

# Thioamide-Based Fluorescent Protease Sensors

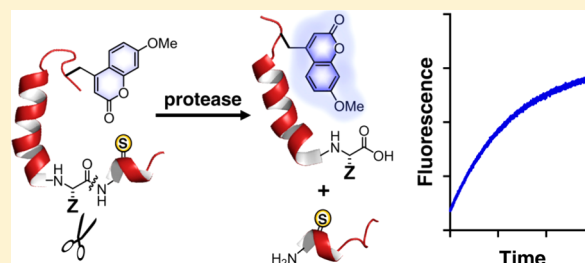
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## Supporting Information

**ABSTRACT:** Thioamide quenchers can be paired with compact fluorophores to design “turn-on” fluorescent protease substrates. We have used this method to study a variety of serine-, cysteine-, carboxyl-, and metallo-proteases, including trypsin, chymotrypsin, pepsin, thermolysin, papain, and calpain. Since thioamides quench some fluorophores red-shifted from those naturally occurring in proteins, this technique can be used for real time monitoring of protease activity in crude preparations of virtually any protease. We demonstrate the value of this method in three model applications: (1) characterization of papain enzyme kinetics using rapid-mixing experiments, (2) selective monitoring of cleavage at a single site in a peptide with multiple proteolytic sites, and (3) analysis of the specificity of an inhibitor of calpain in cell lysates.



## INTRODUCTION

Proteases are a diverse class of enzymes that catalyze the hydrolysis of peptide bonds with varying degrees of specificity. They serve important physiological roles, especially in metabolic pathways, signaling cascades, and regulatory processes, though they have also been implicated in a variety of disease pathologies, including cancer, hypertension, viral infections (e.g., hepatitis C, HIV, and malaria), and neurodegeneration.<sup>1–4</sup> As such, proteases have received a considerable amount of clinical, commercial, and academic attention, and numerous techniques have been developed to monitor their activity or probe their specificity.<sup>5,6</sup> Chief among these techniques is fluorescence spectroscopy, which can provide results in real time and is amenable to high throughput methodology, although radioactive, chromatographic, and colorimetric assays are also common.<sup>7</sup>

Fluorescence experiments often require that a peptide or protein substrate be labeled with at least one fluorophore that is selectively excitable in the presence of Trp and Tyr, since these residues are common in proteolytic enzymes. The proteolysis of substrates bearing a single fluorescent label can be monitored with fluorescence anisotropy measurements if the signal of the intact substrate is sufficiently different from that of the cleavage product. For this method to be useful, the absolute difference in size between the substrate and cleavage products needs to be rather substantial; short peptides are often too small to provide accurate results.<sup>8</sup> An alternative strategy is based on fluorogenic molecules that can be covalently installed at the P1' site of a protease substrate.<sup>9</sup> Some amidated fluorophores, such as 2-naphthylamides, 4-methyl-7-coumarylamides, rhodamine 110, and various anilides are quenched relative to the corresponding

derivatives bearing free amines, and enzymatic hydrolysis of the amide bond can result in a substantial gain in fluorescence.<sup>10–13</sup> Unfortunately, this type of probe must be installed directly at the site of proteolysis and requires the protease to tolerate a large aromatic dye in its active site. Furthermore, since these probes must be installed at the P1' site, the specificity of the S1' pocket cannot be easily explored—specificity can only be conferred from the S1, S2, S3, and other pockets. Although these constraints can be relaxed to some extent by the use of 6-amino-1-naphthalenesulfonamides, which can allow for the incorporation of alkyl chains as short spacers, these methods are limited in scope by the fact that the P1' site must be a fluorogenic probe.<sup>14,15</sup>

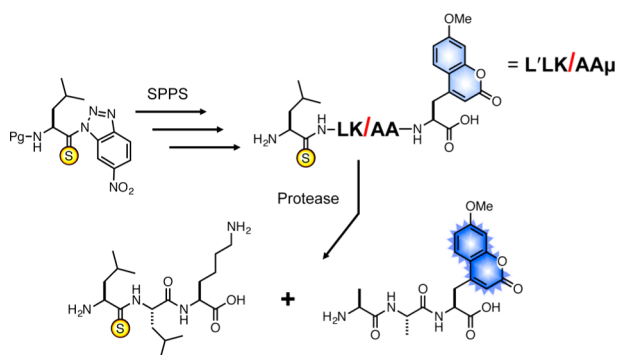
Protease substrates labeled with two chromophores can be used to overcome this restriction. Changes in distance-dependent energy transfer between the labels—either through Förster resonance energy transfer (FRET) or photoinduced electron transfer (PET) mechanisms—can be used to monitor protease activity.<sup>9,16</sup> Typically, these probes are installed on opposite ends of a short peptide sequence and interact through FRET or PET. Upon cleavage of the intervening sequence, the fluorophore interaction is lost as the fragments diffuse through solution. FRET based sensors can sometimes be used as ratiometric probes if both chromophores are fluorescent; PET-quenched substrates typically give a fluorogenic response. A major limitation of these methods, however, is the requirement that the substrate be labeled with two probes, which are often

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bulky.<sup>17–19</sup> The large size of the requisite probes may influence or interfere with the kinetics of proteolysis.

To eliminate the problems associated with bulkier probes, this sort of profluorescent reporter design can be adapted for use with small thioamide quenchers (Figure 1). Previously, we



**Figure 1.** Profluorescent thiopeptides for monitoring protease activity. Thioamide (denoted by the one or three letter code of the corresponding natural amino acid with a prime symbol, e.g., L') substrates can be prepared on solid phase from benzotriazole precursors and fluorescent amino acids such as 7-methoxycoumarin-4-ylalanine ( $\mu$ ). Incubation of a coumarin/thioamide labeled peptide with a protease results in cleavage and a concomitant gain of fluorescence.

have shown that thioamides quench a variety of fluorophores, including 7-methoxycoumarin and fluorescein, through a PET mechanism.<sup>20–23</sup> As probes, thioamides can be installed in peptide backbones as single-atom substitutions in amide bonds, and they are much smaller than almost any other conventional quencher or fluorophore used in this type of experiment. In this way, thioamides can be incorporated in positions where larger probes would not be well tolerated by a protease. In principle, thioamides could be scanned through an entire candidate substrate sequence, thus providing more thorough, and perhaps more accurate, information about proteolysis than other methods would allow.

