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Modulation of *p*-Cyanophenylalanine Fluorescence by Amino Acid Side Chains and Rational Design of Fluorescence Probes of α-Helix Formation[†]

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ABSTRACT: *p*-Cyanophenylalanine is an extremely useful fluorescence probe of protein structure that can be recombinantly and chemically incorporated into proteins. The probe has been used to study protein folding, protein−membrane interactions, protein−peptide interactions, and amyloid formation; however, the factors that control its fluorescence are not fully understood. Hydrogen bonding to the cyano group is known to play a major role in modulating the fluorescence quantum yield, but the role of potential side-chain quenchers has not yet been elucidated. A systematic study of the effects of different side chains on *p*-cyanophenylalanine fluorescence is reported. Tyr is found to have the largest effect followed by deprotonated His, Met, Cys, protonated His, Asn, Arg, and protonated Lys. Deprotonated amino groups are much more effective fluorescence quenchers than protonated amino groups. Free neutral imidazole and hydroxide ion are also effective quenchers of *p*-cyanophenylalanine fluorescence with Stern−Volmer constants of 39.8 and 22.1 M^{-1}, respectively. The quenching of *p*-cyanophenylalanine fluorescence by specific side chains is exploited in developing specific, high-sensitivity, fluorescence probes of helix formation. The approach is demonstrated with Ala-based peptides that contain a *p*-cyanophenylalanine-His or a *p*-cyanophenylalanine-Tyr pair located at positions *i* and *i* + 4. The *p*-cyanophenylalanine-His pair is most useful when the His side chain is deprotonated and is, thus, complementary to the Trp-His pair which is most sensitive when the His side chain is protonated.

Fluorescence spectroscopy is widely used to study proteins and peptides, with the vast majority of studies making use of the naturally occurring fluorophores, Tyr and Trp (*1*, *2*). However, there is considerable interest in the incorporation of novel fluorophores, because they can offer site specific probes and, in many cases, can be chosen to allow selective excitation and detection (*3*−*7*). Recently, *p*-cyanophenylalanine (F_{CN})[1] fluorescence has been developed as a robust probe of protein folding, protein−peptide interactions, protein−membrane interactions, ligand binding, and amyloid formation (Figure 1) (*8*−*15*).

The utility of *p*-cyanophenylalanine is due to three features. First, it can be incorporated into proteins recombinantly, using the so-called 21st pair methodology, or by solid-phase peptide synthesis (*11*, *16*). Second, it represents a relatively conservative substitution for the aromatic amino acids since it is much more similar in shape and size than many other fluorophores and the polarity of the cyano group is between that of a methylene and that of an amide group. This intermediate polarity allows F_{CN} to be accepted in both hydrophobic and hydrophilic environments in a protein. Third, the photophysical properties of F_{CN} nicely complement existing fluorophores. It can be selectively excited in the presence of Trp and Tyr, and it forms a useful resonance energy transfer (RET) probe with both Tyr and Trp (*7*, *14*, *17*, *18*). However, the factors that control its quantum yield are not completely understood. It is known that the fluorescence is high when the cyano group is hydrogen bonded and fluorescence can be quenched by RET to Tyr or Trp, but unfortunately, the effect of other amino acid side chains is not known (*14*, *19*). A detailed understanding of the factors that control F_{CN} fluorescence is required to fully exploit this very promising probe and to avoid misinterpretation. A striking example is provided by our recent application of F_{CN} to study the folding of NTL9, a small globular protein. The fluorescence of F_{CN} in NTL9 was very low in the folded state, suggesting that the cyano group was sequestered from solvent; however, IR measurements showed that it was exposed, and further investigation revealed that the F_{CN} fluorescence was quenched by interactions with a Tyr side chain in the native state and showed that the cyano group was, in fact, exposed to solvent (*14*).

Here we systematically examine the ability of other amino acids and the termini of polypeptides to modulate F_{CN} fluorescence by examining the fluorescence of F_{CN} in a set of peptides of general sequence $GGF_{CN}XA$, where X represents Ala, Cys, His, Lys, Met, Asn, Arg, or Tyr. On the basis of these results, we demonstrate how such interactions can be used as a highly sensitive probe of helix formation. We also show that free imidazole and hydroxide ion are effective quenchers of F_{CN} fluorescence.

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[1]Abbreviations: F_{CN}, *p*-cyanophenylalanine; RET, resonance energy transfer; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; CD, circular dichroism; K_{SV}, Stern−Volmer constant.

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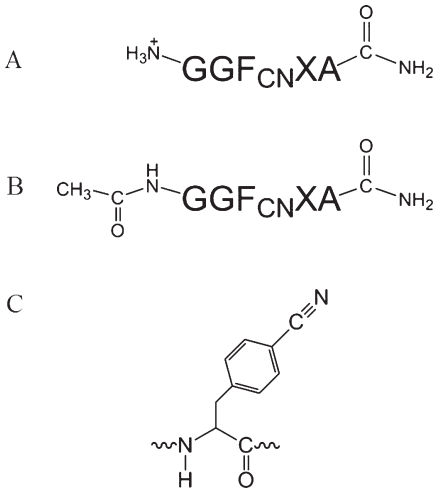

FIGURE 1: General sequence of the peptides studied. (A) Several variants have an amidated C-terminus and a free N-terminus. (B) Three additional peptides ($GGF_{CN}AA$, $GGF_{CN}HA$, and $GGF_{CN}KA$) were synthesized with an acetylated N-terminus. (C) Structure of the *p*-cyanophenylalanine side chain.

