic and hydrophilic environments present in proteins, respectively. Figure 3 shows that the azide asymmetric stretch vibration of pN_3Phe shifts from 2115.5 cm^{-1} to 2128.6 cm^{-1} upon going from DMSO to water as the solvent, resulting in a blue shift of 13.1 cm^{-1}. Similarly, the azide asymmetric stretch vibration of pN_3CH_2Phe shifts from 2097.7 cm^{-1} to

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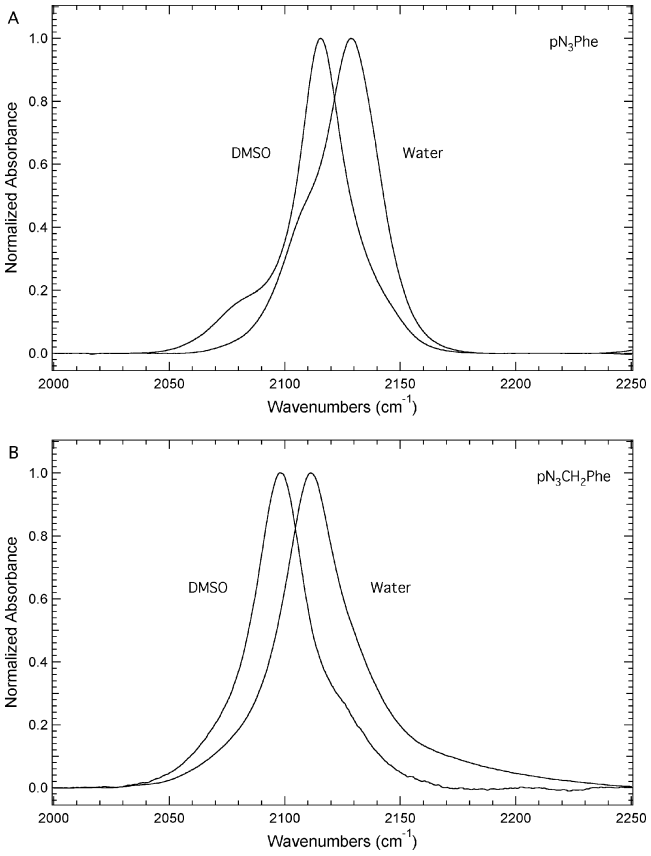

Figure 3. Transmission FTIR absorbance spectra of pN_3Phe (panel A) and pN_3CH_2Phe (Ppanel B) dissolved in either DMSO or water. The UAAs were dissolved to a concentration of ~50 mM. The spectra were recorded at 25 °C, baseline corrected, and intensity normalized.

2111.2 cm^{-1} upon going from DMSO to water as the solvent, resulting in a blue shift of 13.5 cm^{-1}. The full-width at half-maximum (fwhm) of this band increases from 28 cm^{-1} to 33 cm^{-1} for pN_3CH_2Phe upon going from DMSO to water as the solvent. These solvent-induced frequency shifts are significantly larger than the shift of the nitrile symmetric stretch vibration of pCNPhe in similar solvents. For instance, the nitrile symmetric stretch vibration was found to shift from 2228.5 cm^{-1} for Fmoc-pCNPhe in THF to 2237.2 cm^{-1} for pCNPhe in water,[9] a blue shift of 8.7 cm^{-1}, which is roughly two-thirds the size of that observed for pN_3Phe and pN_3CH_2Phe. The direction and magnitude of the solvent-induced frequency shift of the azide asymmetric stretch vibration of pN_3Phe and pN_3CH_2Phe is similar to the previously reported solvent-induced frequency shifts observed for this vibration in 5-azido-1-pentanoic acid.[43]

Structure of wt-sfGFP. Figure 1A shows the structure of the 247 residue β-barrel protein wt-sfGFP with residues 75 (magenta) and 134 (cyan) highlighted. This monomeric protein consists of 47% β-sheet and 10% helical structure. Residues 75 and 134 are both located in loop regions of the protein; however, these two residues represent two distinct local environments in the protein as illustrated by the solvent accessible surface area (SASA) of these two residues. The SASA for Y75 and D134 in wt-sfGFP was calculated to be 4 $Å^2$ and 100 $Å^2$, respectively, using the software GETAREA[44] with a probe radius of 1.4 Å. These results illustrate that site 75 represents a buried position in the protein, while site 134 represents a fully solvated position in the protein.

