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Dithioamide Substitutions in Proteins: Effects on thermostability, peptide binding, and fluorescence quenching in calmodulin

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Thioamide substitutions in the backbones of proteins can modulate their structure and thermostability, or serve as spectroscopic probes in fluorescence quenching experiments. Using native chemical ligation, we have produced the first examples of a protein (calmodulin) containing two thioamides. Dithioamide variants were made to explore the effects of combining stabilizing, neutral, and destabilizing single thioamide substitutions. One of the dithioamide calmodulin variants exhibited stabilization greater than any monothioamide variant, although the effect could not easily be anticipated from the results of single substitutions. Each of the calmodulin variants retained the ability to bind a target peptide, and the dithioamide proteins exhibited an increase in fluorescence quenching of tryptophan relative to their single thioamide counterparts. These results show that multiply thioamidated proteins can be synthesized, and that properly placed thioamides can be used to increase protein thermostability or enhance fluorecsence quenching in peptide binding experiments.

Thioamides, oxygen-to-sulfur substitutions of the peptide backbone, are found in a small, but increasing number of identified natural peptides and can be introduced into synthetic peptides and proteins.[1-4] Kiefhaber, Miwa, Fischer, and others have shown that thioamides can have either subtle or dramatic effects on the stability of peptides, depending on their placement.[5-7] Our recent study of thioamide effects on the stability of the α-helical protein calmodulin (CaM), the β-sheet protein GB1, and the polyproline type II helices of collagen model peptides (as well as preceding work by Raines) showed that these effects translate to full-sized proteins.[8, 9] These effects can be used in biophysical studies of protein folding, or to engineer peptide/protein interactions to improve the *in vivo* activity of peptides.[10, 11] Thioamides can also serve as spectroscopic probes: as site-specific circular dichroism (CD) labels, *cis/trans* photoswitches, or as fluorescence quenchers.[12-14] Our laboratory, in particular, has shown that thioamide fluorescence quenching through either Förster resonance energy transfer (FRET) or photo-induced electron transfer can be used to monitor protein folding.[14-16]

Incorporation of multiple thioamides in a protein has the potential to provide advantages over single thioamides in all of these studies. This is especially true in their capacity as fluorescence quenchers; dithioamides show increased fluorescence quenching relative to monothioamides in model peptides containing Pro, Ala, and Gly.[17] Furthermore, additive stabilization or destabilization of protein structure or protein/protein interactions may be possible with dithioamide substitutions, amplifying the effects seen with single thioamides.[8, 10] However, the solid phase peptide synthesis (SPPS) of sequence-diverse dithioamide peptides and their stability during cleavage from resin, high performance liquid chromatography (HPLC) purification, and native chemical ligation (NCL), remain essentially untested.[18] In this work, we describe the semi-synthesis through NCL of six distinct dithioamide variants of CaM (CaM^{SS}). Using CD thermal denaturation experiments, we determined the impact that these substitutions have on CaM thermostability. Sites for thioamide incorporation were chosen to assess the effects of combining single thioamide substitutions previously shown to be stabilizing, neutral, or destabilizing.[8] We also studied the fluorescence quenching properties of dithioamides relative to their single thioamide counterparts (CaM^{S}) in a CaM-peptide binding assay that utilizes a tryptophan variant of a peptide from an olfactory cyclic nucleotide gated channel.[15, 19]

Our design placed the thioamides in isolated positions as well as interacting positions which had the potential for degradation during SPPS extension and cleavage. For example, placing thioamides at the i and i + 1 positions may lead to Edman-like degradation of the i + 1 thioamide due to the enhanced nucleophilicity and electrophilicity of the thioamide.[20] In previous studies of polyproline peptides, we

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successfully synthesized peptides with i, i + 1 thioamides, but here we wished to explore the stability of this motif in more standard secondary structures.[17] Therefore, we focused on the C-terminal loop and helix of CaM, which allowed us to take advantage of our established method for CaM semi-synthesis with an N-terminal fragment (1-134) expressed in *E. coli*, and a C-terminal fragment (135-148) made by SPPS (Fig. 1).[8] We began our studies by synthesizing $CaM_{135\text{-}148}$-$Cys_{135}Phe^{S}_{141}Val^{S}_{142}$ using optimized thioamide coupling procedures and Fmoc deprotection reactions with 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU).[21] It should be noted that the use of DBU rather than piperidine as a deprotection reagent is especially advantageous for peptides containing polythioamides due to the suppression of possible epimerization reactions at each thioamide α-carbon. After HPLC purification, pure $CaM_{135\text{-}148}$-$Cys_{135}Phe^{S}_{141}Val^{S}_{142}$ was obtained in 1% isolated yield (1.5 mg).