MATERIALS AND METHODS

Peptide Synthesis and Purification. A set of peptides with a general sequence $GGF_{CN}XA$ (X is A, C, H, K, M, N, R, or Y) were synthesized using standard Fmoc solid-phase methods on an Applied Biosystems 433A peptide synthesizer. PAL-PEG-PS resin was used which leads to an amidated C-terminus. Unless noted, the peptides have a free N-terminus. Additional $GGF_{CN}XA$ peptides (X is A, H, or K) were synthesized with an acetylated N-terminus and an amidated C-terminus. Two 21-residue Ala-based helical peptides were prepared with the sequence D-P-A-A-K-A-A-A-K-A-A-**X**-A-A-A-**F_{CN}**-A-A-A-K-A, where X is His or Tyr. These peptides were amidated at their C-termini and acetylated at their N-termini. The peptides were cleaved from the resin using 91% (v/v) trifluoroacetic acid (TFA), 3% (v/v) anisole, 3% (v/v) thioanisole, and 3% (v/v) 1,2-ethanedithiol and precipitated using cold ethyl ether, and scavengers were removed under vacuum. The crude peptides were purified via reverse-phase HPLC using a Vydac C18 semipreparative column. An A−B gradient system was used, with buffer A composed of a 0.1% (v/v) solution of TFA and buffer B composed of 90% (v/v) acetonitrile, 9.9% (v/v) water, and 0.1% (v/v) TFA. The gradient was from 0 to 90% B over 90 min. The identities of the purified peptides were confirmed by MALDI-TOF MS. The expected and observed molecular masses for the $GGF_{CN}XA$ peptides are 446.5 and 446.8 Da for $GGF_{CN}AA$, 538.6 and 538.6 Da for $GGF_{CN}YA$, 506.6 and 507.8 Da for $GGF_{CN}MA$, 478.5 and 478.5 Da for $GGF_{CN}CA$, 512.5 and 512.8 Da for $GGF_{CN}HA$, 503.6 and 504.0 Da for $GGF_{CN}KA$, 489.5 and 489.8 Da for $GGF_{CN}NA$, and 531.5 and 531.3 Da for $GGF_{CN}RA$, respectively. The expected and observed molecular masses for the Ac-$GGF_{CN}XA$ peptides are 488.5 and 488.1 Da for Ac-GGF_{CN}-AA, 554.5 and 554.2 Da for Ac-$GGF_{CN}HA$, and 545.6 and 545.3 Da for Ac-$GGF_{CN}KA$, respectively. The observed masses of the Ala-based peptides were 1961.0 Da for the H-F_{CN} peptide and 1987.3 Da for the Y-F_{CN} peptide. The expected masses were 1961.1 Da for the H-F_{CN} peptide and 1987.2 Da for the Y-F_{CN} peptide.

Fluorescence Spectroscopy. Fluorescence emission spectra of the peptides were recorded using an Applied Photon Technologies fluorimeter. The peptide concentration was determined by measuring the absorbance of the sample at 280 nm. The extinction coefficient for F_{CN} is 850 M^{-1} cm^{-1} and for Tyr 1490 M^{-1} cm^{-1} (*9*).

Table 1: Effect of Various Side Chains on *p*-Cyanophenylalanine Fluorescence[a]

side chain	intensity ratio relative to control ($GGF_{CN}XA$:$GGF_{CN}AA$)
Tyr	0.10
His (pH 10)	0.27
Met	0.31
Cys	0.54
His^+ (pH 5)	0.85
Asn	0.93
Lys^+ (pH 5)	1.00
Arg	1.00

[a]Measurements were performed in water at pH 5 if not otherwise indicated. Acetylated peptides were used to obtain the data for both of the His peptides.

Fluorescence was excited at 240 nm, and the emission signal was recorded from 250 to 350 nm. For the pH-dependent fluorescence experiments, the excitation wavelength for the F_{CN} group was 240 nm, and the emission signal was followed at 291 nm. The peptide concentration for both the fluorescence emission spectra and pH-dependent fluorescence experiments was 20 μM.

Emission spectra of the Ala-based peptides were collected in buffer and 8 M urea over the range of 250−350 nm with excitation at 240 nm. The peptide concentration was 25 μM. The Tyr-F_{CN} Ala rich peptide was prepared at pH 5.5 in 10 mM sodium acetate. Fluorescence emission spectra of the His-F_{CN} peptide were recorded as a function of pH. The His-F_{CN} Ala rich peptide was examined in 10 mM sodium acetate (pH 5.5) and 10 mM Tris (pH 8.3).

Circular Dichroism. Circular dichroism spectra of the 21-residue Ala-based peptides were recorded using a Chirascan Applied Photophysics CD spectrophotometer. The peptides were in 10 mM sodium acetate (pH 5.5) or 10 mM Tris (pH 8.3) for studies of the folded form. The unfolded peptides were in 8 M urea and either 10 mM sodium acetate (pH 5.5) or 10 mM Tris (pH 8.3). The urea concentration was determined using refractive index measurements. Wavelength scans were recorded at 25 °C from 190 to 260 nm for the folded peptide and from 210 to 260 nm for the urea-unfolded peptide.