Incorporation of pN_3Phe and pN_3CH_2Phe into Site 75 or Site 134 of sfGFP. The UAAs pN_3Phe and pN_3CH_2Phe were genetically incorporated into the buried (site 75) or the fully solvated position (site 134) in sfGFP in response to an amber codon in an efficient, site-specific manner with high fidelity utilizing an engineered, orthogonal aminoacyl-tRNA synthetase. The incorporation of pN_3Phe into site 75 or site 134 in sfGFP resulted in the production of the protein constructs sfGFP-75-pN_3Phe and sfGFP-134-pN_3Phe, while the incorporation of pN_3CH_2Phe into site 75 or site 134 in sfGFP resulted in the production of the protein constructs sfGFP-75-pN_3CH_2Phe and sfGFP-134-pN_3CH_2Phe. The production of these constructs was verified by SDS-PAGE (see Figure 4) and ESI-Q-TOF mass analysis (see the Supporting Information). The high fidelity of the UAA incorporation was also confirmed by SDS-PAGE (Figure 4, lanes 5 and 8).

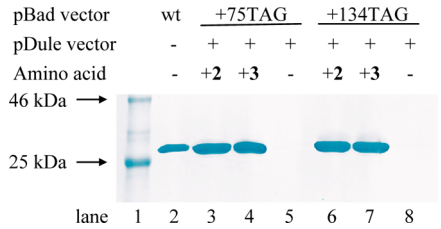

Figure 4. Coomassie blue stained tris-glycine SDS-PAGE illustrating efficient, site-specific incorporation of **2** and **3** with high fidelity into sfGFP. The protein constructs were expressed from *pBad-sfGFP* (wt-sfGFP, lane 2); *pBad-sfGFP-75TAG* and *pDule- pN_3/pN_3CH_2Phe* (lanes 3 − 5) in the presence (lanes 3 and 4) or absence (lane 5) of **2** or **3**, respectively; or *pBad-sfGFP-134TAG* and *pDule-pN_3/pN_3CH_2Phe* (lanes 6−8) in the presence (lanes 6 and 7) or absence (lane 8) of **2** or **3**, respectively.

Sensitivity of pN_3CH_2Phe to Local Protein Environments. Figure 5 shows the linear IR absorbance spectra of sfGFP-75-pN_3CH_2Phe and sfGFP-134-pN_3CH_2Phe in the azide asymmetric stretch vibration region (2000−2250 cm^{-1}). The IR spectrum of sfGFP-75-pN_3CH_2Phe shows a single, fairly symmetrical absorbance band at 2094.3 cm^{-1}. This frequency suggests that the azide group of pN_3CH_2Phe is in a

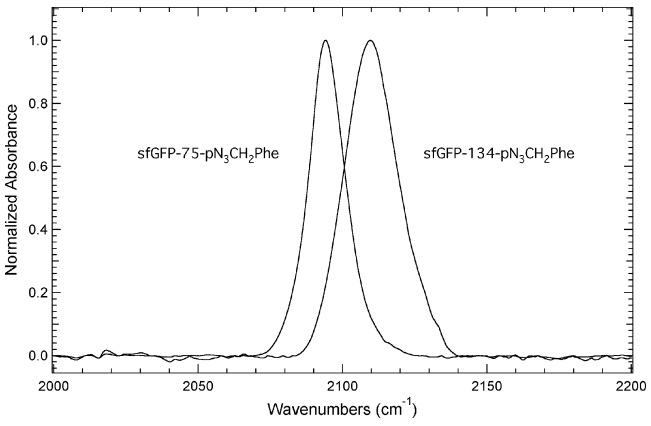

Figure 5. Transmission FTIR absorbance spectra of sfGFP constructs containing **3** at either site 75 or site 134 in the protein. The protein samples were dissolved to a concentration of ~1 mM in a pH 7.3 aqueous buffer containing 50 mM sodium phosphate and 150 mM sodium chloride. The spectra were recorded at 25 °C, baseline corrected, and intensity normalized.