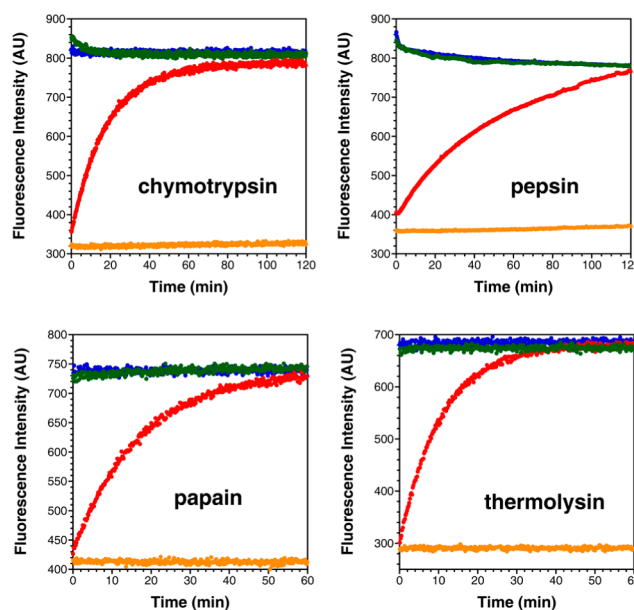
It is important to note that thioamide replacement of the scissile bond in the substrate might affect proteolysis, so care must be taken when examining this position.<sup>24–34</sup> This was seen in attempts to use thioamides as protease inhibitors and in the two previous cases in which thioamides have been used as probes of proteolysis. Bond et al. inserted a thioamide at the scissile bond and monitored carboxypeptidase A activity by disappearance of the thiocarbonyl absorbance at 270 nm.<sup>27</sup> Cho et al. tracked papain activity by the increase in fluorescence from cleavage of a thioacyl anilide.<sup>29</sup> In both cases, placing the thioamide at the site of cleavage disrupted protease activity. Our PET-based design allows one to place the fluorophore and thioamide probes at sites that flank the scissile bond, and is thus less perturbing and very general.

We have recently reported an initial trial of this design using fluorescein as a fluorescent donor.<sup>20</sup> Here, we show that this general strategy can be used to track the activity of a variety of proteases using 7-methoxycoumarin-4-yl-alanine (Mcm;  $\mu$ ) as a donor fluorophore. Mcm is only slightly larger than Trp, and can be selectively excited at 325 nm in the presence of endogenous Trp and Tyr residues in the analyte. Mcm is superior to fluorescein as a donor because it is smaller, can be easily incorporated during solid phase synthesis, and is more quenched by thioamides.<sup>22</sup> We have used Mcm/thioamide

substrates to study a variety of serine-, cysteine-, carboxyl-, and metallo-proteases. Furthermore, we demonstrate the value of this method by showing that it can be used in rapid-mixing experiments, monitoring of cleavage at selective sites, and tracking specific activity in cell lysates.

## RESULTS AND DISCUSSION

We began our investigation by preparing short target peptides labeled with N-terminal thioamides and C-terminal Mcm. Intervening amino acid sequences were chosen such that the peptides would be recognized and cleaved by a variety of proteases, including chymotrypsin, papain, pepsin, thermolysin, and trypsin (Figure 2 and Figure S1, Supporting Information).



**Figure 2.** Protease substrate trials. Representative results for chymotrypsin, pepsin, papain, and thermolysin experiments. The fluorescence of the thiopeptide in the presence (red trace) and absence (orange trace) of protease is shown with the corresponding oxopeptide also in the presence (green trace) and absence (blue trace) of protease. Chymotrypsin: fluorescence of 8.3  $\mu$ M AAF $\mu$  or A'AF $\mu$  in 100 mM Tris-HCl, pH 7.8, at 30  $^{\circ}$ C in the presence and absence of 0.2 mg/mL chymotrypsin. Pepsin: fluorescence of 0.5  $\mu$ M AAF $\mu$  or A'AF $\mu$  in 10 mM HCl at 37  $^{\circ}$ C in the presence and absence of 1.5 mg/mL pepsin. Papain: fluorescence of 8.3  $\mu$ M LLKAA $\mu$  or L'LKAA $\mu$  in 2.0 mM EDTA, 5.00 mM L-cysteine, 300 mM NaCl, pH 6.2, at 25  $^{\circ}$ C in the presence and absence of 2.5  $\mu$ g/mL papain. Thermolysin: fluorescence of 8.3  $\mu$ M AAF $\mu$  or A'AF $\mu$  in 2 mM calcium acetate 10 mM sodium acetate, pH 7.5 at 25  $^{\circ}$ C in the presence and absence of 6  $\mu$ g/mL thermolysin.

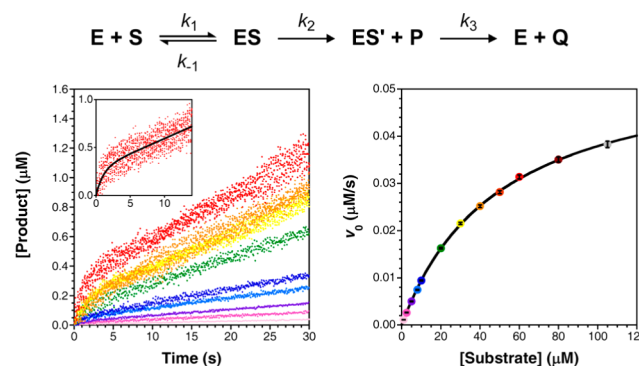
In a typical experiment, we measured the fluorescence of the peptide in the presence and absence of a protease as a function of time. As a control, we also synthesized the corresponding oxoamide version of each peptide and measured the fluorescence of these samples as well. The concentrations of peptide and protease were adjusted so that proteolysis reached completion within 1 to 2 h. In the absence of any proteases, the thioamide peptide A'AF $\mu$  was quenched  $\sim$ 65% relative to the all oxoamide control peptide (AAFA $\mu$ ) and the thioamide peptide L'LKAA $\mu$  was quenched  $\sim$ 40% relative to the control peptide, LLKAA $\mu$ . Thioamide amino acid analogs are denoted by the one or three letter code of the corresponding natural amino acid with a prime (') symbol. The fluorescence of both

peptides remained constant over the timecourse of experiments in the absence of protease, with slight decreases attributed to photobleaching. We observed an immediate gain in fluorescence upon addition of an appropriate protease to the thiopeptides, but almost no change in fluorescence in the oxoamide control experiments.