The dithiopeptide was ligated to $CaM_{1\text{-}134}$-MES (where MES is a C-terminal mercaptoethanesulfonate thioester generated from an intein fusion) in the presence of 6 M guanidinium and thiophenol. The reaction was monitored by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). We observed no thio-to-oxo exchange of the amide or degradation products other than the typical $CaM_{1\text{-}134}$-OH hydrolysis byproduct.[8] To remove this byproduct, the crude reaction was subjected to anion exchange purification, followed by Cys capture and release purification using thiopropyl sepharose resin.[8] The site of the ligation (Cys_{135}) was masked by alkylation with iodoacetamide to create a nearly isosteric mimic (denoted "Cys^{Q}") of the native Gln_{135} residue in CaM. Only singly alkylated protein was detected by MALDI-MS, with no observable thioamide sulfur modifications, in agreement with past preparations of CaM thioproteins.[8] CaM-$Cys^{Q}_{135}Phe^{S}_{141}Val^{S}_{142}$ (denoted $Phe^{S}_{141}/Val^{S}_{142}$, all CaM^{S} and CaM^{SS} proteins are denoted in the same format) was obtained in an isolated yield of 6%. Although this yield was lower than our previous syntheses of CaM^{S} (typically 10-20% yield), this provided evidence that dithioproteins could be synthesized using NCL and our newly developed SPPS protocols.[21] The low yield may be due in part to poor solubility due to aggregation, as observed previously for the Phe^{S}_{141} single thioamide mutant.[8] Thus, although we were unable to measure the stability of $Phe^{S}_{141}/Val^{S}_{142}$ due to aggregation, this study gave us confidence that we could synthesize CaM^{SS} proteins in sufficient amounts for biophysical assays.

For CD studies, CaM^{SS} variants of Val^{S}_{136} and Glu^{S}_{139} proteins were targeted to determine whether single stabilizing mutations could be combined to create hyperstable CaM^{SS} variants and if stabilizing substitutions can recover the effects of destabilizing substitutions. The molar residue ellipticity (MRE) of the holo and apo forms of the protein was measured, along with the MRE of the Cys^{Q} and single thioamide Val^{S}_{136} and Glu^{S}_{139} controls. To more clearly assess thermostability, for each protein, the melting temperature (T_M*, the unfolding midpoint in a three state transition, see ESI for details) and free energy of unfolding (ΔG_U) were determined by performing CD thermal denaturation while monitoring the helicity of the

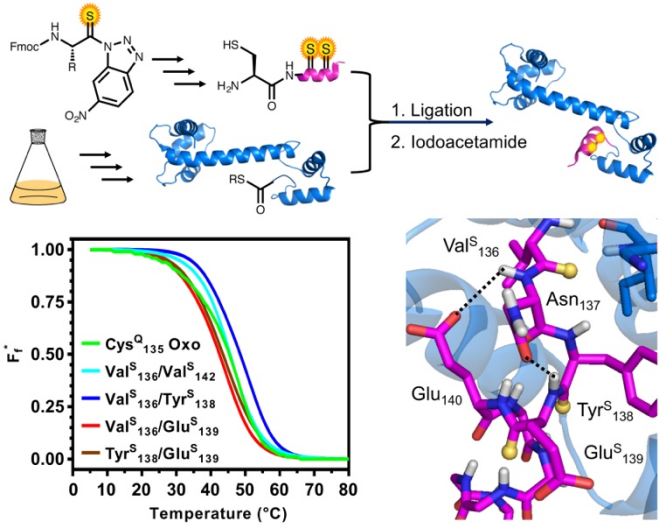

Fig. 1 Thermal Stability of CaM^{SS} Variants. Top: CaM^{SS} semi-synthesis scheme. Bottom Left: CD thermal denaturation of dithioamide CaM variants plotted as weighted fraction folded plots (F_f*) fits.[8] Transformations of raw data to F_f* described in ESI. Bottom Right: Structure of CaM from PDB ID 1QX5 highlighting thioamide positions.[22]

protein at 222 nm. None of the holo CaM^{SS} proteins completely unfolded during thermal denaturation, consistent with previous studies of CaM and CaM^{S} (See ESI, Fig. S8).[8] T_M* and ΔG_U were successfully determined for all of the constructs except for $Tyr^{S}_{138}/Val^{S}_{142}$, which aggregated.