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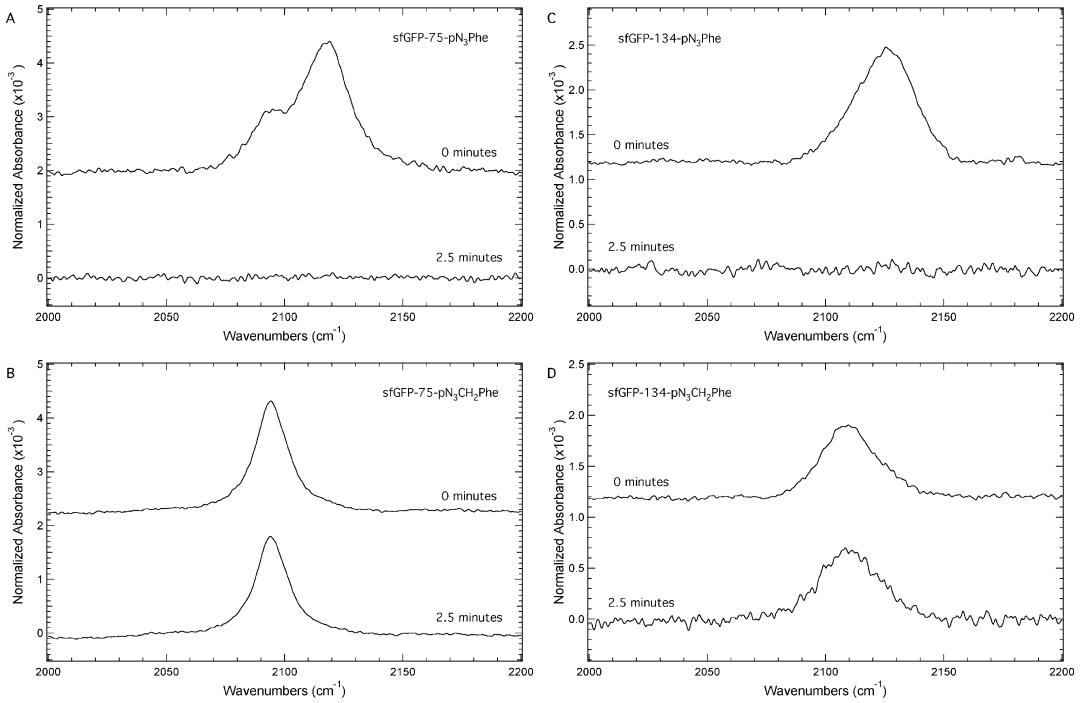

Figure 6. ATR-FTIR absorbance spectra of sfGFP containing either **2** or **3** at site 75 (panels A and B) or at site 134 (panels C and D) in the protein. The protein samples were dissolved to a concentration of ∼1 mM in a pH 7.3 aqueous buffer containing 50 mM sodium phosphate and 150 mM sodium chloride. The protein samples were irradiated for either zero or 2.5 min with a hand-held UV lamp (254 nm, 4 W). The spectra were recorded at 25 °C, baseline corrected, and normalized to the Amide I band.

buried, hydrophobic position in the protein due to the similarity of this frequency to the position of the azide IR absorbance band of pN_3CH_2Phe dissolved in DMSO (Figure 3B) and is in agreement with the SASA calculated for site 75 in sfGFP.

The IR spectrum of sfGFP-134-pN_3CH_2Phe also shows a single, fairly symmetrical IR absorbance band, which is centered at 2109.8 cm^{-1}. This frequency is similar to the frequency of pN_3CH_2Phe dissolved in water (Figure 3B) suggesting that the azide group of pN_3CH_2Phe is in a solvated position in the protein, which is in agreement with the SASA calculated for site 134 in sfGFP. The position of the azide IR absorbance band in these two protein constructs represents a 15.5 cm^{-1} blue shift in the azide asymmetric stretch vibration from site 75 to site 134 and illustrates a high sensitivity of this vibration to local protein environment. This result was expected based upon the direction and magnitude of the shift of the azide asymmetric stretch vibration of pN_3CH_2Phe when dissolved in DMSO and water (Figure 3B).

Photo-Stability of pN_3CH_2Phe in sfGFP. Figure 6 illustrates the significantly decreased photoreactivity of pN_3CH_2Phe relative to pN_3Phe. This figure shows the IR absorbance spectra of sfGFP-75-pN_3Phe (Panel A), sfGFP-75-pN_3CH_2Phe (Panel B), sfGFP-134-pN_3Phe (Panel C), and sfGFP-134-pN_3CH_2Phe (Panel D) in the region 2000−2200 cm^{-1} before and after irradiation of the samples for 2.5 min with a hand-held UV lamp (254 nm). The irradiation conditions were selected to be consistent with previous work[11] involving pN_3Phe and due to the position of the UV absorbance band of pN_3Phe at 251 nm (see the Supporting Information).

