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Sensing pH via *p*-Cyanophenylalanine Fluorescence: Application to Determine Peptide pK_a and Membrane-Penetration Kinetics

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Abstract

We expand the spectroscopic utility of a well-known infrared and fluorescence probe, *p*-cyanophenylalanine, by showing that it can also serve as a pH sensor. This new application is based on the notion that the fluorescence quantum yield of this unnatural amino acid, when placed at or near the N-terminal end of a polypeptide, depends on the protonation status of the N-terminal amino group of the peptide. Using this pH sensor, we are able to determine the N-terminal pK_a values of nine tripeptides and also the membrane penetration kinetics of a cell-penetrating peptide. Taken together, these examples demonstrate the applicability of using this unnatural amino acid fluorophore to study pH-dependent biological processes or events that accompany a pH change.

1. INTRODUCTION

Recently, *p*-cyanophenylalanine (Phe_{CN}) has emerged as a convenient and versatile site-specific fluorescence reporter for various biochemical and biophysical studies [1]. The broad spectroscopic utility of this unnatural amino acid, which is a structural derivative of tyrosine (Tyr) or phenylalanine (Phe) [2], stems from the fact that its fluorescence quantum yield and lifetime are sensitive to environment [3], as well as its easy incorporation into peptides and proteins [4]. For example, dehydration leads to a significant decrease in the fluorescence intensity of Phe_{CN}, thus making it a useful probe of processes that involve exclusion of water, such as protein folding [5,6], binding [7,8], aggregation [9–11], and interaction with membranes [12–15]. In addition, the fluorescence quantum yield of Phe_{CN} can be modulated

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by various metal ions [16] and several amino acid sidechains [17–21]. In particular, its fluorescence can be quenched by a nearby tryptophan (Trp) residue via the mechanism of fluorescence resonance energy transfer (FRET) [22–24]. Since this FRET pair has a Förster radius of approximately 16 Å, it has become a very useful tool to probe protein conformational changes over a relatively short distance [25]. Furthermore, Raleigh and coworkers have shown that the fluorescence intensity of Phe_{CN}, when placed at or near the N-terminus of a peptide, depends on the protonation status of the N-terminal amine group of the peptide [26]. However, the potential utility of this pH dependence of the Phe_{CN} fluorescence has not been demonstrated. Herein, we show that this unnatural amino acid can be used as a pH sensor to study pH-dependent biological processes or events that accompany a pH change. Specifically, we carry out two experiments to demonstrate the utility of Phe_{CN} fluorescence as a pH sensor; in the first one, we use it to determine the N-terminal p*K_a* values of a series of short peptides and, in the second one, we employ it to characterize the membrane penetration kinetics of a cell-penetrating peptide, the trans-activator of transcription (TAT) peptide derived from HIV-1 [27–30].

Several methods, including high voltage electrophoresis [31], circular dichroism (CD) [32–35] and nuclear magnetic resonance (NMR) spectroscopy [36,37], have been used to determine the N-terminal p*K_a* values of unstructured or α -helical peptides [38]. However, due to various limitations, for instance the CD method relies on the peptide of interest to form a well-folded structure such as an α -helix, applying these methods in practice is not always feasible, straightforward or convenient. Since fluorescence measurements are easier and also accessible to most, if not all, biochemical and biophysical researchers, it would be advantageous to devise a method that allows determination of N-terminal p*K_a* values of peptides and proteins via fluorescence spectroscopy.