Surprisingly, the $Val^{S}_{136}/Glu^{S}_{139}$ construct was even less thermostable than the Cys^{Q} control (ΔT_M* = -2.9 °C, $\Delta\Delta G_U$ = -1.1 kcal mol^{-1} relative to Cys^{Q}) in spite of the fact that both Val^{S}_{136} (ΔT_M = 3.1 °C) and Glu^{S}_{139} (ΔT_M = 1.6 °C) are stabilizing. This destabilization may indicate incompatibility between hydrogen bonding interactions which we hypothesized to stabilize folding for the single thioamide substitutions (Fig. 1). Apo CaM has been structurally characterized by NMR (PDB ID: 1CFD) and X-ray crystallography (PDB ID: 1QX5).[22, 23] The protein conformations in these two structures are generally similar, but differ in the region of our studies so that the N-H of Val_{136} makes a hydrogen bond with the sidechain of Glu_{140} in 1QX5, and the N-H of Glu_{139} makes a hydrogen bond with the sidechain of Asn_{137} in 1CFD (see ESI, Fig. S9-S11 and additional discussion). While the stronger N-H hydrogen bond donation of a thioamide at either position would be stabilizing, the inability to form both interactions simultaneously could lead to net destabilization, with the protein sampling both 1QX5 and 1CFD conformations.

In contrast, the stabilizing effects of the Val^{S}_{136} mutation were maintained with Val^{S}_{142}, a mildly destabilizing substitution (ΔT_M = -1.5 °C). $Val^{S}_{136}/Val^{S}_{142}$ showed a similar T_M* to the Cys^{Q} control (see Table 1), yet underwent a significantly less cooperative unfolding transition ($\Delta\Delta G_U$ = -1.0 kcal mol^{-1}). Furthermore, we found that both stabilizing substitutions, Val^{S}_{136} and Glu^{S}_{139}, could recover the massive destabilization induced by Tyr^{S}_{138} (ΔT_M = -6.2 °C). Unlike all of the other CaM^{SS} variants, which exhibited diminished MRE values in CD

wavelength scans, the $Val^{S}_{136}/Tyr^{S}_{138}$ CD signature matched that of the Val^{S}_{136} monothioamide protein (see ESI, Figs. S4-S5). In addition, CaM $Val^{S}_{136}/Tyr^{S}_{138}$ was more thermally stable when compared to Cys^{Q}_{135} (ΔT_M = 3.1 °C) and had a more cooperative transition during unfolding ($\Delta\Delta G_U$ = 2.2 kcal mol^{-1}) than Tyr^{S}_{138} or Val^{S}_{136} (see ESI, Figs. S6-S7).

Table 1. CaM^{S} and CaM^{SS} Thermodynamic Values

CaM Variant	T_M* [a] (°C)	ΔT_m* (°C)	ΔG_U [b] (kcal mol^{-1})	$\Delta\Delta G_U$ (kcal mol^{-1})
Cys^{Q}_{135} Oxo	45.5 ± 0.4	---	5.9 ± 0.1	---
Val^{S}_{136}	48.6 ± 0.7	3.1	6.5 ± 0.1	0.6
Tyr^{S}_{138}	39.3 ± 0.8	-6.2	4.1 ± 0.2	-1.8
Glu^{S}_{139}	47.0 ± 0.1	1.6	6.4 ± 0.1	0.5
Val^{S}_{142}	44.0 ± 0.7	-1.5	5.8 ± 0.2	-0.1
$Val^{S}_{136}/Tyr^{S}_{138}$	48.6 ± 0.2	3.1	8.1 ± 0.3	2.2
$Val^{S}_{136}/Glu^{S}_{139}$	42.6 ± 0.1	-2.9	4.8 ± 0.2	-1.1
$Val^{S}_{136}/Val^{S}_{142}$	45.6 ± 0.6	0.1	4.9 ± 0.1	-1.0
$Tyr^{S}_{138}/Glu^{S}_{139}$	43.6 ± 0.2	-2.2	4.6 ± 0.1	-1.3

[a]Weighted melting temperature from three-state unfolding fit. Single thioamide values were calculated from a previously published study.[8] [b]ΔG_U calculated from three-state unfolding fit as outlined in equations S2-S5 and Table S6 (see ESI).