Panels A and C show an azide IR absorbance band for sfGFP-75-pN_3Phe and sfGFP-134-pN_3Phe at 2118.9 cm^{-1} and 2125.7 cm^{-1}, respectively, before irradiation. These frequencies correspond to the maximum intensity of the absorbance band. The position of the bands and the direction of the shift between the bands were expected based upon the effect of solvent on the position of this band in pN_3Phe (Figure 3A). After irradiation at 254 nm for 2.5 min, the azide IR absorbance band is absent from the spectra as expected based upon previous studies of pN_3Phe.[11−13]

By contrast, the azide IR absorbance band of sfGFP-75-pN_3CH_2Phe (Panel B) and sfGFP-134-pN_3CH_2Phe (panel D) at 2094.3 cm^{-1} and 2109.8 cm^{-1}, respectively, remain after irradiation at 254 nm for 2.5 min, exhibiting only a slight (∼10%) decrease in intensity after the irradiation. This significantly decreased photoreactivity of pN_3CH_2Phe relative to pN_3Phe was expected due to the significant decrease in absorbance at 254 nm in pN_3CH_2Phe compared to pN_3Phe (see the Supporting Information).

■ CONCLUSIONS

The UAA 4-azidomethyl-L-phenylalanine (pN_3CH_2Phe) has been successfully synthesized and represents a much improved vibrational reporter of local protein environments compared to the UAA vibrational reporters 4-cyano-L-phenylalanine (pCNPhe) and 4-azido-L-phenylalanine (pN_3Phe). Similar to the nitrile symmetric stretch vibration of pCNPhe, the azide asymmetric stretch vibration of pN_3CH_2Phe occurs in a relatively clear region of the infrared. However, the extinction coefficient of the azide asymmetric stretch vibration of pN_3CH_2Phe is ∼1.7 times greater than the nitrile symmetric stretch vibration of pCNPhe, which will ultimately allow lower protein concentrations to be utilized when pN_3CH_2Phe is incorporated into protein constructs compared to pCNPhe. Additionally, the azide asymmetric stretch vibration of pN_3CH_2Phe is more sensitive to local environment than the nitrile symmetric stretch vibration of pCNPhe as illustrated by

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the blue shift of 13.5 cm^{-1} of this vibration from DMSO to water.

pN_3CH_2Phe was also successfully genetically incorporated individually into two distinct sites of sfGFP in response to an amber codon in an efficient, site-specific manner with high fidelity utilizing an engineered, orthogonal aminoacyl-tRNA synthetase. The azide asymmetric stretch vibration of pN_3CH_2Phe was found to blue shift 15.5 cm^{-1} upon going from a buried position (site 75) in the protein to a solvated position (site 134), illustrating the high sensitivity of this vibrational mode to local protein environment.

The selection of pN_3CH_2Phe as the target azide analogue of pCNPhe was made to take advantage of the favorable azide vibrational properties, compared to that of the nitrile, while attempting to minimize the unfavorable photoreactivity of pN_3Phe (the direct azide analogue of pCNPhe). Our results show that the inclusion of the methylene spacer between the azide group and the phenyl ring of pN_3CH_2Phe, although reducing the intensity of the azide asymmetric stretch vibration of pN_3CH_2Phe relative to pN_3Phe, greatly reduced the photoreactivity of pN_3CH_2Phe compared to pN_3Phe, while minimizing the increase in size of pN_3CH_2Phe relative to pN_3Phe. Thus the photostability of pN_3CH_2Phe will permit the azide vibrational reporter to be utilized to effectively study local protein environments in a relatively noninvasive manner.

In addition to serving as effective vibrational reporters, azides can also undergo bioorthogonal click cycloaddition reactions with terminal alkynes.[45−47] Thus pN_3CH_2Phe can serve as both a vibrational reporter of local protein environments and a conduit to many other probes such as fluorescent dyes, nitroxide spin labels or metal carbonyl vibrational probes.

■ ASSOCIATED CONTENT

Supporting Information

Sequence of wt-sfGFP, ESI-Q-TOF mass analysis of the sfGFP protein constructs, equilibrium UV/vis absorbance spectra of pN_3Phe and pN_3CH_2Phe in water. 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.

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