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Electronic Interactions of i, i+1 Dithioamides: Increased Fluorescence Quenching and Evidence for n-to-π* Interactions

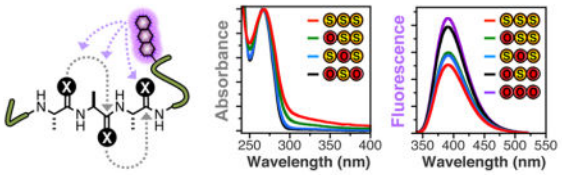
Yun Huang[a,b], **John J. Ferrie**[a], **Xing Chen**[a], **Yitao Zhang**[a], **D. Miklos Szantai-Kis**[a], **David M. Chenoweth**[a], and **E. James Petersson**[a,*]

[a]Department of Chemistry, University of Pennsylvania, 213 South 34th Street, Philadelphia, PA 19104, USA

Abstract

Thioamide residues can be effective, minimally-perturbing fluorescence quenching probes for studying protein folding and proteolysis. In order to increase the level of quenching, we have here explored the use of adjacent dithioamides. We have found that they are more effective fluorescence quenchers, as expected, but we have also observed unexpected changes in the thioamide absorption spectra that may arise from n-to-π* interactions of the thiocarbonyls. We have made use of the increased quenching to improve the fluorescence turn-on of thioamide protease sensors.

Graphical abstract



Fluorescence quenching can be very effectively used to study dynamic biological processes, both *in vitro* and in living systems.[1] For this purpose, a fluorophore and a quencher are needed to label the target protein or other biomolecule. In such applications, it is important to maintain the intrinsic structural and functional characteristics of the protein of interest. Thus, much effort is continuously put into the development of new fluorophores and quenchers. Our laboratory has developed the thioamide functional group as a minimally perturbing fluorescent quencher to monitor protein conformational changes as well as proteolysis.[2–5]

The thioamide is a single atom substitution of the native peptide bond, with relatively small changes to its key functional features. For example, the thioamide is a stronger hydrogen bond donor, but a slightly weaker acceptor than the native amide bond, sharing a similar

ejpetersson@sas.upenn.edu; Tel: 1-215-746-2221.

[b]Current address: Department of Physiology and Biophysics, Weill Cornell Medical College, Cornell University, 1300 York Avenue, New York, NY 10065, USA

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planar structure with a rotational barrier of 15–20 kcal/mol.[–] Importantly, the thioamide has been shown to be broadly compatible with α-helix, β-sheet, and polyproline type II (PPII) secondary structures, although position-dependent increases or decreases in stability have been reported.[–] A strong π-π* transition band around 270 nm makes the thioamide a good photoswitch and a good Förster resonance energy transfer (FRET) acceptor for short wavelength fluorophores such as *p*-cyanophenylalanine (F*, Fig. 1).[,] Additionally, thioamides are able to quench fluorophores such as tryptophan, 7-methoxycoumarin-4-ylalanine (μ, Fig. 1), acridon-2-ylalanine (δ, Fig. 1) and fluorescein via photo-induced electron transfer (PET).[] However, the quenching efficiency for longer wavelength fluorophores is relatively limited. For instance, the maximum observed quenching by 65 mM thioacetamide is 84% for μ and 63% for δ. Further improvement of the quenching ability of the thioamide is of great interest, since this would bring greater sensitivity to protein folding and proteolysis studies.

According to a collision-based description of dynamic electron-transfer, placing two thioamides near each other would increase the probability of quenching by effectively increasing the radius of the quenching moiety. This increase in quenching efficiency might be further modulated by an electronic interaction between the two thioamide moieties that could change the optical or electrochemical properties of the dithioamide relative to the corresponding single thioamides. To investigate these possibilities, we examined quenching by multiple thioamides in two model systems.

First, we designed a series of peptides in which an N-terminal μ was separated by two or four proline residues from two consecutive thioamide amino acid analogs in order to provide rigid spacers to ensure that interactions between the fluorophore and the quencher occurred primarily through space. We also synthesized control peptides containing single thioamides at each of the considered residues and an all oxoamide peptide. It was found that yields of the dithiopeptides were inconsistent when using standard 20% piperidine in DMF for deprotection, and that multiple products of identical mass (presumably attributable to epimerization) were observed. A recent report on the solid-phase synthesis of dithioamide α–amino acid peptides advocated the use of dry dichloromethane in coupling thioacylbenzotriazole precursors. In our hands, the use of dry dichloromethane did not suppress the production of these sideproducts. However, when deprotection conditions consisting of 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 1% hydroxylbenzotriazole (HOBt) in DMF were used a single product was observed (ESI, Scheme S2, and Fig. S10). The peptide sequences are shown in Fig. 1, where the superscript “S” is used to denote the positions of thioamide residues.