RESULTS AND DISCUSSION

A set of peptides with the general sequence GGF_{CN} XA (X is A, C, H, K, M, N, R, or Y) were synthesized to probe the ability of different side chains to modulate F_{CN} fluorescence. These side chains include all of the potential quenching groups in proteins with the exception of Trp which has already been analyzed. Ser was not examined because it is known that hydrogen bonding to the cyano group leads to high fluorescence. The potential effects of protonated and deprotonated carboxylic acid were examined by a Stern−Volmer analysis of the quenching by acetic acid and sodium acetate (Supporting Information). All of the peptides have an amidated C-terminus. Two $GGF_{CN}AA$ peptides were prepared, one with a free N-terminus and the other with an acetylated N-terminus (Figure 1). The structure of F_{CN} is shown in Figure 1. The $GGF_{CN}AA$ peptide sequence is the parent (reference) peptide and was chosen as such since the Ala side chain will not quench the fluorescence (Table 1).

To determine the potential effects of the N-terminus, we examined the fluorescence of the $GGF_{CN}AA$ variant with a free N-terminus as a function of pH (Figure 2). The fluorescence

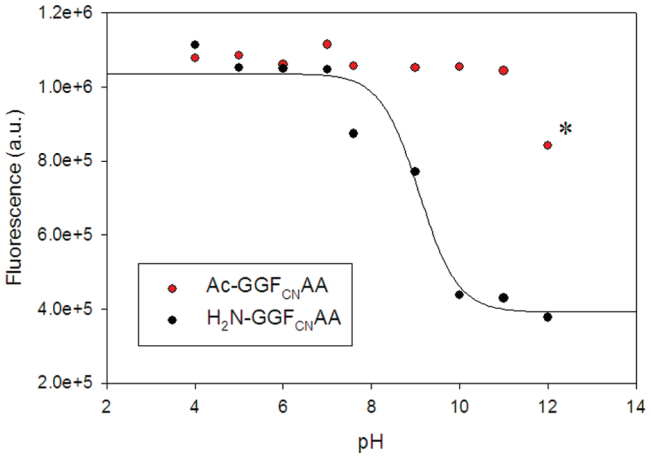

FIGURE 2: Comparison of the pH dependence of the fluorescence emission intensity for the GGF$_{CN}$AA peptide with a free N-terminus (black) and with an acetylated N-terminus (Ac-GGF$_{CN}$AA, red). The solid black line is a fit of the data to the Henderson−Hasselbalch equation with an apparent pK_a of 9.0. The decrease in intensity for the Ac-GGF$_{CN}$AA peptide at pH >11 is due to quenching by hydroxide ion (labeled with an asterisk). Fluorescence was excited at 240 nm, and the emission was collected at 291 nm. The peptide concentration was 20 μM. The experiments were conducted in water at the indicated pH values.

intensity titrates with a pK_a of 9.0, consistent with that of the amino group, decreasing as the amino group is deprotonated. This observation is consistent with reports about benzonitrile fluorescence quenching with diethylamine (*20*). As a control, a parent peptide with an acetylated N-terminus was also examined and showed no change in fluorescence up to pH 12. The decrease at pH 12 is reproducible and is due to quenching by hydroxide ion (Supporting Information).

The ability of individual side chains to modulate F_{CN} fluorescence was examined in the context of the GGF$_{CN}$AA peptides at pH 5. The peptides have a free N-terminus, but the pH-dependent studies of the parent peptide with the acetylated and unblocked N-terminus demonstrate that a protonated amino group has no effect, thus alleviating any potential concerns about the interpretation of the spectra, since quenching is dominated by the side chain. Figure 3 displays fluorescence emission spectra of various peptides. Each panel of the figure includes the spectrum of the parent peptide, GGF$_{CN}$AA, for comparison. At pH 5.0, the rank order of effectiveness in reducing the F_{CN} fluorescence is as follows: Y > M > C > H^+ > N > R = K^+ (where H^+ denotes a positively charged His side chain and K^+ a positively charged Lys side chain). Arg and positively charged Lys had no effect on F_{CN} fluorescence.

The pH-dependent data collected for the GGF$_{CN}$AA peptide suggest that it is worthwhile to examine the pH dependence of the quenching by His and Lys. To examine the effect of the protonation state of the His and Lys side chains, we recorded fluorescence emission spectra of N-terminally acetylated variants of the GGF$_{CN}$AA, GGF$_{CN}$HA, and GGF$_{CN}$KA peptides. Acetylation blocks the N-terminus, and the resulting amide linkage is not an effective quencher. This allows the effect of the side chain to be probed without complications from quenching by a deprotonated N-terminal amino group at high pH. The data indicate that a deprotonated His side chain is a much more effective quencher than a protonated one (Figure 4). Figure 5 displays the fluorescence emission of His and Lys peptides with an acetylated N-terminus as a function of pH. In the His peptide, the F_{CN} fluorescence decreases as the His side chain is deprotonated at higher pH and the intensity versus pH profile is well fit to an apparent pK_a of 7.3. It is