While the stability of $Val^{S}_{136}/Tyr^{S}_{138}$ may seem counter-intuitive, we can provide a reasonable explanation based on the available structural information (see ESI, Figs. S9-S11 and Table S7). If the N-H of Val^{S}_{136} forms a hydrogen bond with the sidechain of Glu^{S}_{140}, this would favor a 1QX5-like conformation which has a relatively large gap between helical turns (3.1 Å) that can more readily accommodate the larger size of the Tyr^{S}_{138} thiocarbonyl. With the Tyr^{S}_{138} thiocarbonyl no longer being structurally perturbing in this conformation, net stabilization is achieved by the Tyr^{S}_{138} participating in a hydrogen bond with the sidechain of Asn_{137}, which is also observed in the 1QX5 structure (Fig. 1). Clearly, high resolution structures of the thioamide proteins themselves would improve our understanding of these effects. Of course, one alternative explanation is that the CaM^{SS} proteins are simply adopting an alternate fold. To investigate this possibility, we determined whether the CaM^{SS} variants can participate in protein-protein interactions that are part of CaM's native signaling function.

Calcium bound CaM is known to bind several peptides derived from various protein regulatory domains.[24] Our laboratory has previously used derivatized sequences of the pOCNC peptide taken from an olfactory cyclic nucleotide gated ion channel as a model system for studying distance dependent fluorescence quenching phenomena in CaM.[19, 25, 26] In the following studies, a pOCNC derivative containing an N-terminal Trp residue (denoted W-pOCNC) was used as a fluorescent probe that bound to CaM and could be selectively excited in the presence of the Tyr residues in our CaM variants. All of the thioamide positions are close enough for Trp quenching to occur through short-range electron transfer (see Fig. 2 and ESI, Table S8).[26] These studies had two objectives: (1) to establish that CaM^{S} and CaM^{SS} proteins are able to bind W-pOCNC, and (2) to show that increased fluorescence quenching by dithioamides can occur in a folded protein.

CaM/W-pOCNC mixtures were prepared in 15 mM HEPES, 140 mM KCl, 6 mM $CaCl_2$, pH 6.7 buffer in 0.5:1 and 2:1 molar ratios (1 µM W-pOCNC) of each CaM^{S} or CaM^{SS} construct as well as WT CaM and the Cys^{Q}_{135} oxoamide control protein. Fluorescence emission spectra were measured to quantitatively assess binding and the additivity of quenching. Excitation of tryptophan was performed at 295 nm and emission spectra were collected from 305 nm to 450 nm. In buffer, the W-pOCNC peptide fluorescence exhibited a single peak with a maximum at 350 nm. When completely bound to WT CaM or Cys^{Q}_{135}, the emission blue-shifted to 330 nm, again displaying a single maximum. The emission profiles of the bound and free W-pOCNC samples were used to determine the fraction of free and bound peptide in each of the W-pOCNC binding assays as described in the ESI. Quenching efficiency (Q_E) was subsequently calculated by comparing the fluorescence of the bound fraction to the fluorescence of a comparable level of W-pOCNC bound to Cys^{Q}_{135}. Since Q_E accounts for the amount of peptide bound, the quantity is concentration independent. Therefore, the Q_Es reported in Fig. 3 are the average Q_Es across all ratios for each CaM mutant.

All of the dithioamide constructs bound W-pOCNC and quenched fluorescence to a degree comparable to or greater than their single thioamide congeners (Fig. 3). The $Val^{S}_{136}/Val^{S}_{142}$ CaM construct demonstrated the best example of the additive contributions of each thioamide to Trp quenching (see Fig. 3). Complete Trp quenching was observed for $Val^{S}_{136}/Val^{S}_{142}$, and 100% of W-pOCNC was bound at the 2:1 CaM^{SS}:W-pOCNC ratio, comparable to Cys^{Q}_{135}. This is greater than the percent of W-pOCNC bound for either Val^{S}_{136} or Val^{S}_{142} (Fig. 3). Interestingly, in spite of its thermostability, $Val^{S}_{136}/Tyr^{S}_{138}$ does not bind W-pOCNC dramatically better than its single thioamide counterparts and exhibits little additional quenching. Both $Val^{S}_{136}/Glu^{S}_{139}$ and $Tyr^{S}_{138}/Glu^{S}_{139}$ showed a significant increase