To ensure that the increases in fluorescence were the result of protease activity, we repeated these experiments with heat-deactivated proteases. The fluorescence of the thiopeptide in the presence of a heat-deactivated protease was identical to that of the peptide in pure buffer (Figure S3, Supporting Information). To further confirm that the observed changes were the result of proteolysis and not some other coincidental process, and to demonstrate that the oxoamide control peptides underwent the same proteolytic degradation, we analyzed the papain reactions by high-performance liquid chromatography (HPLC). Here, we sampled each reaction at various time points. After precipitating the protein, we analyzed the supernatant by HPLC (Figure S2, Supporting Information). We saw essentially no change in the chromatograms of the peptides that were not treated with papain. For the peptides that were treated with papain, we saw a decrease in starting material signal and an increase in signal for the expected cleavage products, which were identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI MS). Cleavage occurred at approximately the same rate in the thiopeptide and in the oxoamide control peptide. Additional experiments with labeled peptide substrates that were not expected to be cleaved by papain provided further evidence that the presence of a thioamide probe does not stimulate proteolysis (Figure S4, Supporting Information).

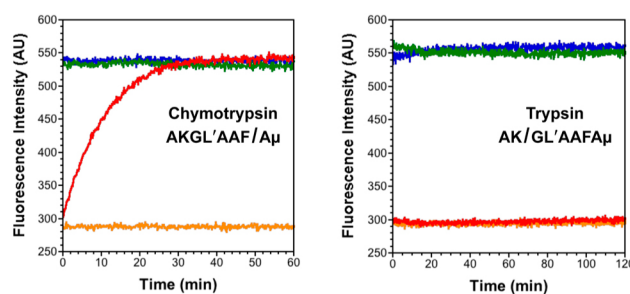
To demonstrate the suitability of this method for studying enzyme kinetics, we conducted stopped-flow experiments with papain and variable initial concentrations of L'LKAA $\mu$ . Papain catalysis can be described by a three step reaction scheme (Figure 3, top). An expression for the rate of proteolysis can be developed from this scheme and globally fit to the series of stopped flow experiments (Figure S6, Supporting Information). We obtained the following kinetic parameters:  $K_S = k_1/k_{-1} = 921 \mu\text{M}$ ,  $k_2 = 0.92 \text{ s}^{-1}$ ,  $k_3 = 0.057 \text{ s}^{-1}$ . These can be used to determine  $k_{\text{cat}} = 0.054 \text{ s}^{-1}$  and  $K_{\text{M,app}} = 53.9 \mu\text{M}$ . The concentration dependence of papain activity can be seen in plots of the stopped-flow primary data and the initial rate (Figure 3, bottom). Nearly identical values for  $k_{\text{cat}}$  and  $K_{\text{M}}$  of  $0.057 \pm 0.001 \text{ s}^{-1}$  and  $50.2 \pm 1.1 \mu\text{M}$ , respectively, can also be obtained by fitting this initial rate data to the standard Michaelis–Menten equation (Supporting Information). The enzyme efficiency,  $k_{\text{cat}}/K_{\text{M}} = 1.00 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ , is similar to that previously reported for papain for the substrate lysylnitroanilide ( $2.57 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ ).<sup>35</sup> Wide variations in rates are often observed for fluorogenic substrates used in studying the kinetics of the same enzyme.<sup>36–38</sup> The small size of the thioamide allows us to place it at a variety of locations while keeping our reporter peptides nearly identical to the actual substrate, which should ensure that the rates obtained in rapid mixing experiments are relevant to the actual protein substrates.

The ability to place the thioamide at different locations without disrupting the native peptide fold can be particularly valuable where one would like to distinguish cleavage at one of two sites, both of which may be bound near the protease active site. To test our ability to carry out such a study, the peptide AKGL'AAFA $\mu$  was labeled such that chymotrypsin cleavage



**Figure 3.** Papain kinetics as determined from stopped-flow fluorescence measurements. Top: kinetic scheme for papain catalysis in terms of enzyme (E), substrate (S), enzyme–substrate complexes (ES and ES'), and products (P and Q) with rate constants as defined. Left: primary stopped-flow data for the proteolysis of L'LKAA $\mu$  by papain (2.5  $\mu\text{g}/\text{mL}$ ) in 2.0 mM EDTA, 5.00 mM L-cysteine, 300 mM NaCl, pH 6.2, at 25  $^{\circ}\text{C}$ . Only a subset of data for selected concentrations are shown for clarity. The inset shows a representative fit to the burst equation for the 60  $\mu\text{M}$  data set. Fits for each data set are shown in the Supporting Information. Right: Michaelis–Menten plot of initial rates determined from primary data. Data are colored according to initial substrate concentration: pink 1.0  $\mu\text{M}$ ; magenta 2.5  $\mu\text{M}$ ; purple 5.0  $\mu\text{M}$ ; azure 8.0  $\mu\text{M}$ ; royal blue 10  $\mu\text{M}$ ; green 20  $\mu\text{M}$ ; yellow 30  $\mu\text{M}$ ; orange 40  $\mu\text{M}$ ; red 60  $\mu\text{M}$ .

after the Phe residue should lead to a turn on of fluorescence, but trypsin cleavage after the Lys residue should not, since intramolecular quenching would be maintained in the resulting GL'AAFA $\mu$  fragment. This is indeed what was observed, as incubation with chymotrypsin gave a robust and rapid increase in fluorescence intensity until it reached the levels observed in the oxopeptide, AKGLAAFA $\mu$  (Figure 4, left). As before, this occurred only for the thiopeptide, and only in the presence of the protease. Incubation in the presence of sequencing grade trypsin produced no change in fluorescence over 2 h (Figure 4, right). However, HPLC and MALDI MS analysis of the



**Figure 4.** Specific monitoring of chymotrypsin activity in a dual substrate peptide. Incubation of a thioamide/7-methoxycoumarin labeled peptide with both chymotrypsin and trypsin cleavage sites results in a gain of fluorescence only in the presence of chymotrypsin. Representative results for chymotrypsin and trypsin experiments. The fluorescence of the thiopeptide in the presence (red trace) and absence (orange trace) of protease is shown with the corresponding oxopeptide also in the presence (green trace) and absence (blue trace) of protease. Chymotrypsin: fluorescence of 8.3  $\mu\text{M}$  AKGLAAFA $\mu$  or AKGL'AAFA $\mu$  in 100 mM Tris-HCl, pH 7.8, at 25  $^{\circ}\text{C}$  in the presence and absence of 5  $\mu\text{g}/\text{mL}$  chymotrypsin. Trypsin: fluorescence of 8.3  $\mu\text{M}$  AKGLAAFA $\mu$  or AKGL'AAFA $\mu$  in 67 mM sodium phosphate, pH 7.6, at 25  $^{\circ}\text{C}$  in the presence and absence of 10  $\mu\text{g}/\text{mL}$  trypsin.