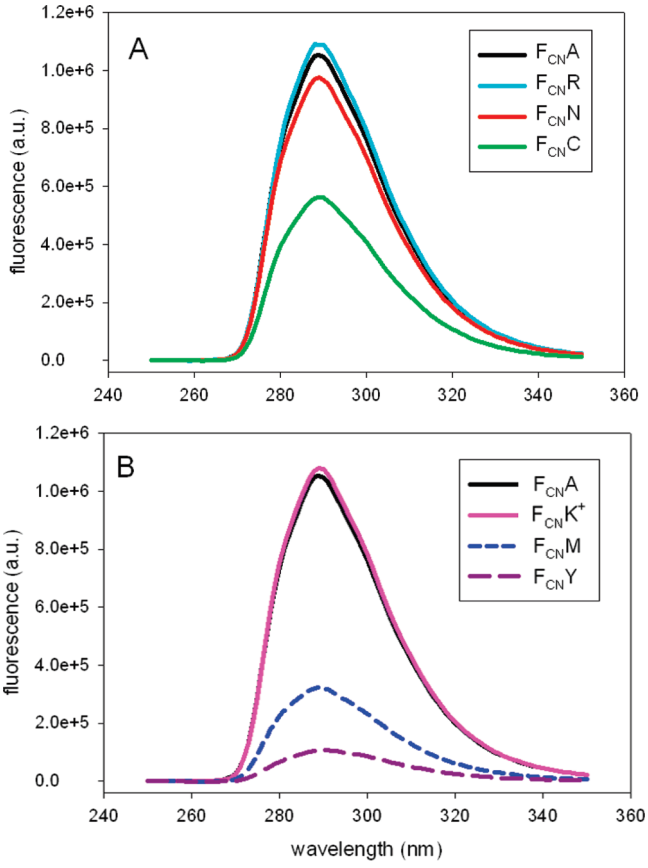

FIGURE 3: Fluorescence emission spectra of GGF$_{CN}$XA peptides with a free N-terminus at pH 5, X being (A) Ala, Arg, Asn, or Cys and (B) Ala, Met, Lys, or Tyr. Fluorescence was excited at 240 nm. The experiments were conducted in water.

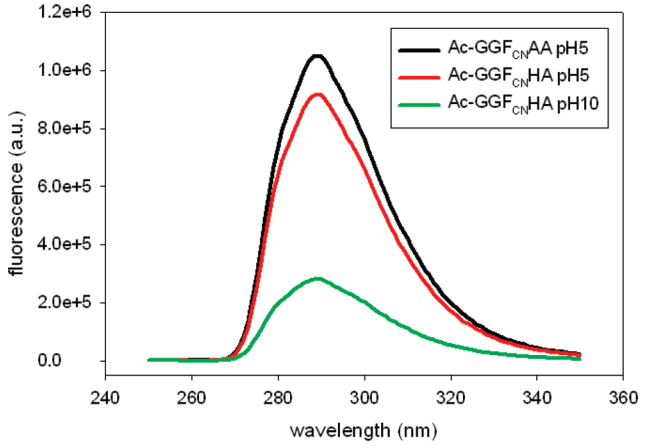

FIGURE 4: Comparison of the effects of a protonated and deprotonated His side chain on F_{CN} fluorescence measured for the GGF$_{CN}$XA peptides with an acetylated (blocked) N-terminus: (black) X is Ala at pH 5, (red) X is His at pH 5, and (green) X is His at pH 10. All spectra were recorded in water at the indicated pH. The sample concentration was 20 μM, and excitation was at 240 nm.

interesting to note that protonated His is a much more effective quencher of Trp fluorescence than neutral His, but the situation is reversed here (*21*, *22*). Likewise, for the Lys peptide, the F_{CN} fluorescence intensity does not change until the pH approaches the pK_a of the Lys side chain and then decreases. It was not possible to accurately monitor the F_{CN} fluorescence of the Lys peptide over the entire pH range, because quenching by hydroxide is significant at pH ≥12. However, our observation that a deprotonated N-terminus

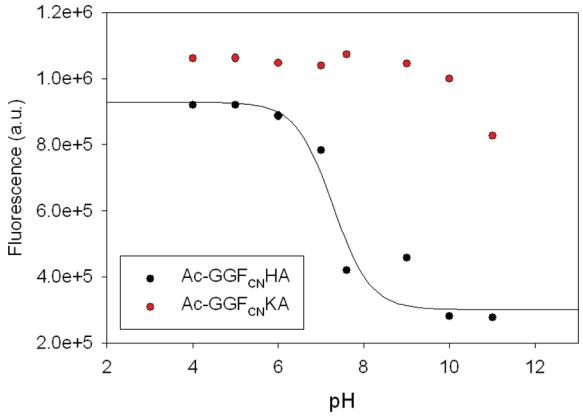

FIGURE 5: Fluorescence emission intensity of the Ac-GGF$_{CN}$HA (black) and Ac-GGF$_{CN}$KA (red) N-teminal acetylated peptides as a function of pH. The F$_{CN}$ fluorescence is quenched as the pH approaches the pK_a of His, indicating that deprotonated His is a better quencher of F$_{CN}$ fluorescence than protonated His. A decrease in the fluorescence intensity of the Ac-GGF$_{CN}$KA peptide is observed as the pH approaches the pK_a of Lys. Data are not reported above pH 11 because quenching by OH^- is significant. The solid curve is a fit of the His data to the Henderson–Hasselbalch equation and yields an apparent pK_a of 7.3.

is an effective quencher indicates that a deprotonated Lys side chain will be a better quencher than a protonated Lys side chain. Of course, the effect is likely to be of little practical significance in most studies of proteins given that the intrinsic pK_a of Lys is > 10. We examined the ability of protonated and deprotonated carboxylic acids to quench F$_{CN}$ fluorescence by conducting Stern–Volmer plots of the quenching by acetic acid and sodium acetate. Both are inefficient quenchers with Stern–Volmer constants of 0.31 and 0.25 M^{-1}, respectively (Supporting Information).

