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Quenching of *p*-Cyanophenylalanine Fluorescence by Various Anions

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Abstract

To expand the spectroscopic utility of the non-natural amino acid *p*-cyanophenylalanine (Phe_{CN}), we examine the quenching efficiencies of a series of commonly encountered anions toward its fluorescence. We find that iodide exhibits an unusually large Stern-Volmer quenching constant, making it a convenient choice in Phe_{CN} fluorescence quenching studies. Indeed, using the villin headpiece subdomain as a testbed we demonstrate that iodide quenching of Phe_{CN} fluorescence offers a convenient means to reveal protein conformational heterogeneity. Furthermore, we show that the amino group of Phe_{CN} strongly quenches its fluorescence, suggesting that Phe_{CN} could be used as a local pH sensor.

Introduction

Because of its distinctive spectroscopic characteristics, relatively small size, and ease of incorporation, the non-natural amino acid, *p*-cyanophenylalanine (Phe_{CN}), has recently emerged as a useful spectroscopic probe of protein structure, dynamics and electrostatics [1–4]. In particular, several photophysical properties of Phe_{CN} make it an attractive fluorescence reporter of many biological processes, such as protein folding [5], binding [6–10] and aggregation [11,12]. This is because (1) the fluorescence quantum yield of Phe_{CN} is sensitive to its environment [5,7], such as the degree of hydration, (2) the fluorescence lifetime of Phe_{CN} correlates linearly with the hydrogen bonding ability of many protic solvents [13], making it possible to use it to quantitatively determine local hydrogen bonding strength, (3) Phe_{CN} fluorescence can be selectively excited even in the presence of other naturally occurring aromatic amino acids [7], such as tyrosine (Tyr) and tryptophan (Trp), and (4) Phe_{CN} can be used as a fluorescence resonance energy transfer (FRET) donor to Trp and other natural or non-natural amino acid fluorophores [14–19], further demonstrating its versatility as a fluorescence reporter. In principle, the application of Phe_{CN} fluorescence can be further extended to structural and dynamic studies by exploiting the mechanism of dynamic quenching by, for example, a halide ion. To facilitate this potential application and to identify practically useful anionic quenchers, herein we determine the quenching efficiencies of a series of sodium salts on the fluorescence of Phe_{CN}, either as a free amino acid or in a peptide environment. In addition, we demonstrate, using the villin

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headpiece subdomain (HP35) as a model, how quenching of Phe_{CN} fluorescence can be used to scrutinize the conformation heterogeneity of proteins, even under native conditions.

The technique of fluorescence quenching has long been used in protein structural studies [20]. For example, quenching of Trp fluorescence [21,22] by either iodide or acrylamide [23] has been widely employed to reveal the relative accessibility of the indole ring in the system of interest, thus providing useful structural information. While we fully anticipate that Phe_{CN} can be used in similar applications, we emphasize that the current study is not aimed to show that Phe_{CN} can replace Trp, but rather to demonstrate another potential utility of this versatile spectroscopic probe. Because for amino acid fluorophores, such as Trp and Tyr, quenching of fluorescence by many anions typically occurs through the mechanism of dynamic quenching [20], without involving static complex formation, we expect that those quenchers that have been shown to be effective in decreasing the fluorescence quantum yields of Trp and Tyr would also be effective for Phe_{CN} fluorescence. Thus, in the current study we have chosen the following quenchers: NaCl, NaBr, and NaI. In addition, to determine the effect of commonly used salts in biological studies on the fluorescence quantum yield of Phe_{CN}, we have also included NaH₂PO₄, Na₂CO₃, NaHCO₃, NaSCN and Na₂S₂O₃. Our results indicate that among these anions, iodide exhibits the largest Stern-Volmer quenching constant, making it a convenient dynamic quencher of Phe_{CN} fluorescence in protein conformational studies. Indeed, using iodide quenching of Phe_{CN} fluorescence we are able to show, in agreement with previous studies [24,25], that HP35 samples at least two conformations in its folded basin.

Experimental

2.1. Sample preparation

p-Cyanophenylalanine was purchased from Bachem Americas (Torrance, CA). The sodium salts were purchased from either Fisher Scientific or Acros Organics. They were the highest grade available and thus used without further purification. All solution samples were prepared by directly dissolving an appropriate amount of the targeted solute in Millipore water of appropriate volume. Protein synthesis was achieved on a PS3 automated peptide synthesizer (Protein Technologies, MA) using standard Fmoc protocols.