Cell-penetrating peptides (CPPs) are short cationic peptides which can spontaneously translocate across cell membranes and, as such, are ideal vehicles to deliver exogenous cargos into cells [39]. In spite of many previous efforts, however, several aspects of the translocation actions of CPPs are not well understood or characterized. For example, measurements of the intrinsic membrane penetration kinetics of CPPs are often done by measuring the fluorescence signal of a dye molecule attached to the CPP of interest. However, the fluorescent dyes used in this type of studies are typically large in size and, consequently, may have a significant effect on the CPP's penetration rate. Here we show, using TAT as an example, that this concern can be alleviated by using Phe_{CN} fluorescence as a probe to follow the kinetics of CPP membrane penetration. Specifically, we replaced the N-terminal Tyr residue of TAT with Phe_{CN}, (the resultant peptide is hereinafter referred to as F_{CN}-TAT), which is expected to cause only a minimum perturbation to the peptide as Tyr and Phe_{CN} are similar in size, and we exploited the pH-dependence of the Phe_{CN} fluorescence to quantitatively assess the penetration kinetics of TAT across a model membrane [40]. Interestingly, we find that under our experimental conditions TAT first translocates across the membrane on a timescale of minutes and then causes membrane leakage on a timescale of hours.

Experimental

Peptide synthesis and sample preparation

Peptides were synthesized on a PS3 automated peptide synthesizer (Protein Technologies, MA) using standard 9-fluorenylmethoxy-carbonyl (Fmoc) solid phase synthesis protocols and Fmoc-protected amino acids from either Bachem Americas (Torrence, CA) or AnaSpec (Fremont, CA). Before use, all peptide samples were further purified by reverse-phase high-performance liquid chromatography and verified by mass spectrometry. All peptide samples used in the pH titration measurements were prepared by dissolving lyophilized peptides into either acidic (25 mM H₃PO₄) or basic (25 mM NaOH) Millipore water, and the final concentration of each sample (20 μM) was determined optically using the absorbance of Phe_{CN} at 280 nm and a molar extinction coefficient of 850 M⁻¹ cm⁻¹ [7].

Fluorescence measurement and pH titration

Fluorescence spectra of the Phe_{CN}-containing peptides were measured at 25 °C on a Fluorolog 3.10 spectrofluorometer (Horiba, NJ) using a 1 cm quartz cuvette, a spectral resolution of 1 nm, an excitation wavelength of 275 nm, and an integration time 1 s/nm. During a specific pH titration experiment, the concentration of the peptide under consideration was maintained at 20 μM and the pH of the solution was varied between 2 and 12. This was achieved by mixing two 20 μM peptide stock solutions, one prepared in 25 mM H₃PO₄ and the other in 25 mM NaOH aqueous solution, in a cuvette at an appropriate volume ratio to achieve the desired pH, which was further measured using a pH meter (Orion/ThermoScientific, MA). Additionally, in each case a background spectrum, obtained with the buffer solution, was subtracted. Because the shape of the Phe_{CN} fluorescence spectrum is insensitive to environment, only the peak intensity was used to generate the pH titration curves. To determine the N-terminal p*K_a* of each peptide, the corresponding fluorescence titration curve was fit to the following equation:

$$F(x) = \frac{(b_1 + m_1 x)(10^{-x}) + (b_2 + m_2 x)(10^{-pK_a})}{10^{-pK_a} + 10^{-x}}, \quad (1)$$

where $F(x)$ is the fluorescence intensity at pH x , b_i and m_i are the intercept and slope of the linear base lines ($i = 1$ for acidic and $i = 2$ for basic).

Measurement of the membrane penetration kinetics of F_{CN}-TAT

The 100-nm, large unilamellar vesicles (LUVs) used in the peptide penetration experiments were composed of either 100% DOPG or a mixture of DOPC, DOPG and cholesterol (3:1:1) and prepared following previously published procedures [41] from stock solutions of the respective lipids (purchased from Avanti Polar Lipids Inc., AL). Briefly, the respective lipid solution in chloroform was first dried under a flow of nitrogen, allowing a lipid film to form, which was followed by a 30-minute lyophilization to remove any remaining solvent. The resultant lipid film was then rehydrated with Millipore water and the pH of the sample was adjusted to 3.5 – 4.0 using H₃PO₄ and NaOH. This sample was then subjected to seven rounds of slow vortexing, freezing and thawing to form LUVs. The resulting vesicle solution was then extruded 11 times through an extruder (Avanti Polar Lipids Inc., AL) equipped

with a 100 nm membrane. After extrusion, the LUV solution was diluted to 100 μM (lipid concentration) with Millipore water and the pH was adjusted between 10.0 – 10.5 with H_3PO_4 and NaOH. This process resulted in LUV's with acidic pH inside the vesicle and basic pH outside. All LUV solutions were stored at 4°C and used within one week after preparation.