Examining the UV/Vis spectra of the thioamide containing peptides showed that the absorbance of the i, i+1 dithioamide was different than the sum of the absorbances of the two corresponding monothioamides. For example, by analyzing the spectra of peptides **1**-**7**, one can see that placing thioamides at adjacent i, i+1 positions induces broadening and red-shifting of the thioamide absorption. The spectrum of μP_3PAAG (**1**) shows a typical absorption spectrum of a μ-containing peptide (Fig. 1, Left). The spectra of μP_3P^SAAG (**2**), μP_3PA^SAG (**3**), and μP_3PAA^SG (**4**) are all typical of μ- and thioamide-containing peptides, with slight changes in the spectral shape based on position. However, in comparing the

spectra of μP_3PA^{S}A^{S}G (**5**), an adjacent i, i+1 dithioamide, and μP_3P^{S}AA^{S}G (**6**), a "skipped" i, i+2 dithioamide, one can see a clear difference in their spectra. Normalizing the absorbance at 270 nm for peptides **5** and **6** allows one to more clearly see the increased relative absorbance of **5** in the 300–350 nm range (Fig. 1, Left Inset). Examination of the spectrum of trithiopeptide μP_3P^{S}A^{S}A^{S}G (**7**) shows that this red-shifting and spectral broadening can even spread into the 400 nm range.

Since the unexpected polythioamide electronic interaction overlapped with the μ absorbance maximum at 325 nm, we prepared peptides **8**-**12**, containing an N-terminal F* residue. F* has no significant absorbance above 250 nm, so the red-shifted shoulder that arises for adjacent i, i+1 thioamides in dithiopeptide F*P_3PA^{S}A^{S}G (**11**) and trithiopeptide F*P_3P^{S}A^{S}A^{S}G (**12**) is very clear (Fig. 1, Right Inset). The effect is independent of chromophore identity. Indeed, spectral broadening was also observed in δ-containing peptides **17**-**20** (ESI, Fig. S13).

While the focus of our work is in using the dithioamides to improve quenching assays, we were intrigued by this spectral red-shifting. Raines and coworkers have assembled a large body of evidence showing that the previously unappreciated n-to-π* interaction is a common and significant stabilizing force in protein secondary structure, particularly in PPII helices.[–] Importantly, they have shown in model systems that n-to-π* interactions involving thioamides can be stronger than the corresponding interactions amongst oxoamides. We believe that our data provide the first direct spectroscopic evidence for the n-to-π* interaction. The n-to-π* interaction would be difficult to discern for an oxoamide since the oxoamide carbonyl absorption occurs at ~220 nm. We note that an n-to-π* transition within a single thioamide carbonyl occurs with an absorption at ~ 340 nm, which is different from the red-shift of the π-π* absorption seen here. Preliminary *ab initio* calculations using model dithiopeptides constrained to form an n-to-π* interaction show a sharing of electron density between the two thiocarbonyls and a decrease in the energy for the π-π* transition consistent with our experimental spectra (see ESI, Fig. S17–S18). We have carried out some preliminary denaturation studies to determine whether the PPII folds of our polythiopeptides are stabilized by n-to-π* interactions among the thioamides, but the peptides are short enough that secondary structures are difficult to discern by circular dichroism (data not shown). We will pursue the study of this thioamide-thioamide interaction in more depth in systems such as proline oligomers or collagen fibrils where stability effects will be easier to observe.

Comparing the emission intensities of peptides **1**-**7** as well as shorter peptides **13**-**16** demonstrated that μ fluorescence was quenched in a position dependent manner, with greater quenching from the proximal thioamides, consistent with previous measurements of thioamide-based PET quenching of Trp in model peptides (Fig. 2). As expected, the di- and trithioamide peptides showed higher levels of quenching. The steady state quenching efficiencies (E_Q^{SS}) are summarized in Table S3 (see ESI). It is interesting to note that the putative n-to-π* interaction does not have a dramatic effect on quenching. This can be seen by a comparison of E_Q^{SS} for the adjacent dithioamide **5** and the skipped dithioamide **6**. We have also observed increased quenching by dithioamides in peptides containing F*, δ, and fluorescein (see ESI, Table S3 and Figs. S12, S13, S14).