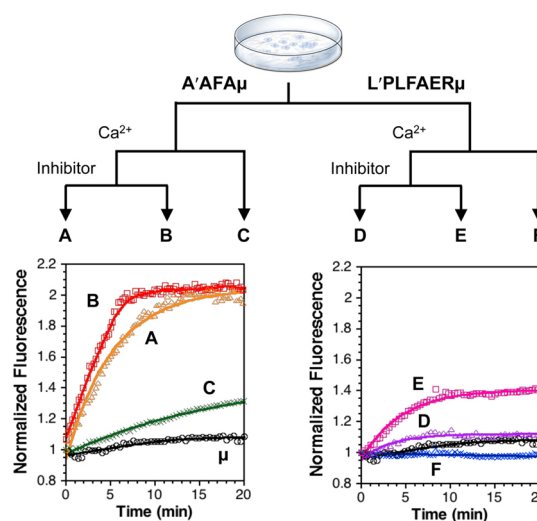
reaction mixtures indicated that cleavage had indeed taken place at the expected location (Figure S8, Supporting Information). HPLC analysis of both chymotrypsin and trypsin cleavage showed that the thiopeptide and oxopeptide were cleaved at roughly the same rates, but that only the intended chymotrypsin activity was detectable by a fluorescence increase. It is interesting to note that when lower grade trypsin was used, trace chymotrypsin activity reported by the manufacturer could be observed as a turn on of fluorescence (Figure S7, Supporting Information). The results observed with AKGL/AFAF $\mu$  should be general, in that thiopeptides could be labeled so that a specific cleavage event can be monitored in real time in an otherwise native sequence.

As a final demonstration of the use of thioamides as protease probes, we evaluated a fluorogenic substrate for monitoring calpain activity in cell lysates. Cell lysates contain a host of different proteases, challenging the specificity of our probes. Insoluble or fluorescent material can contribute to background signal, which demands robustness from the assay. Thus, we viewed this as a stringent test of the utility of the fluorophore/thioamide strategy. We designed a thiopeptide, L'PLFAER $\mu$ , to serve as a reporter of calpain activity and to be used in screening calpain inhibitor efficacy in crude cell lysates. Calpains 1–15 are Ca<sup>2+</sup>-activated cysteine proteases that have been implicated in several neuronal processes, insulin secretion, cell survival, and the regulation of blood vessels.<sup>39–42</sup> It has recently been shown that malarial parasites hijack calpain activity in order to digest heme proteins to serve as metabolites during their exit from host red blood cells.<sup>43</sup> Calpain activity has also been tied to Alzheimer's disease via the regulation of APP proteolysis and to breast cancer.<sup>44,45</sup> As a consequence, there is substantial current interest in discovering reporters and modulators of calpain activity.<sup>46–48</sup> We felt that calpains would be a valuable test of our technology as the substrate peptide adopts an unusual helical conformation in the active site, different than the extended structures typical of many proteases. We therefore believed that taking advantage of the natural substrate sequences to confer specificity for calpains on our probes would show their value.

Prior to any usage of thiopeptide protease sensors in cell lysates, we performed important control experiments that evaluated the stability of thioamides toward nonproteolytic degradation. Although backbone thioamides are found in several peptide natural products and have in fact shown some resistance to metabolism, we were able to find only one previous assessment of their stability that we considered applicable to our studies.<sup>49–51</sup> Here, we refer to only acyclic thioamides of the type incorporated in our peptides, not the well-studied thiazolene rings in molecules such as thiostrepton, which are also commonly referred to as "thiopeptides."<sup>52</sup> To evaluate thioamide stability in cell lysates, we synthesized a biotin-labeled thiopeptide composed of D-amino acids, Biotin~F'aa, which should not be recognized by proteases, but should report on any nonstereo-specific metabolic degradation of the thioamide bond. When this probe was incubated with PBS at 25 °C, recovered using neutravidin beads, and analyzed by HPLC, it was found to be completely intact after 1 h and 94  $\pm$  2% recoverable after 24 h (as compared to a biotinylated coumarin control dipeptide) (Figure S10, Supporting Information). Moreover, when the same experiments were performed with mouse serum, the peptide was recovered in 85  $\pm$  2% yield after 1 h and 80  $\pm$  2% yield after 4 h. We note that most of our protease assays are

completed in 0.5–2 h. While we continue to pursue experiments to understand the nature of this 15–20% degradation, we take confidence from these experiments that thiopeptide applications in cell lysates are viable.

During initial proteolytic testing using purified calpain 1 in buffer, we only observed an increase in fluorescence when the L'PLFAER $\mu$  thiopeptide was incubated with calpain that had been activated by the addition of Ca<sup>2+</sup>. No increase was observed in the oxopeptide (LPLFAER $\mu$ ), or in the thiopeptide when calpain activity was inhibited by the inclusion of the Ca<sup>2+</sup> chelator EDTA in the buffer (Figure S11, Supporting Information). We then tested the probes using mouse embryonic fibroblast (MEF) cells, which endogenously express calpains 1 and 2. When the cell lysates were incubated with the thiopeptide L'PLFAER $\mu$  in the presence of added Ca<sup>2+</sup>, we indeed observed an increase in fluorescence (Figure 5, right).



**Figure 5.** Inhibition of calpain by calpastatin peptide in cell lysate. Top: scheme for experimental setup wherein MEF cell lysate is incubated with a reporter peptide (either A'FAF $\mu$  or L'PLFAER $\mu$ ) in the presence or absence (EDTA) of Ca<sup>2+</sup>, used to activate calpain proteases. Ca<sup>2+</sup>-activated samples were incubated in the presence or absence of a peptide inhibitor derived from calpastatin. Left: relative fluorescence of A'FAF $\mu$  in MEF cell lysate in the presence of Ca<sup>2+</sup> and inhibitor peptide (A, yellow), in the presence of Ca<sup>2+</sup> (B, red), or in the presence of EDTA (C, green). The increase of fluorescence of 7-methoxycoumarin in Ca<sup>2+</sup>-loaded lysate is also shown ( $\mu$ , black). Right: relative fluorescence of L'PLFAER $\mu$  in MEF cell lysate in the presence of Ca<sup>2+</sup> and inhibitor peptide (D, purple), in the presence of Ca<sup>2+</sup> (E, pink), or in the presence of EDTA (F, blue). All traces are normalized to the fluorescence of the peptide at time 0 min.