The membrane penetration kinetics of $\text{F}_{\text{CN}}\text{-TAT}$ was initiated by manually adding an appropriate aliquot of a concentrated peptide solution (350 μM , pH 7.0) to a 2.0 mL LUV solution described above. The final peptide concentration was 1.0 μM , resulting in a 1:100 peptide to lipid ratio. The time dependent fluorescence intensity of $\text{F}_{\text{CN}}\text{-TAT}$ at 298 nm was collected at 25 °C using the time based acquisition function of the Fluorolog 3.10 spectrofluorometer with time intervals of 2 seconds for the first 1,000 seconds, then every 10 seconds for the next 10,000 seconds and then 100 seconds for the remainder of the experiment. These data were then binned and the initial kinetic phase corresponding to the membrane penetration process was fit to a single-exponential function. To remove the contribution of water's Raman scattering to the fluorescence signal, most of the kinetic traces were collected using an excitation wavelength of 240 nm. In other cases, an excitation wavelength of 275 nm was used. The pH of the solution was recorded throughout the duration of the experiment.

The fluorescence quenching assay used to estimate the timescale of membrane leakage was modified from a liposome flux assay [42]. Briefly, the LUVs were prepared using the abovementioned procedures, with the exception that a membrane impermeable fluorescent dye, pyranine (Alfa Aaser, MA), was added to the lipid solution. The concentration of pyranine was 4 μM in phosphate buffer (pH 7.5, 30 mM sodium phosphate and 100 mM NaCl). After extrusion of the lipid solution, the dye molecules that were not encapsulated inside LUVs were separated from the LUVs by passing the mixture through a pD-10 column (GE Healthcare, NJ). The dye-encapsulated LUV solution was then diluted into the same phosphate buffer that also contains 25 μM *p*-xylene-bis-pyridinium bromide (DPX) (Sigma-Aldrich, MO), a pyranine fluorescence quencher, to yield a final lipid concentration of 100 μM . The pyranine fluorescence was excited at 417 nm (isosbestic point) and monitored at 515 nm with and without the presence of $\text{F}_{\text{CN}}\text{-TAT}$ peptide.

Results and Discussion

Effect of the N-terminal amine group on Phe_{CN} fluorescence

Previous studies has suggested that the fluorescence of a Phe_{CN} residue, at or near the N-terminus of a peptide, can be significantly quenched by the deprotonated neutral form of the N-terminal amino group [16,26]. To further validate this notion, we measured the Phe_{CN} fluorescence spectra of a tripeptide consisting of glycine (G) and Phe_{CN} (F_{CN}) with a sequence of $\text{GF}_{\text{CN}}\text{G-CONH}_2$, having either a free amino N-terminal end (the corresponding peptide is hereafter referred to as $\text{GF}_{\text{CN}}\text{G}$) or an acetylated N-terminus (the corresponding peptide is hereafter referred to as $^*\text{GF}_{\text{CN}}\text{G}$). As shown (Figure 1), the Phe_{CN} fluorescence intensity of $\text{GF}_{\text{CN}}\text{G}$ shows a drastic decrease when the pH of the peptide solution is increased from 2.1 to 10.0. As the N-terminal pK_a of peptides is typically smaller than 9.0 and, thus, at pH 10.0 the N-terminus of $\text{GF}_{\text{CN}}\text{G}$ is expected to be deprotonated, this result

suggests that the N-terminal amino group is an effective quencher of the Phe_{CN} fluorescence in this case. In support of this conclusion, the Phe_{CN} fluorescence of *GF_{CN}G does not show such a pH dependence within the pH range studied (Figure 1 inset). Taken together, these results indicate that a Phe_{CN} residue, when placed at the second position in a peptide sequence, can be used as a pH sensor.