2.2. Spectroscopic measurements

The absorption spectra were collected on a Perkin Elmer Lambda 25 UV/Vis spectrometer at room temperature. The fluorescence spectra were collected on a Jobin Yvon Horiba Fluorolog 3.10 spectrofluorometer using a 1 cm quartz cuvette at 25 °C, with a spectral resolution of 1 nm and an integration time of 1 s/nm. The excitation wavelength was 275 nm. For the free Phe_{CN} quenching experiments, the concentration of the fluorophore was maintained at a constant value of 165 μM, whereas for other quenching experiments the peptide and protein concentrations were in the range of 10 – 20 μM. In addition, the Raman band of water has been subtracted from the raw fluorescence spectra and, whenever necessary, the inner-filter effect was considered and corresponding corrections were made.

2.3. Data analysis

Unless otherwise specified, all fluorescence quenching data were fit to the standard Stern-Volmer equation [26],

$$\frac{F_0}{F} = (1 + K_{sv} [Q]) e^{V[Q]} \quad (1)$$

where F and F_0 represent the fluorescence intensities of Phe_{CN} obtained with and without the presence of the quencher Q, respectively, and $[Q]$ is the molar concentration of the quencher. In addition, K_{SV} is the Stern-Volmer constant for the dynamic quenching process and V is an effective volume constant representing the effect of sphere-of-action [27].

Results and discussion

3.1. Quenching of free Phe_{CN} fluorescence by halide ions

Halide ions, especially iodide, are commonly used in fluorescence quenching studies of biological systems [28]. For example, iodide is an efficient quencher of Trp fluorescence, with a Stern-Volmer quenching constant (K_{SV}) of 11.6 M^{-1} at neutral pH [28]. As shown (Figures 1 and 2), the fluorescence quantum yield of free Phe_{CN} in aqueous solution is also sensitive to the presence of iodide: a quantitative analysis of the fluorescence quenching data yields a K_{SV} of $58.6 \pm 2.6 \text{ M}^{-1}$. As expected (Figure 2 and Table 1), bromide is also an efficient quencher of Phe_{CN} fluorescence ($K_{SV} = 54.9 \pm 2.4 \text{ M}^{-1}$), whereas in comparison chloride is far less effective ($K_{SV} = 10.6 \pm 2.0 \text{ M}^{-1}$). This trend is consistent with the well documented notion that dynamic fluorescence quenching often arises from the heavy-atom effect [27], which increases the yield or rate of the intersystem crossing process [29]. Perhaps more importantly, in comparison to quenching experiments carried out with Trp, both iodide and bromide exhibit an unusually large quenching efficiency towards Phe_{CN} fluorescence, suggesting that both are excellent candidates for quenching Phe_{CN} fluorescence in practice. A previous study by Serrano *et al.* [25] has shown that solute-solvent interactions have a large effect on the non-radiative decay rate of the fluorescent state of free Phe_{CN}, suggesting that internal conversion is the major non-radiative decaying channel. On the other hand, it has been shown that Trp has a relatively large intrinsic triplet state quantum yield (>0.1) [30]. Thus, it seems reasonable to attribute the difference between the iodide quenching efficiencies of Phe_{CN} fluorescence and Trp fluorescence to the difference in their intrinsic quantum yields of triplet state formation.

Taken together, the above results suggest that, due to its large K_{SV} value, iodide is potentially a very useful Phe_{CN} fluorescence quencher in protein conformational studies. To validate this potential utility of iodide, we further conducted fluorescence quenching experiments using a tripeptide, Gly-Phe_{CN}-Gly. As shown (Figure 3 and Table 1), the Phe_{CN} fluorescence quenching efficiency of iodide obtained in this case, as judged by the value of K_{SV} , is almost identical to that obtained with free Phe_{CN}, indicating that the large effect of iodide on Phe_{CN} fluorescence stems from its collisions with the sidechain of Phe_{CN} and therefore can be used as a sensitive and convenient Phe_{CN} fluorescence quencher in protein conformational studies.