Combining the peptide data with the pH-dependent studies shows that the rank order of quenching is as follows: Y > H° > M > deprotonated N-terminus > C > H^+ > N > R = K^+ (where H° denotes a neutral His side chain). As noted, the quenching by a neutral Lys side chain could not be measured, but it is expected to be comparable to that of the deprotonated N-terminus.

The results with the His peptide indicate that free imidazole should be a quencher of F$_{CN}$ fluorescence and suggest that the neutral form should be more efficient than the protonated form. This was confirmed by Stern–Volmer analysis of quenching data for the acetylated GGF$_{CN}$AA peptide (Figure 6). A Stern–Volmer plot of the ratio of the F$_{CN}$ fluorescence in the absence and presence of the quencher (F_0/F) versus quencher concentration is linear. The slope of the curve, the Stern–Volmer constant (K_{SV}), is a measure of the efficiency of a quencher. K_{SV} for imidazole at pH 5 is 18.3 M^{-1} and at pH 9 is 39.8 M^{-1}, confirming that neutral imidazole is a significantly better quencher of F$_{CN}$ fluorescence than protonated imidazole. For comparison, the K_{SV} value for hydroxide ion is 22.1 M^{-1} (Supporting Information). Chloride ion is the only other reported quencher of F$_{CN}$ fluorescence, and its K_{SV} value is 9.3 M^{-1} (*9*, *23*).

F$_{CN}$-Based Site Specific Probes of Helix Formation. The short-range nature of the quenching suggests that suitable X-F$_{CN}$ pairs could be used to probe local structure formation. We tested this hypothesis by examining de novo designed helical peptides that contained either a Tyr-F$_{CN}$ pair or a His-F$_{CN}$ pair located one helical turn apart. Two 21-residue Ala-based peptides were designed to test the ability of F$_{CN}$ fluorescence quenching to probe helix formation. The designed peptides each contain an Asp residue at position 1 since it is an excellent capping residue

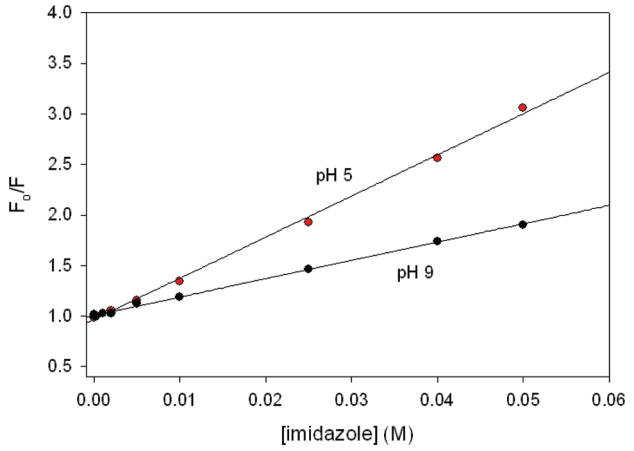

FIGURE 6: Stern–Volmer plots of the quenching of F$_{CN}$ fluorescence of the acetylated GGF$_{CN}$AA (Ac-GGF$_{CN}$AA) peptide by imidazole at pH 5 (red) and pH 9 (black). The Stern–Volmer constants are 18.3 M^{-1} at pH 5 and 39.8 M^{-1} at pH 9.

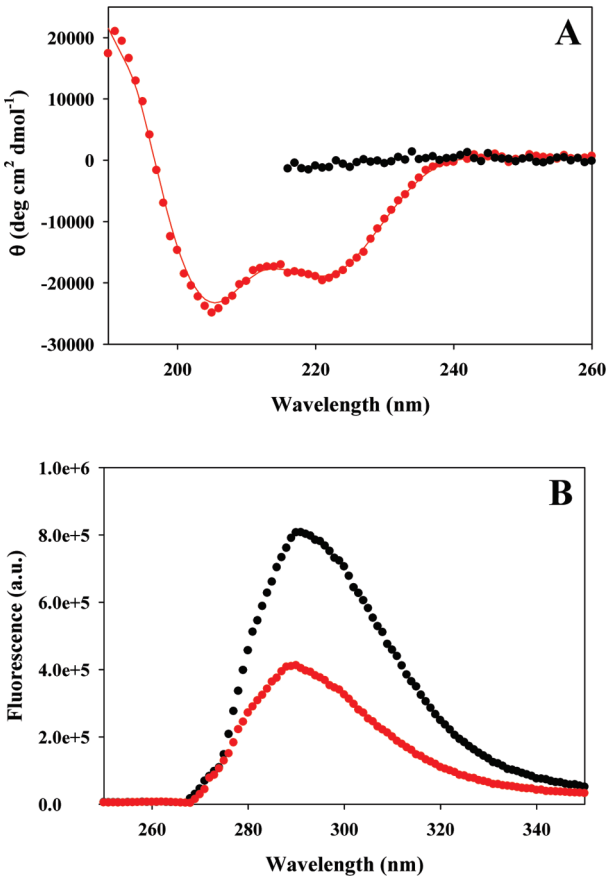

FIGURE 7: (A) CD spectra of the Tyr-F$_{CN}$ peptide indicate that it is partially helical in buffer (red) but unfolded in 8 M urea (black). Spectra were recorded at 25 °C and pH 5.5. (B) Fluorescence emission spectra confirm that the Tyr-F$_{CN}$ pair provides a probe of helix formation. Fluorescence emission spectra of the Tyr-F$_{CN}$ peptide in the partially helical state (red) and in the urea-unfolded state (black) are shown.