To demonstrate its application in this regard, we first used Phe_{CN} fluorescence to determine the N-terminal p*K_a* of GF_{CN}G. As shown (Figure 2), the intensity of Phe_{CN} fluorescence of GF_{CN}G displays a sigmoidal dependence on pH, characteristic of an acid-base titration. As expected, the peptide *GF_{CN}G, whose N-terminus is acetylated, does not show such a transition. Further fitting the fluorescence titration curve of GF_{CN}G to a two-state model (i.e., eq. 1) yielded a p*K_a* of 7.99, which is similar to the N-terminal p*K_a* (7.85) of the amino group of an antimicrobial peptide [36], melittin, which has a Gly residue at the N-terminus. Interestingly, as indicated (Figure 2), swapping of the first two amino acids in GF_{CN}G (the resulting peptide is hereafter referred to as F_{CN}GG) leads to a decrease in not only the N-terminal p*K_a* value (i.e., 6.97) but also the quantum yield of Phe_{CN} fluorescence. The latter is suggestive of a fluorescence quenching mechanism that is electron transfer in nature.

Determining N-terminal p*K_a* values of a series of tripeptides

The results obtained with GF_{CN}G and F_{CN}GG indicate that the dynamic range of the Phe_{CN} as a pH sensor can be tuned by changing the identity of the first amino acid. To this end, we carried out acid-base titrations on eight additional peptides having the following sequence: NH₂-(XF_{CN}G)-CONH₂, where X represents a different amino acid in each case. Specifically, we chose several amino acids with varying sidechain properties, including positively charged (K), negatively charged (D), polar (N, S, G), or nonpolar (G, M, P, V, A) sidechains, to demonstrate the sensitivity of the fluorescence of Phe_{CN}. As shown (Figure 3), the corresponding pH titration curves of these peptides measured via Phe_{CN} fluorescence intensity have similar sigmoidal shapes but different mid-points (i.e., p*K_a* values). Indeed, the N-terminal p*K_a* values of these peptides, determined by fitting the respective acid-base titrations curves to eq. 1, are spread over two units of pH, ranging from 6.7 to 8.6 (Table 1). In particular, the N-terminal p*K_a* (7.99) of the AF_{CN}G tripeptide agrees well with that (8.0) determined for two alanine-based pentapeptides (NH₂-AAEAA-Ac and NH₂-AAHAA-Ac [43]). Because of the scarcity of N-terminal p*K_a* values of short peptides, however, making more comparisons with other studies is not possible. On the other hand, it is clear that the N-terminal p*K_a* value of the XF_{CN}G peptide is, in most cases, smaller than that determined for an α -helical peptide with the same N-terminal residue (Table 1), suggesting that the peptide structure has a significant effect on the electronic property of the N-terminal amino group. In addition, as indicated (Figure 4), except for the proline (P) and serine (S) variants, the N-terminal p*K_a* values of other peptides show a weak linear correlation with those (i.e., p*K₂*) of the corresponding free amino acids. While further confirmation is needed, the large deviation observed for proline and serine likely results from their interactions with the Phe_{CN} sidechain. Regardless of the exact mechanism that controls the N-terminal p*K_a* values of these tripeptides, the data in Figure 3 indicates that by changing the N-terminal residue, the useful dynamic range of Phe_{CN} fluorescence can be extended over five units of pH, from approximately 5 to 10.