3.2. Quenching of free Phe_{CN} fluorescence by other anions

In order to better determine the conditions under which Phe_{CN} fluorescence can be used as an effective spectroscopic reporter, we also studied the Phe_{CN} fluorescence quenching effects of the following sodium salts: NaH₂PO₄, NaSCN, Na₂S₂O₃, and Na₂CO₃. These salts were chosen because they are commonly used in biological studies to serve either as a buffer, denaturant, or precipitant [31]. As shown (Figure 4 and Table 1), both SCN^- and $\text{S}_2\text{O}_3^{2-}$ can effectively quench the fluorescence of free Phe_{CN}. On the other hand, phosphate, which is commonly used in biological buffers, has little, if any, effect on the fluorescence quantum yield of Phe_{CN}. What is surprising, however, is that Na₂CO₃ seems to show an unexpectedly larger quenching efficiency towards Phe_{CN} fluorescence (Figure 5). Since Na₂CO₃ is a relatively strong base ($\text{p}K_b = 4.67$), which increases the pH of the aqueous Phe_{CN} solution to about 10.2 at 1 mM concentration, it is possible that the large quenching effect observed arises from deprotonation of the amine group of the amino acid, which for

phenylalanine has a pK_a value of 9.13. Indeed, as shown (Figure 5), adjusting the pH of the Phe_{CN} solution using NaOH to 9.9 achieved the same quenching effect, whereas at 1 mM concentration NaHCO₃, which is a weaker base than Na₂CO₃, only showed a small degree of fluorescence quenching. Thus taken together, these results validate the above hypothesis. In addition, the results obtained with Gly-Phe_{CN}-Gly and Na₂CO₃, which demonstrate that Na₂CO₃ shows appreciable fluorescence quenching only at high concentrations (data not shown), also corroborate this notion. Taken together, these results suggest that the –NH₂ group is an efficient quencher of the fluorescence of free Phe_{CN}. This finding is particularly interesting as it suggests that Phe_{CN} fluorescence could be potentially useful as a pH sensor, for example, by appending a Phe_{CN} residue to the N-terminus of a peptide or protein.

3.3. Application to HP35

To demonstrate the utility of quenching Phe_{CN} fluorescence by iodide, we used it to characterize the native state conformational heterogeneity of HP35. As shown (Figure 6), the NMR structure of HP35 indicates that it has a well organized hydrophobic cluster with Phe58 being situated inside the resultant hydrophobic core. However, two recent studies, one using two-dimensional infrared (2D IR) spectroscopy [24] and another employing time-resolved fluorescence energy transfer (FRET) [25], provided strong evidence suggesting that even in the native state of HP35 Phe58 can sample two different environments, one of which is accessible by water molecules. Thus, HP35 constitutes a good model system to test the feasibility of using iodide quenching of Phe_{CN} fluorescence in protein conformational studies. To do so, we mutated the Phe58 residue to Phe_{CN} and also Trp64 to alanine (the resultant doubled mutant is referred to as HP35-Phe_{CN}-WA) because Trp is known to quench Phe_{CN} fluorescence through the Förster mechanism [6]. Since this double mutation has been shown to decrease the stability of the native fold [25], we conducted the fluorescence quenching experiments in aqueous solutions that contain 20% (v/v) trifluoroethanol (TFE). As shown (Figure 6), it is clear that the fluorescence quenching data can not be fit by the standard Stern-Volmer equation, which suggests that the Phe_{CN} residue samples two or more inequivalent environments. Indeed, the iodide quenching data can be well described by the following model, which assumes, based on previous studies [25], that HP35-Phe_{CN}-WA can sample two different conformations in the native basin:

$$\frac{F_0}{F} = \frac{1}{\frac{\alpha}{(1+K_{SV1}[Q])e^{V_1[Q]}} + \frac{1-\alpha}{(1+K_{SV2}[Q])e^{V_2[Q]}}} \quad (2)$$