When the lysates were incubated with the thiopeptide in a buffer containing EDTA, no increase in fluorescence was observed (Figure 5, right). However, the interpretation of these results is not quite as simple as it might seem; a small increase in fluorescence was also observed for the 7-methoxycoumarin fluorophore itself, induced by nonspecific aggregation in the Ca<sup>2+</sup>-loaded lysates (Figure 5, left and right). When a saturating concentration of a high affinity calpain-specific peptide inhibitor (a 27mer peptide derived from the endogenous inhibitor calpastatin) was added to the lysates,<sup>53</sup> the timecourse of the fluorescence increase was nearly identical to the nonspecific increase observed for 7-methoxycoumarin alone, showing that all calpain activity had been inhibited. (Figure 5, Right)

In contrast, when the nonspecific thiopeptide reporter A'AFAM was used, an increase in fluorescence was again observed upon addition to lysates, but only a small portion of this increase could be inhibited by the calpastatin peptide (Figure 5, left). Moreover, a substantial increase in fluorescence was observed in the lysates even in the presence of EDTA, indicating clearly that non-calpain proteases were also acting on the A'AFAM thiopeptide (Figure 5, left). Taken together, these data show that thiopeptides can be used as fluorescence reporters even in cell lysates and that through judicious sequence design, they can be made highly specific for use in monitoring the activity of only a select protease toward an essentially native sequence.

## ■ CONCLUSIONS

In summary, we have shown that thioamides can be used to prepare quenched substrates that become fluorescent upon cleavage by a protease. These probes can be used to monitor kinetics in real time and should be compatible with most proteolytic enzymes. Since thioamides are small, and since they can conceivably be scanned through a protein backbone with minimal perturbation to native structure, thioamide-based probes should allow investigators to study protease activity with more detail than current methods allow. However, the design of such substrates requires two important considerations. First, that quenching by PET is distance dependent so that the fluorophore and thioamide must be placed relatively close to each other in three-dimensional space. We have shown that they can be placed at least six amino acids apart, and we will continue to explore the trade-off between fluorophore/thioamide spacing and the increase in fluorescence upon cleavage. The second consideration is one of the nonperturbing nature of the thioamide substitution. Here, we have shown that in the case of the papain substrate, placement of the thioamide two residues away from the scissile bond does not impact the cleavage rate relative to the corresponding oxoamide peptide. Further study of thioamide placement with papain and other proteases will allow us to provide more general guidance on how closely one can place the thioamide to the cleavable bond. Given the success of our cell lysate experiments, we expect to be able to extend this method to applications in cell culture and we are currently exploring strategies for delivering appropriately labeled peptides into cells.

Since there are many existing strategies for monitoring proteolysis through fluorescence, a comparison of these approaches to our thioamide method is valuable. A primary concern in any fluorescent assay is sensitivity, especially with respect to signal/noise, signal/background, and the limit of detection. For our steady state (i.e., not rapid mixing) measurements, we found signal/noise ratios that were typically >100 and signal/background ratios that were typically >150 (See Supporting Information). These values are comparable to most other fluorophore reporting systems. Turn-on values for typical FRET or PET probes routinely vary by substrate and range from ~2 to >190-fold.<sup>9</sup> Most of the thioamide examples presented here have a ~2-fold fluorescence increase, which is at the low end of the observed range, but more than sufficient to extract reliable kinetic data given the low background and high signal/noise of our probes. In principle, thioamide quenchers should have limits of detection similar to other fluorescent probes, given that they can be paired with very bright fluorophores such as Alexa Fluor 488 that can even be used in single molecule studies.<sup>20,54</sup> Of course, the chief advantage of

thioamides is their small size, which we believe is a significant improvement over current methods. Although there are certainly situations in which the placement of the fluorophores on the substrate does not appear to interfere with enzyme kinetics, there are also many examples, such as with papain, in which fluorophore placement disturbs the observed kinetics, sometimes by as much as 50-fold.<sup>55–57</sup> Placing the fluorophores far away from the scissile bond can obviate such problems, but results in a loss of information about which bond is being hydrolyzed. Here, we show that thioamides can be incorporated at positions close to the site of cleavage to retain some of this information that would otherwise be lost. The sensitivity of fluorescent protease sensing is not changed by our innovation, but its resolving power is increased substantially. Ultimately, thioamide probes will not be appropriate for every experiment, but should find use in a number of applications in which the use of larger probes might interfere with enzyme activity.

Finally, while short reporter peptides of the type used here can be valuable for simple assays, we also envision future applications of this technology to analyzing proteolysis in full-sized proteins. We have shown that native chemical ligation (NCL) reactions can be applied to synthesize full-sized proteins containing thioamides, taking advantage of ligations to expressed proteins to minimize unnecessary protein synthesis.<sup>58</sup> Furthermore, we have recently shown that NCL can be coupled to unnatural amino acid (Uaa) mutagenesis to generate double labeled proteins wherein the fluorophore is incorporated during ribosomal protein synthesis.<sup>59</sup> We have applied a combination of NCL and Uaa methods to the synthesis of labeled versions of  $\alpha$ -synuclein ( $\alpha$ S), a neuronal protein that forms fibrillar aggregates that are believed to contribute to Parkinson's disease pathology. Several proteases have been reported to act on  $\alpha$ S, and cleavage of the C-terminal tail of  $\alpha$ S has been shown to accelerate fibrillization.<sup>60–63</sup> Moreover, there has been a report of autoproteolysis of  $\alpha$ S.<sup>64</sup> It is difficult to imagine studying the mechanism of such a reaction without access to labeled versions of the full-length protein. Clearly short peptides would not be appropriate model systems. Synthesis of appropriately labeled versions of  $\alpha$ S using NCL and Uaa techniques will allow us to investigate such questions using thioamide-quenching methods. Many other proteins undergo autoproteolysis, or are only cleaved in multiprotein complexes. These proteins also lend themselves to investigation with fluorophore/thioamide pairs where probes could be placed at appropriate locations to monitor specific proteolytic events for which maintaining the native conformation of the protein is important. We are pursuing such applications while we further study fundamental aspects of fluorophore/thioamide placement to design optimal probes for certain types of cleavage events.

## ■ EXPERIMENTAL PROCEDURES

**Peptide Synthesis.** Peptides were synthesized on solid phase using standard Fmoc chemistry and purified to homogeneity by reverse-phase high performance liquid chromatography (HPLC). Thioamide benzotriazole precursors were either commercially available or synthesized according to literature precedent.<sup>21</sup> Explicit protocols are provided in the Supporting Information.