The quenching of F* by thioamides is FRET-based, due to spectral overlap between F* emission and thioamide π-π* absorption. The broadening of this absorption band due to thioamide-thioamide interactions alters the spectral overlap integral, and thus the distance dependence of F* quenching. The Förster radius (R_0), the distance of half-maximal energy transfer, increases from 14–16 Å for a typical F*-containing monothiopeptide (e.g., **9**) to 18.0 Å for dithioamide **11**, to 20.5 Å for trithioamide **12** (see ESI for R_0 calculations). In contrast to F*, quenching of μ, δ, or fluorescein is PET-based for monothioamides, since the emissions of these fluorophores lack any spectral overlap with thioamide absorption. The polythioamide broadening effect observed in the absorption spectra of μ-containing peptides **5**-**7**, and **16** dictates that a FRET-based mechanism will now contribute to the quenching. For example, if we assume that the three thioamides act as one chromophore unit, R_0 for peptide **7** is 18.3 Å (see ESI for other R_0 values). However, for all of the polythioamide peptides, it is likely that the n-to-π* conformers are only a subset of the conformational ensemble, so that there will also be contributions from conformers where they act as isolated monothioamides. It should also be noted that interactions between the thioamide units are likely to change their oxidation potentials, which would impact the free energy of electron transfer governing PET processes (FRET and PET discussed in more detail in the ESI). Further characterization will be necessary to determine whether we can deconvolute the various FRET and PET components to extract distance information in fluorophore/polythioamide interactions.

It is possible that the change in absorption spectrum also results from the formation of a ground-state complex between the polythioamide and the fluorophore, resulting in static quenching. Previous studies of the quenching of a variety of fluorophores in monothiopeptides had shown thioamide PET quenching to be predominantly dynamic, in which the fluorophore is excited, and then electron transfer for quenching.[9] We used fluorescence lifetime measurements to characterize the quenching efficiencies (E_Q^{τ}) for peptides **13**-**24**. Consistent with the steady state measurements, the lifetimes of dithiopeptides were shorter than those of the corresponding monothiopeptides (Fig. 2, Right, and ESI, Table S3). However, the lifetimes were not completely correlated with the steady state fluorescence intensities, which may result from a small static quenching component. We have investigated quenching at multiple concentrations in the μM range using peptides **17**-**24** (ESI, Fig. S19). We see no evidence for intermolecular static quenching effects or aggregation.

In a proof-of-principle demonstration, we took advantage of the higher levels of quenching by dithioamides to increase the sensitivity of fluorescent thiopeptide-based protease sensors. We designed a peptide substrate for trypsin (**28**) featuring μ at the C-terminus and two thioamide bonds in the N-terminus. The corresponding monothioamide and oxoamide control peptides (**25**-**27**) were also generated. As in the proline containing peptides, steady state and fluorescence lifetime data showed quenching by monothioamides in a distance dependent manner and stronger quenching in the dithioamide peptide (Fig. 3 and ESI, Table S2 and Fig. S16). Upon hydrolysis by trypsin, the fluorescence of the oxoamide peptide **25** showed no change, while both the mono- and dithioamide peptides showed strong increases in μ fluorescence (Fig. 3). The timecourse and identity of the peptide fragments were

verified by HPLC and mass spectrometry analysis (ESI, Fig. S17). It should be noted that although the thioamide substitution in **26** (between P4 and P3 in protease nomenclature) does not perturb proteolytic kinetics, the thioamide between in **27** (between P3 and P2) slows hydrolysis slightly (ESI, Fig. S17). We do not observe any additional slowing of reaction kinetics in dithiopeptide **28**.

Rapid-mixing experiments using a stopped-flow apparatus allowed us to use the increased quenching of the dithioamide in making kinetic measurements of the cleavage of **28** by trypsin (see ESI, Fig. S18). Initial cleavage rates were measured for concentrations of **28** ranging from < 5 μM to > 50 μM. These data were fit to a standard Michaelis-Menten kinetic model to obtain k_{cat} and $K_{m,app}$ values of 0.547 s^{-1} and 66.7 μM, respectively (see ESI). These values are consistent with previous studies of trypsin using similar substrates.