and a Pro at position 2 since prolines are favorable at this position in a helix (*24*). Several Lys residues were included to ensure solubility, and the termini were capped to avoid unfavorable interactions with the helix dipole. The method will be easiest to interpret quantitatively in terms of helix formation when the F$_{CN}$ group is exposed to solvent in both states since the fluorescence

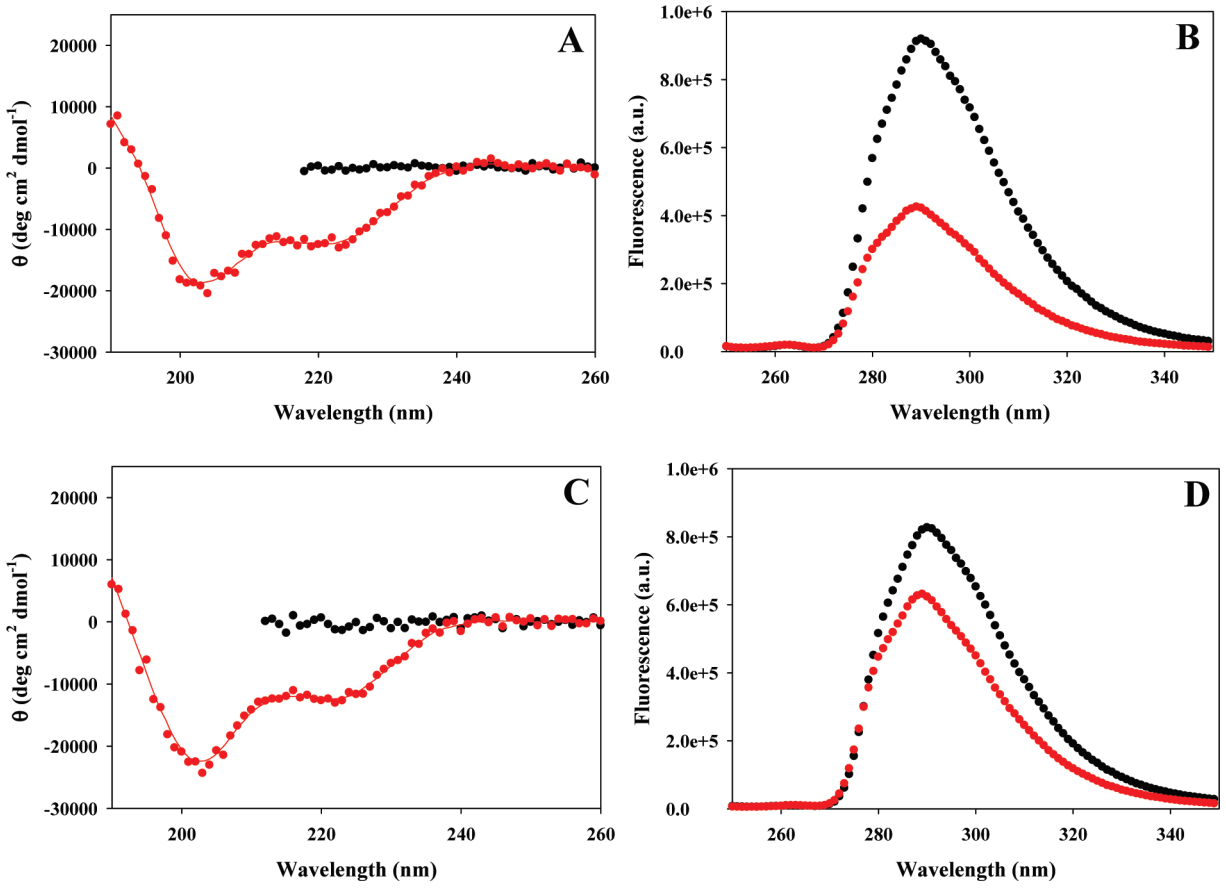

FIGURE 8: (A) CD spectra of the His-F_{CN} peptide at pH 8.3 indicate that it is partially helical in buffer (red) but unfolded in 8 M urea (black). The red curve in the CD spectra is included as a visual aid. (B) Fluorescence emission spectra confirm that the His-F_{CN} pair provides a sensitive probe of helix formation when the His side chain is neutral. Fluorescence emission spectra of the His-F_{CN} peptide in the partially helical state (red) and in the urea-unfolded state (black) are shown at pH 8.3. (C) CD spectra of the His-F_{CN} peptide at pH 5.5 indicate that it is partially helical in buffer (red) but unfolded in 8 M urea (black). (D) Fluorescence emission spectra confirm that the His-F_{CN} pair is a less sensitive probe of helix formation when the His side chain is charged. Fluorescence emission spectra of the His-F_{CN} peptide in the partially helical state (red) and in the urea-unfolded state (black) are shown at pH 5.5.

quantum yield is influenced by hydrogen bonding to the cyano group as well as by quenching from other protein side chains. If the F_{CN} group were buried in one conformation, but not in the other, then the observed intensity change would be a convolution of the effects due to changes in side-chain quencher interactions and the effects of changes in solvation (*18*). However, note that both quenching and side-chain burial reduce F_{CN} fluorescence. Thus, the pair can still be used as a probe of global folding even if the side chain is buried.