**Fluorescence Spectroscopy.** Steady-state fluorescence measurements were collected with a Tecan M1000 plate reader, the kinetics module of a Varian Cary Eclipse fluorometer, or a Photon Technologies International QuantaMaster fluorometer outfitted with multicell Peltier sample holders. For the fluorometers, the excitation wavelength was 325 nm and the excitation slit width was 5 nm. The emission wavelength was 391 nm and the slit width was 5 nm. The

averaging time was 0.100 s. Samples were stirred in 1.00 cm quartz cuvettes. Peptide concentrations were determined by absorbance at 325 nm ( $\epsilon_{325} = 12\,000\text{ M}^{-1}\text{cm}^{-1}$ ).<sup>65</sup> Plate reader measurements were recorded with black plates with optically transparent bottoms (Greiner Bio-One No. 675096; Frickenhausen, Germany) as the average of nine 20  $\mu\text{s}$  reads per well with an excitation wavelength of 330 nm and emission wavelength of 385 nm with 5 nm slit widths and a 400 Hz flash frequency with 30 s shaking before each kinetics run unless otherwise indicated.

**Steady-State Protease Assays.** At least three independent trials of each assay were conducted to ensure reproducibility (Figure S1, Supporting Information). Concentrated stock solutions of each peptide and protease in the appropriate buffer were used to prepare fresh samples immediately prior to each experiment. Chymotrypsin assay (30 °C): Chymotrypsin (type II from bovine pancreas;  $\geq 40$  units/mg protein) was dissolved in 1 mM HCl, 10 mM  $\text{CaCl}_2$  and diluted to a working concentration of 0.2 mg/mL immediately prior to use such that each sample contained 8.3  $\mu\text{M}$  AFAF $\mu$  or A'FAF $\mu$  in 100 mM Tris-HCl, pH 7.8. Pepsin assay (37 °C): Pepsin (from porcine gastric mucosa; 3200–4500 units/mg protein) was diluted to a working concentration of 1.5 mg/mL in samples that were 0.5  $\mu\text{M}$  AFAF $\mu$  or A'FAF $\mu$  in 10 mM HCl. Papain assay (25 °C): Papain (crude, from papaya latex; 1.5–10 units/mg solid) was diluted to a working concentration of 2.5  $\mu\text{g/mL}$  such that samples also contained 8.3  $\mu\text{M}$  LLKAA $\mu$  or L'LKAA $\mu$  in 2.0 mM EDTA, 5.00 mM L-cysteine, 300 mM NaCl, pH 6.2. Thermolysin assay (25 °C): Thermolysin (from *Bacillus thermoproteolyticus* rokko; 50–100 units/mg protein) was diluted to a working concentration of 6  $\mu\text{g/mL}$  such that the samples contained 8.3  $\mu\text{M}$  AFAF $\mu$  or A'FAF $\mu$  in 2 mM calcium acetate, 10 mM sodium acetate, pH 7.5. Trypsin assay (25 °C): Stock solutions of trypsin (type II from porcine pancreas; 1000–2000 units/mg dry solid) in cold 1 mM HCl were diluted with 67 mM sodium phosphate, pH 7.6, to a working concentration of 25  $\mu\text{g/mL}$  such that the samples also contained 8.3  $\mu\text{M}$  LLKAA $\mu$  or L'LKAA $\mu$ .

**Stopped-Flow Kinetics.** Stopped-flow kinetics measurements were obtained with a KinTek SF-120 instrument (KinTek Corporation; Austin, TX, USA). One syringe was filled with 2  $\mu\text{g/mL}$  papain in 4.0 mM EDTA, 10 mM L-cysteine, 600 mM NaCl, pH 6.2. The second syringe was filled with LLKAA $\mu$  or L'LKAA $\mu$  in water at concentrations ranging from 2 to 200  $\mu\text{M}$ , as determined by absorbance at 325 nm. After mixing, data were collected for 2 min at a rate of 5,000 points/min. The excitation wavelength was 310 nm and the emission was recorded as the integrated signal for wavelengths  $> 320$  nm. Data sets for each concentration were collected as the average of at least three trials. The determination of the concentrations of the cleaved thiopeptides as a function of time and the kinetic analysis of the data are presented in Supporting Information. Primary stopped flow data are shown with the results of global fitting (Figure S6, Supporting Information).

**Multisite Substrate Assay.** Multisite substrate assays were conducted in a fashion similar to that described for other steady-state measurements in the presence and absence of enzyme. Concentrated stocks of the peptides AKGLAAFAF $\mu$  and AKGL'AAFAF $\mu$  were prepared in 67 mM sodium phosphate buffer, pH 7.6 for experiments with trypsin. Sequencing grade modified trypsin (Promega; Madison, WI, USA) was dissolved in 50 mM acetic acid and stored at  $-80$  °C. For the trypsin assay, samples were prepared such that the enzyme concentration was 1  $\mu\text{g/mL}$  and the peptide concentration was 8.3  $\mu\text{M}$ . Fluorescence was monitored as described above at 30 °C. Preliminary experiments with technical grade trypsin (25  $\mu\text{g/mL}$ ) resulted in a slight turn on of fluorescence for the AKGL'AAFAF $\mu$  sample in the presence of enzyme (Figure S7, Supporting Information). We attribute this observation to chymotrypsin-like activity of the lower grade enzyme as indicated by the manufacturer; this activity was not observed in with sequencing-grade enzyme. For chymotrypsin experiments, concentrated stocks of AKGLAAFAF $\mu$  and AKGL'AAFAF $\mu$  were prepared in 100 mM Tris buffer, pH 7.8, and lyophilized chymotrypsin was dissolved in a solution of 1 mM HCl and 10 mM  $\text{CaCl}_2$  immediately prior to use. For the chymotrypsin assay, the enzyme concentration was 5  $\mu\text{g/mL}$

and the peptide concentration was 8.3  $\mu\text{M}$ . Fluorescence was monitored as described above at 25 °C.

The reactions (120  $\mu\text{L}$  total volume) were quenched at 1 h (chymotrypsin) or 2 h (trypsin) by addition of 1080  $\mu\text{L}$  of cold ethanol to precipitate the protease. The samples were then cooled at  $-20$  °C for 1 h and then centrifuged at 13 200 rpm at 4 °C for 20 min. The supernatant was transferred to a clean microcentrifuge tube and dried in a vacuum centrifuge. Each sample was brought up in 800  $\mu\text{L}$   $\text{H}_2\text{O}$  and analyzed by HPLC (Figure S8, Supporting Information).

**Cell Culture.** Mouse embryonic fibroblast (MEF) cells were grown in Dulbecco's modified eagle medium (DMEM) (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1:100 penicillin and streptomycin (Gibco) at 37 °C. Cells were grown to 90% confluency then trypsinized, pelleted, and frozen.

