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Thioamides as Fluorescence Quenching Probes: Minimalist Chromophores To Monitor Protein Dynamics

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Abstract: Decreasing the size of spectroscopic probes can afford higher-resolution structural information from fluorescence experiments. Therefore, we have developed *p*-cyanophenylalanine (Cnf) and backbone thioamides as a fluorophore/quencher pair. Through the examination of a series of thiopeptides, we have determined the working distance for this pair to be 8−30 Å. We have also carried out a proof-of-principle protein-folding experiment in which a Cnf/thioamide-labeled version of villin headpiece HP35 was thermally unfolded while the Cnf/thioamide distance was monitored by fluorescence. For a given protein, thioamide substitutions could be used to track motions with a much greater number of measurements than for current fluorescence probes, providing a dense array of data with which to model conformational changes.

One of the great challenges facing biochemists is understanding the rapid and complex structural dynamics of proteins. Fluorescence measurements can be made on the nanosecond time scale, and distance-dependent interactions such as Förster resonant energy transfer (FRET) can be used to determine the separation of chromophore labels to glean time-resolved structural information on protein motions.[1] However, the relatively large size of common fluorophores precludes the assignment of these motions with atomic resolution. If optical probes could be made sufficiently small, they could provide the time and structural resolution necessary to truly dissect protein motions. Here we demonstrate that *p*-cyanophenylalanine (Cnf), which is structurally similar to Phe, and a thioamide obtained by a single-atom substitution in the peptide backbone can be used to monitor structural changes in proteins.

The most widely used method for fluorescent tagging of proteins employs genetic fusion of a variant of the green fluorescent protein from *Aequorea victoria* (GFP) to the protein of interest. GFP fusions are easy to prepare by standard cloning methods, but the large size of GFP (268 amino acids) limits its utility in observing the motions of proteins, which are often of comparable size.[2] Labeling proteins with a small organic molecule such as fluorescein either during or after translation introduces a less structurally perturbing probe.[3] However, many positions in the protein cannot accommodate polycyclic organic chromophores. The bulk of GFP and fluorescein derivatives not only limits the number of places that the label can be placed but also raises the concern that the label itself may alter the observed motion. On the other hand, sulfur replacement of oxygen in the peptide backbone provides a fluorescence quencher that is extremely small and compatible with virtually any position in a protein sequence.

Peptides with one or more thioamides in the backbone have been used previously in a small number of applications. These have primarily focused on photoisomerization of the thioamide[4,5] and its impact on folding in simple secondary-structure motifs.[6,7] We note one preliminary exploration of thioamide as a resonance energy acceptor, but the pairing with Trp was reported to be viable only under nonphysiological solvent conditions (neat propylene glycol).[8] We chose to use Cnf as a donor chromophore because its fluorescence emission was expected to overlap well with thioamide absorption and its extinction coefficient is much greater than that of Tyr in the 230−250 nm range.[9] Cnf has previously been used as a donor in FRET experiments with both Trp and Tyr acceptors,[10,11] which also have spectral overlap with Cnf emission. Since deconvolution of Cnf and thioamide interactions with these chromophores could be very complex, we limited our initial experiments to cases without Trp or Tyr residues in order to more easily characterize the interactions with the thioamide.

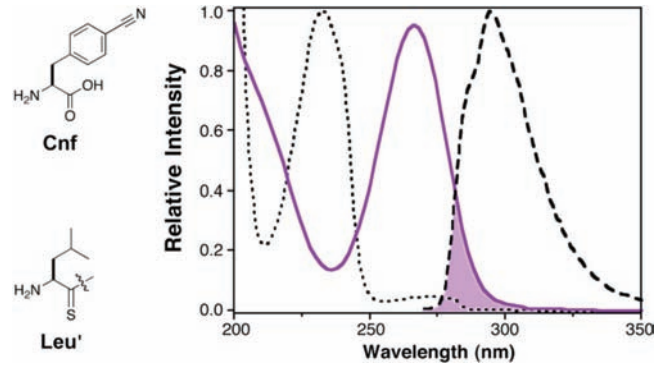

Figure 1. Thioleucylalanine ester (Leu′Ala) and *p*-cyanophenylalanine (Cnf) spectra. Absorption spectra of Cnf (dotted black line) and Leu′Ala (solid purple line) with relative absorption intensities normalized to the extinction coefficients (13 000 M^{-1} cm^{-1} at 232 nm and 12 400 M^{-1} cm^{-1} at 266 nm, respectively) are shown.[4,9] The fluorescence spectrum of Cnf (dashed black line) arbitrarily normalized to the absorption maximum of Cnf is also shown. The shaded area indicates the spectral overlap that contributes to FRET.