We first examined the use of a Tyr-F_{CN} pair. In our case, Tyr was incorporated at residue 12 and F_{CN} at residue 16. The distance between the Tyr and F_{CN} residues, when folded into a helical conformation, is well within the Förster distance of 12 Å (see the Supporting Information for the Förster distance calculation), and the cyano group is exposed to solvent. The CD spectrum indicates that the Tyr-F_{CN} peptide is partially helical in buffer. The spectrum shows the characteristic helical trend in ellipticity at 208 and 222 nm. The mean residue ellipticity is -19300 deg cm^2 dmol^{-1} at 222 nm, which corresponds to a helical content of 55%, as estimated using the method of Baldwin (Figure 7A) (*25*). CD reveals, as expected, that the peptide is unstructured in 8 M urea. The change in the conformation of the peptide is reflected by significant changes in its fluorescence properties. The fluorescence intensity is high in the urea-induced unfolded state but significantly reduced in the partially folded state in buffer, because of helix formation which brings the Tyr-F_{CN} pair into greater proximity (Figure 7B). The ratio of the fluorescence of the partially folded form to that of the unfolded form is 0.50. The data demonstrate that a solvent-exposed Tyr-F_{CN} pair can be used as a local fluorescence probe of helix formation.

The data collected for the GGF_{CN}HA peptide suggest that a solvent-exposed His-F_{CN} pair could also be exploited to provide a fluorescence-based probe of helix formation. Our analysis of the small peptides indicates that the method should be more sensitive at pH values where the His side chain is neutral. The quenching of Trp by protonated His has been used to probe protein conformation changes but is limited to pH values at which His is protonated (*26*). Thus, a His-F_{CN} pair should be complementary since it will be most effective when the His side chain is neutral. We examined the fluorescence properties of the His-F_{CN} pair-containing Ala-based peptide at pH 5.5, where the His side chain is predominantly protonated, and at pH 8.3, where it is largely deprotonated. The peptide is partially helical in buffer at both pH 5.5 and 8.3 as judged by CD (Figure 8). The helical content is lower than that observed for the Tyr-containing peptide, but it is still significant. The shape of the spectra suggests that the His-containing peptide is slightly more helical at the higher pH, although the helical content is on the order of 37−42% for both pHs. The addition of 8 M urea disrupts the helical structure (Figure 8). The fluorescence is lower in the partially folded state than in the unfolded state at both pHs; however, the change is much larger at pH 8.3, where the ratio of

the fluorescence of the partially folded form to that of the unfolded form is 0.46 (Figure 8B). At pH 5.5, the ratio is 0.74 (Figure 8D). The data confirm that solvent-exposed F_{CN}-His pairs can be effectively exploited in probing helical structure and demonstrate that the method is most sensitive when the His side chain is neutral.