Using Phe_{CN} fluorescence to probe the membrane-penetrating kinetics of TAT

In this example, we seek to demonstrate that Phe_{CN} can serve as a pH sensor to monitor the rate of membrane penetration of CPPs. Specifically, we employed a well-studied CPP, TAT (YGRKKRRQRRR-CONH₂) as our model peptide. As discussed above, the Phe_{CN} pH sensor was introduced via a Tyr to Phe_{CN} mutation. Previous studies have shown that the Tyr residue plays a minimal, if any, role in facilitating TAT translocation across membranes. Thus, we expect that the mutant peptide (i.e., F_{CN}-TAT) will exhibit similar, if not identical, membrane penetration kinetics as the wild type peptide. As expected (Figure 5 inset), the Phe_{CN} fluorescence intensity of F_{CN}-TAT depends on pH. To utilize this dependence to report the membrane penetration kinetics of F_{CN}-TAT, we prepared a DOPG vesicle solution where the pH inside the vesicle was 3.5 and the pH outside the vesicle was 10.0. As shown (Figure 5), upon mixing this vesicle solution with a F_{CN}-TAT solution at pH 10.0, the Phe_{CN} fluorescence increases with time in two well separated kinetic phases. This increase in Phe_{CN} fluorescence is consistent with the idea that a certain fraction of the peptide molecules has translocated across the DOPG membranes and, thus, are experiencing a significant decrease in pH. However, repetitive measurements indicate that while the first or fast kinetic phase is well defined and reproducible, the second phase appears to be ill-defined and occurs on a much longer timescale. These differences prompt us to assign the fast phase to peptide internalization in vesicles (or membrane penetration) and the slow one to membrane leakage. This is based on the notion that, unlike the penetration process, a number of peptide molecules need to work together, for example, through the formation of transmembrane pores [44,45], to cause the membrane to leak; as a result, the leakage kinetics are intrinsically more stochastic like and statistically more sensitive to the experimental uncertainties (e.g., variations in the peptide and vesicle concentrations). To confirm this assignment, we measured the pH of the peptide-vesicle solution at different reaction times and found that after the completion of the fast kinetics phase (e.g., at 50 minutes) the pH of the system was practically unchanged (i.e., 10.0 to 9.9), whereas after 5 hours the pH of the solution was decreased to 7.4. On the other hand, adding the GF_{CN}G tripeptide to the same DOPG vesicle solution did not cause any appreciable change in the pH value after 12 hours. Thus, taken together these results support the aforementioned assignment. Moreover, the notion that the slow kinetic phase reports on peptide induced membrane leakage is further corroborated by the data obtained from a dye leakage experiment. As shown (Figure 5), under similar experimental conditions (i.e., same peptide and lipid concentrations) the fluorescence intensity of a dye (pyranine) that is initially encapsulated inside the DOPG vesicle shows a significant decrease (approximately 50% without counting photobleaching) over 10 hours, due to membrane leakage and consequently fluorescence quenching by a quencher (DPX) initially existing outside the vesicles. As indicated (Figure 6), the fast or peptide penetration kinetic phase can be fit by a single-exponential function with a time constant of 10.5 ± 2.2 minutes. This value is similar to those reported in the literature for TAT [40,46,47], providing further supporting evidence for our assignment.

Using 240 nm photons to excite the Phe_{CN} fluorescence may lead to undesirable heating effect. To verify that this is not the case, we also carried out fluorescence kinetic measurements using an excitation wavelength of 275 nm. As shown (Figure 7), the resultant

penetration kinetics has a time constant of 12.8 ± 2.0 minutes, indicating that the photo-excitation induced heating affects minimally, if any, the kinetic results. Similarly, to better mimic eukaryotic cell membranes, we also conducted the penetration experiment using a ternary mixture of DOPC, DOPG, and cholesterol [48,49]. As indicated (Figure 7), the F_{CN}-TAT penetration kinetics obtained with the mixed lipid membrane are comparable to those measured with DOPG vesicle, with a time constant of 11.9 ± 1.5 minutes.

Conclusion

The unnatural amino acid, *p*-cyanophenylalanine (Phe_{CN}), has unique infrared (IR) and fluorescent properties. As such, it has been used as both IR and fluorescence probes in a wide range of applications. Here, we further show that, for peptides having a Phe_{CN} residue at or near the N-terminus, the neutral form of the N-terminal amino group is much more effective than the protonated form in quenching the Phe_{CN} fluorescence. As a result, such peptides can be used as pH sensors. By examining the pH-dependence of the Phe_{CN} fluorescence of nine tripeptides (i.e., X-Phe_{CN}-Gly, where X represents either Asn, Met, Lys, Pro, Val, Asp, Ala, Gly or Ser), we are able to show that the dynamic range of such pH sensors can be tuned to cover several units of pH as the N-terminal p*K*_a of these peptides depends on the identity of the N-terminal amino acid. In addition, using TAT as an example, we demonstrate that the aforementioned pH-dependence of Phe_{CN} fluorescence can be exploited to measure penetration kinetics of cell-penetrating peptides across model membranes.