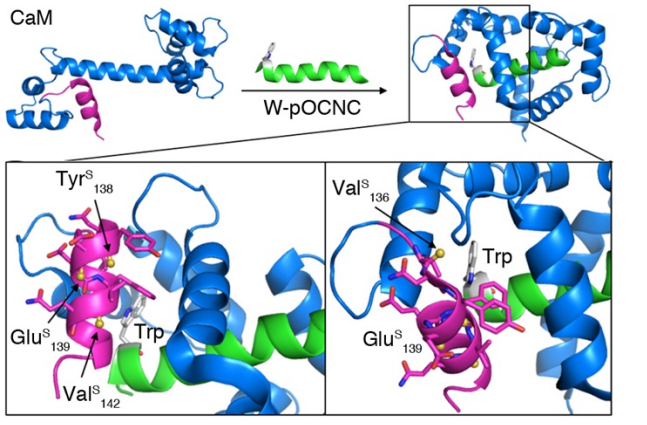

Fig. 2 Quenching of tryptophan by CaM thioproteins. CaM binding of W-pOCNC peptide with thioamide substitution positions highlighted relative to the modelled Trp residue.[25] Bound structure based on PDB ID 1SY9.[25]

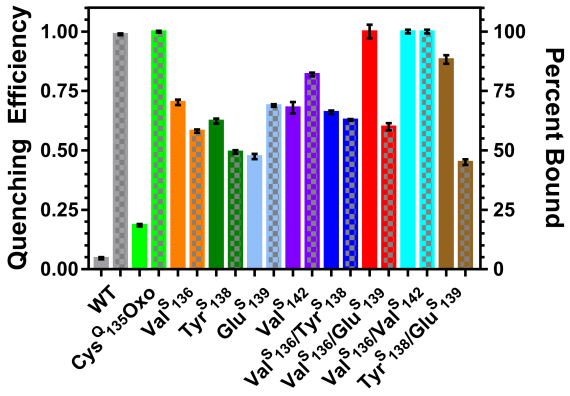

Fig. 3 Quenching of tryptophan by CaM thioproteins. Percent W-pOCNC bound (solid bars) and quenching efficiency (Q_E, checked bars) of Trp by each thioprotein. Percent binding was determined based on Trp spectral shift upon binding to CaM. Q_E is calculated as percent quenching of Trp divided by the percent of WpOCNC bound. The sample concentrations were 1 μM CaM with varying concentrations of W-pOCNC. Fitting procedures for processing of raw spectra can be seen in equations S6-S8 and Figs. S12-S17 in the ESI.

in Q_E compared to their single thioamide congeners, but bound the peptide at a comparable or lesser level. Although quantitative thermostability analysis was only performed on the calcium-free forms of these constructs, these peptide binding results are sensible as these two CaM^{SS} variants are significantly destabilized compared to Cys$^{Q}_{135}$.

In this work, we have undertaken the initial steps towards understanding how to best utilize dithioamide substitutions in a full-length protein. The CD studies performed show that multiple thioamide substitutions within the same protein domain can have unexpected effects, and it appears that successfully combining two thioamide modifications requires considering the mutual compatibility of the interactions in which they participate, rather than simply summing the thermodynamic effects of the individual substitutions. These CaM^{SS} proteins were tested as fluorescence quenchers to monitor protein/peptide interactions, and all of them bound the W-pOCNC peptide with Q_E greater than or equal to Q_E of the corresponding CaM^{S} proteins. Notably, Val$^{S}_{136}$/Val$^{S}_{142}$ bound W-pOCNC comparably to WT CaM. Taken together, these results show that multiple thionations have the potential to improve protein thermostability and stabilize a native, functional fold while simultaneously providing increased levels of fluorescence quenching for better sensors of protein interactions. Future work in our laboratory will center on understanding the structural basis for these effects to enable the rational design of polythioamide proteins and aid the study of thioamide natural products.

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Conflicts of interest

There are no conflicts to declare.

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