Our study of Cnf/thioamide spectroscopy began with the calculation of the Förster distance R_0, the distance at which resonant energy transfer between two chromophores is half-maximal. This calculation requires determination of the spectral overlap integral from the absorption spectrum of the thioamide acceptor and the fluorescence emission spectrum of the Cnf donor[12] (Figure 1). Using the reported quantum yield for Cnf fluorescence (0.11)[9] and the common assumption that the orientation of the chromophore transition dipoles is random on the time scale of energy transfer (i.e., $\kappa^2 = {}^2/_3$), we calculated R_0 to be 15.6 Å (see the Supporting Information). This value implied that the pair could be useful in measuring distance changes on the scale of a protein.

To further characterize the Cnf/thioamide pair, we prepared peptides with an N-terminal thioleucine (Leu′) and a C-terminal Cnf separated by 2−10 proline residues, analogous to the classic experiments of Stryer and Haugland.[13] We obtained fluorescence

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spectra of 12 μM solutions of these peptides in phosphate buffer and observed a strong dependence of the fluorescence intensity on the number of intervening Pro residues. To correlate the fluorescence intensity with interchromophore distance, we carried out a series of 10 ns molecular dynamics (MD) simulations of Leu-Pro$_n$-Phe ($n = 2-10$) in explicit water boxes. The distances between the thioamide (using the Leu amide as a proxy) and Cnf (using Phe as a proxy) were time-averaged over the unrestrained portions of the simulations (see the Supporting Information). Normalizing the fluorescence emission (F) across the proline series to the emission of Cnf in an oxoamide control peptide (F_0), we computed the efficiency of fluorescence quenching as $E_Q = 1 - F/F_0$. This allowed us to plot E_Q as a function of distance for the proline series and compute an R_0 value of 16.5 Å by fitting the data to a $1/R^6$ function (Figure 2, solid line), in reasonable agreement with the value determined from spectroscopic data alone.

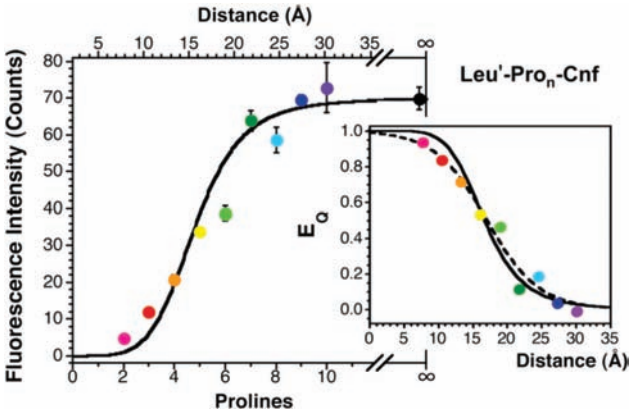

Figure 2. Fluorescence emission as a function of chromophore spacing. The fluorescence emission of Leu′-Pro$_n$-Cnf ($n = 2-10$) at 293 nm is shown (three trials per peptide, bars represent standard errors). The "∞" data point indicates the fluorescence of Leu-Pro$_2$-Cnf. The solid line indicates the distance dependence predicted by Förster theory with $R_0 = 15.6$ Å (~5.1 prolines). The inset shows E_Q as a function of the computed Leu′−Cnf interchromophore distance for the proline series. The data were fit to Förster (solid line, $r^2 = 0.973$) and Dexter (dashed line, $r^2 = 0.986$) distance dependences.

Several observations regarding this result are worth noting. First, although the distance dependence is comparable to the prediction from Förster theory, for such short distances one must also consider Dexter energy transfer via direct orbital overlap.[12] Dexter transfer has a $1/e^R$ distance dependence, which provided a slightly better fit to the Pro series data (Figure 2, dashed line). In fact, both mechanisms probably contribute to thioamide quenching and may be difficult to deconvolute. Second, as thioamides have previously been used in photoisomerization experiments,[4,5] we investigated the possibility that energy transfer might cause cis/trans isomerization. After 1 h of irradiation, our most efficient energy transfer peptide, Leu′-Pro$_2$-Cnf, showed no significant change in HPLC, UV−vis, or circular dichroism (CD) assays (see the Supporting Information). The lack of isomerization seems to be inconsistent with either energy transfer mechanism and will be investigated further. Regardless of the mechanism, our data confirm that the working distance for the Cnf/thioamide pair is 8−30 Å, complementing longer-range pairs like Trp/dansyl ($R_0 \approx 22$ Å) and fluorescein/tetramethylrhodamine ($R_0 \approx 50$ Å).[14] Finally, although a proximal N-terminal amine can quench Cnf fluorescence,[15] acetylation of Leu-Pro$_2$-Cnf and Leu′-Pro$_2$-Cnf had no effect on the fluorescence at pH 7.0 (see the Supporting Information). This gives us confidence that the quenching (relative to the parent oxoamide) is due to the O-to-S substitution alone.