**Calpain Cell Lysate Assay.** The MEF cells were both hypotonically lysed and lysed via freeze/thaw (3 $\times$ ) in liquid nitrogen in hypotonic lysing buffer (10 mM dithiothreitol (DTT), 5 mM  $\text{KH}_2\text{PO}_4$ , and 6 mM EGTA, pH 7.5).<sup>66</sup> Total protein concentration was  $\sim 4$  mg/mL as determined by bicinchoninic acid assays (Thermo Scientific/Pierce; Rockford, IL, USA).<sup>67</sup> The LPLFAER $\mu$  and L'PLFAER $\mu$  substrate concentrations were 8  $\mu\text{M}$ . The inhibitor, calpastatin peptide (sequence: Biotin-Asp-Pro-Met-Ser-Thr-Tyr-Ile-Glu-Glu-Leu-Gly-Lys-Arg-Glu-Val-Thr-Ile-Pro-Pro-Lys-Tyr-Arg-Glu-Leu-Ala- $\text{NH}_2$ ), was used at 0 and 25  $\mu\text{M}$  concentrations. Calpains were activated by the addition of  $\text{CaCl}_2$  via a multichannel pipet to a final concentration of 10 mM. In negative control experiments, buffer without calcium was added instead. All assays were done at a total well volume of 100  $\mu\text{L}$  in 96-well black flat bottom plates (Greiner Bio-One No. 655209). Fluorescence was read in a Tecan M1000 plate reader over 30 min. The excitation wavelength was 330 nm and the emission wavelength was 390 nm. All other settings were as described above.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Additional data for protease screening, detailed peptide synthesis information, HPLC analysis of protease reactions and thiopeptide stability, details of papain kinetic modeling. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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## ■ REFERENCES