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Figure 1. Normalized Phe_{CN} fluorescence spectra of GF_{CN}G obtained at different pH values, as indicated. Shown in the inset are the normalized Phe_{CN} fluorescence spectra of *GF_{CN}G (i.e., the N-terminal acetylated version of GF_{CN}G) collected under acidic and basic pH conditions.

Figure 2. Normalized Phe_{CN} fluorescence intensity versus pH of three tripeptides, as indicated. In each case, the smooth line represents the best fit of the corresponding fluorescence titration curve to Eq. 1 and the resulting pK_a value is listed in Table 1.

Figure 3. Normalized Phe_{CN} fluorescence intensity versus pH of XF_{CN}G peptides, as indicated by the X amino acid. Smooth lines correspond to the best fits of these fluorescence titration curves to Eq. 1 and the resulting pK_a values are given in Table 1. In addition, the significantly decreased fluorescence intensity of MF_{CN}G is due to the previously verified quenching effect of methionine sidechain toward Phe_{CN} fluorescence [26].

Figure 4.

Comparison between the N-terminal pK_a of $\text{XF}_{\text{CN}}\text{G}$ with the pK_2 of the corresponding free amino acid X. Except for proline and serine, a correlation ($R^2 = 0.98$) between the pK_a and pK_2 values exists for the amino acids studied.

Figure 5.

Four representative Phe_{CN} fluorescence kinetic traces (black, purple, green and blue) obtained upon mixing a F_{CN} -TAT solution with a DOPG LUV solution. The excitation wavelength was 240 nm. The pink lines are the time-dependent fluorescence intensities of the pyranine dye initially encapsulated inside the DOPG vesicles obtained in the presence (thick line) and absence (thin line) of the F_{CN} -TAT peptide and DPX quencher. In other words, the thin pink line measures the photobleaching rate of the pyranine dye, whereas the thick pink line reports the peptide induced membrane leakage kinetics. Shown in the inset is the normalized Phe_{CN} fluorescence of F_{CN} -TAT as a function of pH.

Figure 6.

The first 2000-second portions of the same Phe_{CN} fluorescence kinetic traces in Figure 5. The red line represents the best fit of the averaged data of these four curves to a single-exponential function with a time constant of 10.5 ± 2.2 minutes.

Figure 7.

The thin red line represents the F_{CN} -TAT penetration kinetics obtained with DOPG LUVs and an excitation wavelength of 275 nm, and the thick and smooth red line represents the best fit of this kinetic trace to a single-exponential function with a time constant of 12.8 ± 2.0 minutes. The thin blue line represents the F_{CN} -TAT penetration kinetics obtained with the ternary lipid membrane described in the text, and the thick and smooth blue line represents the best fit of this kinetic trace to a single-exponential function with a time constant of 11.9 ± 1.5 minutes.

Table 1

The N-terminal pK_a value of the XF_{CN}G tripeptide determined from the current experiment, the pK_2 value of the corresponding free amino acid X [46], and the N-terminal pK_a value (pK_a^H) of an α -helical peptide with X at the N-terminus [32]. The uncertainty of the measured pK_a values is ± 0.1 .

X	pK_a	pK_a^H	pK_2
N	6.69	7.07	8.8
M	7.04	7.83	9.2
K	7.25	8.12	9.2
P	7.28	8.85	10.6
V	7.29	8.14	9.6
D	7.70	8.25	10.0
A	7.99	8.35	9.7
G	7.99	8.51	9.8
S	8.59	7.63	9.2
F _{CN} GG	6.97		9.1