We also carried out one proof-of-principle experiment to demonstrate the utility of the thioamide/Cnf pair in monitoring a conformational change. We incorporated Leu′ at the N-terminus and Cnf at the C-terminus of the villin headpiece HP35 variant, which was originally described by Kim and co-workers.[16] The fraction folded (f_f) as a function of temperature was determined for the thioamide version HP35-Leu′$_1$Cnf$_{35}$ and the corresponding oxoamide control protein HP35-Cnf$_{35}$ using CD spectroscopy (Figure 3). The two proteins had comparable T_m values (i.e., temperatures at which $f_f = 0.5$), indicating that incorporating the thioamide at this position had no dramatic effect on protein unfolding. The quenching efficiency was determined by comparing the fluorescence of the thioamide to that of the oxoamide. Since Cnf fluorescence has been shown to vary strongly with temperature and local environment,[10,17] determining E_Q by comparison to the oxoamide HP35-Cnf$_{35}$ was essential to control for effects on Cnf emission that are independent of the thioamide interaction. E_Q ranged from 0.19 at 5 °C to <0.02 at 75 °C.[18] The change in E_Q implies a distance change from 20−21 Å in the folded state (19.2 Å observed in 10 ns MD simulations) to greater than the detectable range of our probe pair (32 Å) in the unfolded state. As Figure 3 shows, converting E_Q to distance using the Förster and Dexter interpretations gave comparable results (root-mean-square deviations of 0.8 Å over the 70 °C temperature range). The data show that Leu′$_1$/Cnf$_{35}$ dissociation is coupled to global unfolding even though it is found on the periphery of HP$_{35}$.

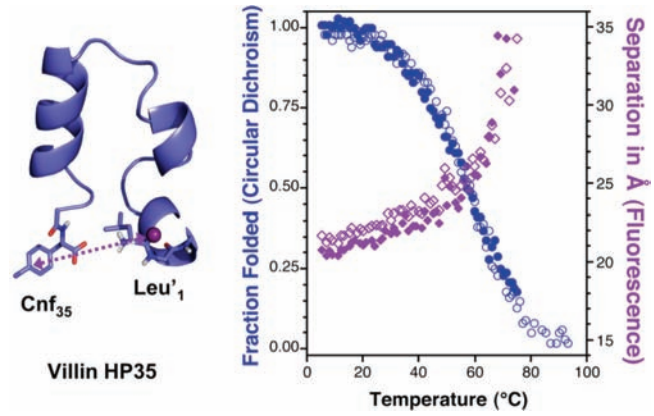

Figure 3. Villin HP35 unfolding monitored by Cnf/thioamide FRET. (left) Villin HP35 structure taken from PDB entry 1VII[19] modified with the Cnf nitrile on Phe$_{35}$ [image rendered in PyMOL (Delano Scientific, LLC, South San Francisco, CA)]. (right) Fraction folded as determined from temperature-dependent CD spectroscopy for HP35-Leu′$_1$Cnf$_{35}$ (●) and HP35-Cnf$_{35}$ (○) and temperature dependence of the Leu′$_1$/Cnf$_{35}$ separation determined by comparison of E_Q computed from the HP35-Leu′$_1$Cnf$_{35}$/HP35-Cnf$_{35}$ fluorescence ratio with the proline series distance dependence computed using either the Förster (◆) or Dexter (◇) equation.

In summary, we have identified a novel fluorophore/quencher pair and demonstrated its distance dependence and application to monitoring of the unfolding of a small protein. Since one can conceivably replace any amino acid in a synthetically accessible protein by its thioamide analogue, this quencher could be applied anywhere in a protein sequence. We are currently exploring the use of the Cnf/thioamide system in proteins containing Trp or Tyr to determine its generality and further studying the mechanism of fluorescence quenching by thioamides.

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Supporting Information Available: Descriptions of peptide synthesis, purification, and characterization; fluorescence, UV−vis spectroscopy, and CD experiments; and data fitting and calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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