CONCLUSIONS

The data presented here provide a comprehensive catalog of the effect of protein side chains on F_{CN} fluorescence. The short range of the quenching by His and Tyr can be exploited to design fluorescence-based sensors of specific elements of secondary structure. The strategy was demonstrated here using α-helical peptides but can obviously be applied to globular proteins as well. In this case, the F_{CN}-Tyr/His pair should be located in solvent-exposed sites so that the change in fluorescence intensity upon unfolding is dominated by changes in the quencher−fluorophore distance and not by changes in the solvation of the cyano group, which may arise if the probe were incorporated into a site that is buried in the native state but exposed in the unfolded state. The change in fluorescence should be even larger than that observed for the Ala rich peptides, since the helical structure will be fully formed in a folded globular protein but is only partially formed in the peptides. The approach is clearly not limited to helices and could be used to follow β-hairpin or β-sheet formation. The quenching studies of the pentapeptides indicate that a F_{CN}-Met pair could also be exploited to follow local structure formation.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Fluorescence emission spectrum of F_{CN}, absorbance spectrum of Tyr, Förster distance calculation for the F_{CN}-Tyr pair, and Stern−Volmer plots of F_{CN} fluorescence quenching by hydroxide ion, acetic acid, and sodium acetate. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Lakowicz, J. R. (2006) Principles of Fluorescence Spectroscopy, Springer Science+Business Media, LLC, New York.
2. Beechem, J. M., and Brand, L. (1985) Time-resolved fluorescence of proteins. *Annu. Rev. Biochem. 54*, 43–71.
3. Ross, J. B. A., Szabo, A. G., and Hogue, C. W. V. (1997) Enhancement of protein spectra with tryptophan analogs: Fluorescence spectroscopy of protein-protein and protein-nucleic acid interactions. *Fluoresc. Spectrosc. 278*, 151–190.
4. Wong, C. Y., and Eftink, M. R. (1998) Incorporation of tryptophan analogues into staphylococcal nuclease, its V66W mutant, and Δ137−149 fragment: Spectroscopic studies. *Biochemistry 37*, 8938–8946.
5. Twine, S. M., and Szabo, A. G. (2003) Fluorescent amino acid analogs. *Methods Enzymol. 360*, 104–127.
6. Wang, L., Xie, J., and Schultz, P. G. (2006) Expanding the genetic code. *Annu. Rev. Biophys. Biomol. Struct. 35*, 225–249.
7. Rogers, J. M. G., Lippert, L. G., and Gai, F. (2010) Non-natural amino acid fluorophores for one- and two-step fluorescence resonance energy transfer applications. *Anal. Biochem. 399*, 182–189.
8. Tucker, M. J., Getahun, Z., Nanda, V., DeGrado, W. F., and Gai, F. (2004) A new method for determining the local environment and orientation of individual side chains of membrane-binding peptides. *J. Am. Chem. Soc. 126*, 5078–5079.
9. Tucker, M. J., Oyola, R., and Gai, F. (2006) A novel fluorescent probe for protein binding and folding studies: p-Cyano-phenylalanine. *Biopolymers 83*, 571–576.
10. Tucker, M. J., Tang, J., and Gai, F. (2006) Probing the kinetics of membrane-mediated helix folding. *J. Phys. Chem. B 110*, 8105–8109.
11. Aprilakis, K. N., Taskent, H., and Raleigh, D. P. (2007) Use of the novel fluorescent amino acid p-cyanophenylalanine offers a direct probe of hydrophobic core formation during the folding of the N-terminal domain of the ribosomal protein L9 and provides evidence for two-state folding. *Biochemistry 46*, 12308–12313.
12. Marek, P., Gupta, R., and Raleigh, D. P. (2008) The fluorescent amino acid p-cyanophenylalanine provides an intrinsic probe of amyloid formation. *ChemBioChem 9*, 1372–1374.
13. Miyake-Stoner, S. J., Miller, A. M., Hammill, J. T., Peeler, J. C., Hess, K. R., Mehl, R. A., and Brewer, S. H. (2009) Probing protein folding using site-specifically encoded unnatural amino acids as FRET donors with tryptophan. *Biochemistry 48*, 5953–5962.
14. Taskent-Sezgin, H., Chung, J., Patsalo, V., Miyake-Stoner, S. J., Miller, A. M., Brewer, S. H., Mehl, R. A., Green, D. F., Raleigh, D. P., and Carrico, I. (2009) Interpretation of p-cyanophenylalanine fluorescence in proteins in terms of solvent exposure and contribution of side-chain quenchers: A combined fluorescence, IR and molecular dynamics study. *Biochemistry 48*, 9040–9046.
15. Liu, J., Strzalka, J., Tronin, A., Johansson, J. S., and Blasie, J. K. (2009) Mechanism of interaction between the general anesthetic halothane and a model ion channel protein, II: Fluorescence and vibrational spectroscopy using a cyanophenylalanine probe. *Biophys. J. 96*, 4176–4187.
16. Schultz, K. C., Supekova, L., Ryu, Y. H., Xie, J. M., Perera, R., and Schultz, P. G. (2006) A genetically encoded infrared probe. *J. Am. Chem. Soc. 128*, 13984–13985.
17. Tucker, M. J., Oyola, R., and Gai, F. (2005) Conformational distribution of a 14-residue peptide in solution: A fluorescence resonance energy transfer study. *J. Phys. Chem. B 109*, 4788–4795.
18. Glasscock, J. M., Zhu, Y. J., Chowdhury, P., Tang, J., and Gai, F. (2008) Using an amino acid fluorescence resonance energy transfer pair to probe protein unfolding: Application to the villin headpiece subdomain and the LysM domain. *Biochemistry 47*, 11070–11076.
19. Serrano, A. L., Troxler, T., Tucker, M. J., and Gai, F. (2010) Photophysics of a fluorescent non-natural amino acid: p-Cyanophenylalanine. *Chem. Phys. Lett. 487*, 303–306.
20. Yoshida, M., Kaneko, H., Kitamura, A., Ito, T., Oohashi, K., Morikawa, N., Sakuragi, H., and Tokumaru, K. (1976) Mechanism for light-induced hydrogen isotope exchange in benzonitrile. *Bull. Chem. Soc. Jpn. 49*, 1697–1700.
21. Chen, Y., and Barkley, M. D. (1998) Toward understanding tryptophan fluorescence in proteins. *Biochemistry 37*, 9976–9982.
22. VanGilst, M., and Hudson, B. S. (1996) Histidine-tryptophan interactions in T4 lysozyme: 'Anomalous' pH dependence of fluorescence. *Biophys. Chem. 63*, 17–25.
23. Marek, P., Mukherjee, S., Zanni, M. T., and Raleigh, D. P. (2010) Residue specific, real time characterization of lag phase species and fibril growth during amyloid formation: A combined fluorescence and IR study of p-cyanophenylalanine analogs of islet amyloid polypeptide. *J. Mol. Biol.* (in press), DOI 10.1016/j.jmb.2010.05.041I.
24. Aurora, R., and Rose, G. D. (1998) Helix capping. *Protein Sci. 7*, 21–38.
25. Luo, P. Z., and Baldwin, R. L. (1997) Mechanism of helix induction by trifluoroethanol: A framework for extrapolating the helix-forming properties of peptides from trifluoroethanol/water mixtures back to water. *Biochemistry 36*, 8413–8421.
26. Kubelka, J., Chiu, T. K., Davies, D. R., Eaton, W. A., and Hofrichter, J. (2006) Sub-microsecond protein folding. *J. Mol. Biol. 359*, 546–553.