- (1) Cory, S.; Adams, J. M. *Nat. Rev. Cancer* **2002**, *2*, 647–656.
- (2) Nicholson, D. W.; Thornberry, N. A. *Trends Biochem. Sci.* **1997**, *22*, 299–306.
- (3) Selkoe, D. J. *Physiol. Rev.* **2001**, *81*, 741–766.
- (4) Visse, R.; Nagase, H. *Circ. Res.* **2003**, *92*, 827–839.
- (5) Edgington, L. E.; Verdoes, M.; Bogoy, M. *Curr. Opin. Chem. Biol.* **2011**, *15*, 798–805.
- (6) Serim, S.; Haedke, U.; Verhelst, S. H. L. *ChemMedChem* **2012**, *7*, 1146–1159.
- (7) Barrett, A. J. *Handbook of Proteolytic Enzymes*; Academic Press: San Diego, CA, 2002.
- (8) Maeda, H. *Anal. Biochem.* **1979**, *92*, 222–227.
- (9) Knight, C. G. *Methods Enzymol.* **1995**, *248*, 18–34.
- (10) Barrett, A. J.; Kirschke, H. *Methods Enzymol.* **1981**, *80*, 535–561.
- (11) Leytus, S. P.; Melhado, L. L.; Mangel, W. F. *Biochem. J.* **1983**, *209*, 299–307.
- (12) Leytus, S. P.; Patterson, W. L.; Mangel, W. F. *Biochem. J.* **1983**, *215*, 253–260.
- (13) Zimmerman, M.; Yurewicz, E.; Patel, G. *Anal. Biochem.* **1976**, *70*, 258–262.
- (14) Butenas, S.; Orfeo, T.; Lawson, J. H.; Mann, K. G. *Biochemistry* **1992**, *31*, 5399–5411.
- (15) Butenas, S.; Ribarik, N.; Mann, K. G. *Biochemistry* **1993**, *32*, 6531–6538.
- (16) Jones, L. J.; Upson, R. H.; Haugland, R. P.; Panchuk-Voloshina, N.; Zhou, M.; Haugland, R. P. *Anal. Biochem.* **1997**, *251*, 144–152.
- (17) Chevalier, A.; Massif, C.; Renard, P. Y.; Romieu, A. *Chem.—Eur. J.* **2013**, *19*, 1686–1699.
- (18) Neefjes, J.; Dantuma, N. P. *Nat. Rev. Drug Discovery* **2004**, *3*, 58–69.
- (19) Shi, L. F.; De Paoli, V.; Rosenzweig, N.; Rosenzweig, Z. *J. Am. Chem. Soc.* **2006**, *128*, 10378–10379.
- (20) Goldberg, J. M.; Batjargal, S.; Chen, B. S.; Petersson, E. J. *J. Am. Chem. Soc.* **2013**, *135*, 18651–18658.
- (21) Goldberg, J. M.; Batjargal, S.; Petersson, E. J. *J. Am. Chem. Soc.* **2010**, *132*, 14718–14720.
- (22) Goldberg, J. M.; Speight, L. C.; Fegley, M. W.; Petersson, E. J. *J. Am. Chem. Soc.* **2012**, *134*, 6088–6091.
- (23) Goldberg, J. M.; Wissner, R. F.; Klein, A. M.; Petersson, E. J. *Chem. Commun.* **2012**, *48*, 1550–1552.
- (24) Yao, S.; Zutshi, R.; Chmielewski, J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 699–704.
- (25) Bartlett, P. A.; Spear, K. L.; Jacobsen, N. E. *Biochemistry* **1982**, *21*, 1608–1611.
- (26) Beattie, R. E.; Elmore, D. T.; Williams, C. H.; Guthrie, D. J. S. *Biochem. J.* **1987**, *245*, 285–288.
- (27) Bond, M. D.; Holmquist, B.; Vallee, B. L. *J. Inorg. Biochem.* **1986**, *28*, 97–105.
- (28) Campbell, P.; Nashed, N. T. *J. Am. Chem. Soc.* **1982**, *104*, 5221–5226.
- (29) Cho, K. *Anal. Biochem.* **1987**, *164*, 248–253.
- (30) Foje, K. L.; Hanzlik, R. P. *Biochim. Biophys. Acta, Gen. Subj.* **1994**, *1201*, 447–453.
- (31) Maziak, L.; Lajoie, G.; Belleau, B. J. *Am. Chem. Soc.* **1986**, *108*, 182–183.
- (32) Mock, W. L.; Chen, J. T.; Tsang, J. W. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 389–396.
- (33) Schutkowski, M.; Jakob, M.; Landgraf, G.; Born, I.; Neubert, K.; Fischer, G. *Eur. J. Biochem.* **1997**, *245*, 381–385.
- (34) Stockel-Maschek, A.; Mrestani-Klaus, C.; Stiebitz, B.; Demuth, H. U.; Neubert, K. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2000**, *1479*, 15–31.
- (35) Angelides, K. J.; Fink, A. L. *Biochemistry* **1979**, *18*, 2363–2369.
- (36) Harris, J. L.; Backes, B. J.; Leonetti, F.; Mahrus, S.; Ellman, J. A.; Craik, C. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7754–7759.
- (37) Melo, R. L.; Alves, L. C.; Del Nery, E.; Juliano, L.; Juliano, M. A. *Anal. Biochem.* **2001**, *293*, 71–77.
- (38) Szabelski, M.; Rogiewicz, M.; Wicz, W. *Anal. Biochem.* **2005**, *342*, 20–27.
- (39) Goll, D. E.; Thompson, V. F.; Li, H. Q.; Wei, W.; Cong, J. Y. *Physiol. Rev.* **2003**, *83*, 731–801.
- (40) Sreenan, S. K.; Zhou, Y. P.; Otani, K.; Hansen, P. A.; Currie, K. P. M.; Pan, C. Y.; Lee, J. P.; Ostrega, D. M.; Pugh, W.; Horikawa, Y.; Cox, N. J.; Hanis, C. L.; Burant, C. F.; Fox, A. P.; Bell, G. L.; Polonsky, K. S. *Diabetes* **2001**, *50*, 2013–2020.
- (41) Tan, Y.; Wu, C.; De Veyra, T.; Greer, P. A. *J. Biol. Chem.* **2006**, *281*, 17689–17698.
- (42) Zatz, M.; Starling, A. *New Engl. J. Med.* **2005**, *352*, 2413–2423.
- (43) Chandramohanadas, R.; Davis, P. H.; Beiting, D. P.; Harbut, M. B.; Darling, C.; Velmourougane, G.; Lee, M. Y.; Greer, P. A.; Roos, D. S.; Greenbaum, D. C. *Science* **2009**, *324*, 794–797.
- (44) Kulkarni, S.; Reddy, K. B.; Esteva, F. J.; Moore, H. C. F.; Budd, G. T.; Tubbs, R. R. *Oncogene* **2010**, *29*, 1339–1350.
- (45) Liang, B.; Duan, B. Y.; Zhou, X. P.; Gong, J. X.; Luo, Z. G. *J. Biol. Chem.* **2010**, *285*, 27737–27744.
- (46) Kelly, J. C.; Cuerrier, D.; Graham, L. A.; Campbell, R. L.; Davies, P. L. *Biochim. Biophys. Acta, Proteins Proteomics* **2009**, *1794*, 1505–1509.
- (47) Jo, H.; Meinhardt, N.; Wu, Y. B.; Kulkarni, S.; Hu, X. Z.; Low, K. E.; Davies, P. L.; DeGrado, W. F.; Greenbaum, D. C. *J. Am. Chem. Soc.* **2012**, *134*, 17704–17713.
- (48) Pietsch, M.; Chua, K. C. H.; Abell, A. D. *Curr. Top. Med. Chem.* **2010**, *10*, 270–293.
- (49) Banala, S.; Sussmuth, R. D. *ChemBioChem* **2010**, *11*, 1335–1337.
- (50) Zacharie, B.; Lagraoui, M.; Dimarco, M.; Penney, C. L.; Gagnon, L. *J. Med. Chem.* **1999**, *42*, 2046–2052.
- (51) Zhang, W.; Li, J.; Liu, L. W.; Wang, K. R.; Song, J. J.; Yan, J. X.; Li, Z. Y.; Zhang, B. Z.; Wang, R. *Peptides* **2010**, *31*, 1832–1838.
- (52) Bagley, M. C.; Dale, J. W.; Merritt, E. A.; Xiong, A. *Chem. Rev.* **2005**, *105*, 685–714.
- (53) Hanna, R. A.; Campbell, R. L.; Davies, P. L. *Nature* **2008**, *456*, 409–U478.
- (54) Torbeev, V. Y.; Myong, S. A.; Ha, T.; Kent, S. B. H. *Isr. J. Chem.* **2011**, *51*, 960–967.
- (55) Gauthier, F.; Moreau, T.; Lalmanach, G.; Brillardbourdet, M.; Ferrerdimartino, M.; Juliano, L. *Arch. Biochem. Biophys.* **1993**, *306*, 304–308.
- (56) Pennington, M. W.; Thornberry, N. A. *Peptide Res.* **1994**, *7*, 72–76.
- (57) Serveau, C.; Juliano, L.; Bernard, P.; Moreau, T.; Mayer, R.; Gauthier, F. *Biochimie* **1994**, *76*, 153–158.
- (58) Batjargal, S.; Wang, Y. J.; Goldberg, J. M.; Wissner, R. F.; Petersson, E. J. *J. Am. Chem. Soc.* **2012**, *134*, 9172–9182.
- (59) Wissner, R. F.; Batjargal, S.; Faden, C. M.; Petersson, E. J. *J. Am. Chem. Soc.* **2013**, *135*, 6529–6540.
- (60) Li, W. X.; West, N.; Colla, E.; Pletnikova, O.; Troncoso, J. C.; Marsh, L.; Dawson, T. M.; Jakala, P.; Hartmann, T.; Price, D. L.; Lee, M. K. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 2162–2167.
- (61) Sevelev, D.; Jiang, P. Z.; Yen, S. H. C. *Biochemistry* **2008**, *47*, 9678–9687.
- (62) Mishizen-Eberz, A. J.; Guttmann, R. P.; Giasson, B. I.; Day, G. A.; Hodara, R.; Ischiropoulos, H.; Lee, V. M. Y.; Trojanowski, J. Q.; Lynch, D. R. *J. Neurochem.* **2003**, *86*, 836–847.
- (63) Choi, D. H.; Kim, Y. J.; Kim, Y. G.; Joh, T. H.; Beal, M. F.; Kim, Y. S. *J. Biol. Chem.* **2011**, *286*, 14168–14177.
- (64) Vlad, C.; Lindner, K.; Karreman, C.; Schildknecht, S.; Leist, M.; Tomczyk, N.; Rontree, J.; Langridge, J.; Danzer, K.; Ciossek, T.; Petre, A.; Gross, M. L.; Hengerer, B.; Przybylski, M. *ChemBioChem* **2011**, *12*, 2740–2744.
- (65) Brun, M. P.; Bischoff, L.; Garbay, C. *Angew. Chem., Int. Ed.* **2004**, *43*, 3432–3436.
- (66) Millholland, M. G.; Chandramohanadas, R.; Pizzarro, A.; Wehr, A.; Shi, H.; Darling, C.; Lim, C. T.; Greenbaum, D. C. *Mol. Cell. Proteomics* **2011**, *10*, No. M111.010678.

(67) Gu, C.; Shannon, D. A.; Colby, T.; Wang, Z. M.; Shabab, M.; Kumari, S.; Villamor, J. G.; McLaughlin, C. J.; Weerapana, E.; Kaiser, M.; Cravatt, B. F.; van der Hoorn, R. A. L. *Chem. Biol.* **2013**, *20*, 541–548.