where K_{SV1} (and V_1) and K_{SV2} (and V_2) represent the corresponding Stern-Volmer quenching parameters for conformations 1 and 2, respectively, and α is the fractional fluorescence contribution of conformation 1. As shown (Figure 6), by fixing the K_{SV1} value to the Stern-Volmer quenching constant of iodide obtained with the Gly-Phe_{CN}-Gly peptide, we were able to quantitatively fit the fluorescence quenching data of HP35-Phe_{CN}-AW to Eq. (2). The resultant fitting parameters are consistent with previous studies [24,25]. For example, the Stern-Volmer constant for the second conformation is about 0.001 M^{-1} , indicating that the Phe_{CN} residue in this conformation is far less solvent-accessible, in comparison to that in the first conformation. In addition, the value of α (0.51) is also in qualitative agreement with the study of Urbanek *et al.* [24], who have estimated, based on parameters obtained by analyzing both the linear and two dimensional infrared spectra, that the population of the solvent exposed Phe_{CN} conformation is 56%. The difference between these two studies most likely arises from one or all of the following factors: (1) in the current case the parameter α does not directly report on the molar fraction of the population of the Phe_{CN} exposed conformation as the fluorescence quantum yield of Phe_{CN} is known to depend on hydration, (2) the addition of 20% TFE changes the equilibrium constant of these two protein conformations, and (3) the two mutations in HP35-Phe_{CN}-WA alter the native

population distribution. Regardless of the origin of this difference, our results nevertheless confirm that iodide quenching of Phe_{CN} fluorescence is a useful tool in the study of protein conformational heterogeneity.

Conclusion

To further expand the spectroscopic utility of the non-natural amino acid Phe_{CN}, herein we investigate the quenching efficiencies of a series of anions to its fluorescence. Our results show that iodide has an unusually large Stern-Volmer quenching constant (i.e., $K_{SV} = 61.8 \pm 3.0 \text{ M}^{-1}$) and thus can be used as an efficient quenching agent in conjunction with Phe_{CN} fluorescence in protein conformational studies. We validate the applicability of this approach by applying it to reveal the native conformational heterogeneity of the villin headpiece subdomain (HP35). Furthermore, we demonstrate that the neutral amino group, but not the positive ammonium group, of the Phe_{CN} amino acid strongly quenches its fluorescence, suggesting that a Phe_{CN} residue located at the N-terminus of a polypeptide sequence could be used as a local pH sensor.

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Highlights

Iodide is an efficient quencher of Phe_{CN} fluorescence

Phosphate does not quench fluorescence

Quenching of Phe_{CN} fluorescence is a useful tool to examine conformational heterogeneity of proteins

Figure 1. Normalized fluorescence spectra of free Phe_{CN} collected at different NaI concentrations, as indicated.

Figure 2. Relative fluorescence intensity of free Phe_{CN} versus concentration of different halide quenchers, as indicated. The smooth lines are fits of these data to the Stern-Volmer equation and the resulting parameters are listed in Table 1.

Figure 3.

Relative fluorescence intensity of Gly-Phe_{CN}-Gly versus $[NaI]$. The smooth line is the fit of these data to the Stern-Volmer equation and the resulting parameters are $K_{SV}=61.8 \pm 3.0$ M^{-1} and $V=0.024 \pm 0.010$ M^{-1} . Shown in the inset are representative normalized fluorescence spectra of this tripeptide collected at different NaI concentrations, as indicated.

Figure 4. Relative fluorescence intensity of free Phe_{CN} versus concentration of different quenchers, as indicated.

Figure 5. Normalized fluorescence spectra of free Phe_{CN} collected with and without the presence of NaH₂PO₄, Na₂CO₃, NaHCO₃, or NaOH, as indicated.

Figure 6.

Relative fluorescence intensity of Phe_{CN} in HP35-Phe_{CN}-WA versus NaI concentration. The smooth lines are fits of these data to Eqs. (1) and (2), as indicated. The fitting parameters obtained with Eq. (2) are $K_{SV1} = 61.8 \pm 3.0 \text{ M}^{-1}$, $V_1 = 3.2 \pm 3.0 \text{ M}^{-1}$, $K_{SV2} = 0.001 \pm 0.001 \text{ M}^{-1}$, $V_2 = 4.8 \pm 3.0 \text{ M}^{-1}$, and $\alpha = 0.51 \pm 0.10$. Shown in the inset is the NMR structure of HP35 with the native Phe and Trp sidechains highlighted.

Table 1Phe_{CN} fluorescence quenching constants for various salts

Salt	K_{SV} (M ⁻¹)	V (M ⁻¹)
NaI	58.6 ± 2.6	4.6 ± 0.5
NaBr	54.9 ± 2.4	1.4 ± 0.5
NaCl	10.6 ± 2.0	0.0
Na ₂ S ₂ O ₃	39.6 ± 4.0	10.0 ± 2.0
NaSCN	35.4 ± 3.0	2.7 ± 0.5