In summary, we have demonstrated that one can easily increase the level of fluorogenicity in thioamide-based sensors by simply introducing an additional thioamide. While the examples here are restricted to short peptides, dithioamides can in principle be inserted into full-length proteins by native chemical ligation to generate fluorophore/dithioamide labeled proteins for folding studies, as our laboratory has done for single thioamides.[–] Additionally, a recent report by Hecht and coworkers describing the incorporation of a dipeptidyl thioamide unit into proteins by mutant ribosomes raises the prospect of incorporating dithioamide units in a similar fashion. One may be concerned about the stability of dithioamides (and we have observed “messier” product distributions during peptide synthesis), but we note that several natural products contain adjacent polythioamides in both α-amino acid and β-amino acid peptides.[–] The impact of multiple thiocarbonyl substitutions can extend beyond natural products and protein analogs to other classes of foldamers such as synthetic β-peptides, α- and β-peptoids, ureas, and azapeptides.[–] We will continue to evaluate the prospects of dithioamides as fluorescence quenching probes as well as their usage in studying n-to-π* interactions in proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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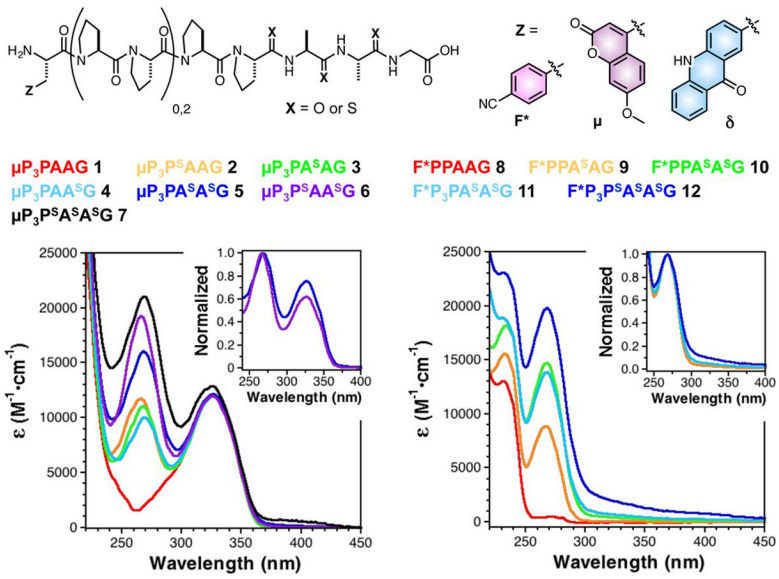

Fig. 1.

UV/Vis spectra of polyproline peptides containing multiple thioamides demonstrate non-additive changes in the thioamide absorption region (250–300 nm). All peptides contained multiple thioamide residues, varying numbers of prolines, and a 7-methoxycoumarin-4-ylalanine (μ) or *p*-cyanophenylalanine (F*) fluorophore. Left: Spectra of μ-containing peptides **1**-**7**. The spectrum of a peptide containing two adjacent thioamides (**5**) is broadened compared to the spectrum of a peptide containing i, i+2 thioamides (**6**). Normalizing the absorbance at 270 nm makes clear the increased relative absorbance of **5** at longer wavelengths (Inset). Spectral broadening of the polythioamides can be even more clearly seen for peptides **8**-**12**, containing F*. Particularly long wavelength features arising from thioamide-thioamide interactions are observed in the spectra of trithioamide peptides **7** and **12**.

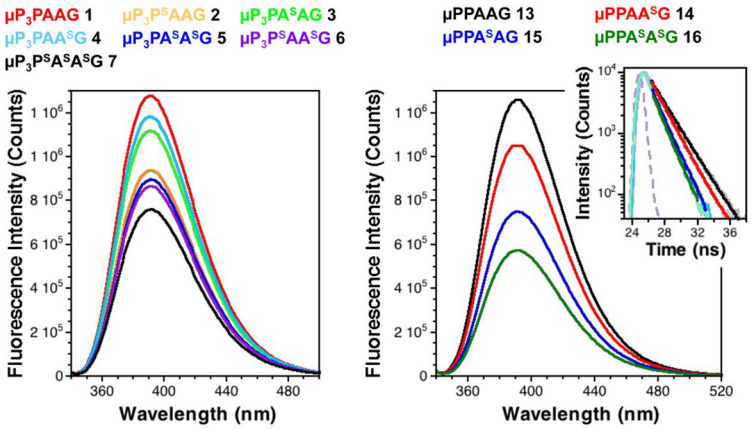

Fig. 2.

Increased fluorescence quenching by dithioamides observed in steady state spectra and fluorescence lifetime measurements. Left: Fluorescence emission spectra (λ_{ex} = 333 nm) of peptides **1**-**7** at 5 μM in 10 mM sodium phosphate, 150 mM NaCl, pH7.0 buffer. Right: Fluorescence emission spectra (λ_{ex} = 333 nm) of peptides **13**-**16** at 5 μM in 10 mM sodium phosphate, 150 mM NaCl, pH7.0 buffer. Inset: Time-correlated single photon counting measurements of (λ_{ex} = 340 nm, λ_{em} = 393 nm) of peptides **13**-**16** at 5 μM in the same buffer, shown fit to single exponential decays (see ESI for details). Instrument response function is shown in light purple.

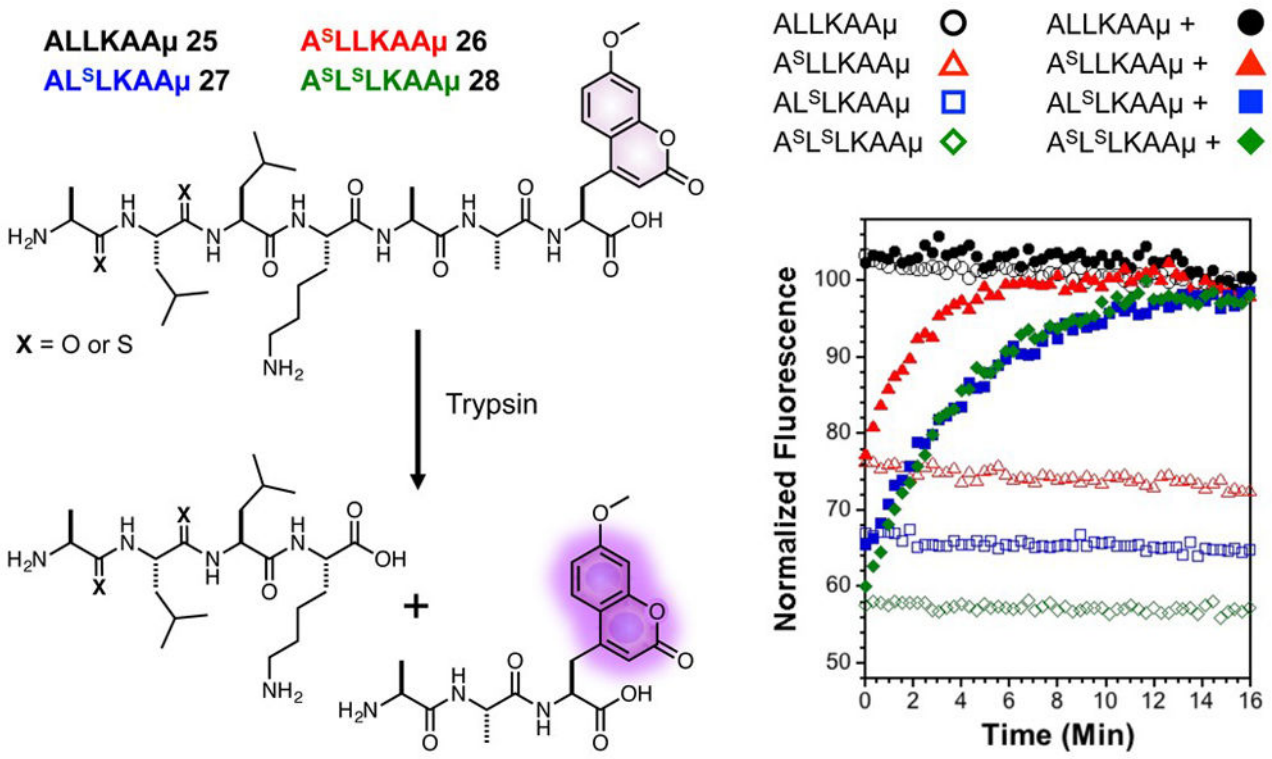

Fig. 3.

Dithioamide peptides provide improved fluorogenicity in a quenching assay. Time-dependent fluorescence measurements of peptides **25-28** (solid symbols) show an increase in fluorescence in the presence of trypsin, which can be used to monitor protease kinetics in real time. No changes in fluorescence are observed in the absence of protease (open symbols).