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A simple fluorescence labeling method for studies of protein oxidation, protein modification, and proteolysis

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ABSTRACT

Proteins are sensitive to oxidation, and oxidized proteins are excellent substrates for degradation by proteolytic enzymes such as the proteasome and the mitochondrial Lon protease. Protein labeling is required for studies of protein turnover. Unfortunately, most labeling techniques involve ^{3}H or ^{14}C methylation, which is expensive, exposes researchers to radioactivity, generates large amounts of radioactive waste, and allows only single-point assays because samples require acid precipitation. Alternative labeling methods have largely proven unsuitable, either because the probe itself is modified by the oxidant(s) being studied or because the alternative labeling techniques are too complex or too costly for routine use. What is needed is a simple, quick, and cheap labeling technique that uses a non-radioactive marker, binds strongly to proteins, is resistant to oxidative modification, and emits a strong signal. We have devised a new reductive method for labeling free carboxyl groups of proteins with the small fluorophore 7-amino-4-methycoumarin (AMC). When bound to target proteins, AMC fluoresces very weakly but when AMC is released by proteinases, proteases, or peptidases, it fluoresces strongly. Thus, without acid precipitation, the proteolysis of any target protein can be studied continuously, in multiwell plates. In direct comparisons, ^{3}H-labeled proteins and AMC-labeled proteins exhibited essentially identical degradation patterns during incubation with trypsin, cell extracts, and purified proteasome. AMC-labeled proteins are well suited to studying increased proteolytic susceptibility after protein modification, because the AMC–protein bond is resistant to oxidizing agents such as hydrogen peroxide and peroxynitrite and is stable over time and to extremes of pH, temperature (even boiling), freeze–thaw, mercaptoethanol, and methanol.

The free radical/oxidative stress field has a long history of papers devoted to lipid peroxidation and DNA oxidation, but protein oxidation and, particularly, altered proteolytic susceptibility have not been studied by very many laboratories. Reasons for this apparent reluctance to measure protein degradation as a consequence of oxidative stress may well include the difficulty, expense, and (even) danger of the available methods. Basically, until now, if one wanted to study how oxidation may change the proteolytic susceptibility of any given purified protein (or mixture of protein substrates), one needed to be willing to use radioactive labels, or tracers. For many laboratories, the complicated protein labeling techniques, radioactive isotope training and licenses or permits, radioactive waste disposal problems, potential dangers to lab workers, and high costs of radioactive techniques have proven to be major barriers to the study of protein oxidation and proteolysis.

The use of ^{3}H and ^{14}C labeling of proteins by in vitro reductive methylation has become the major tool by which to measure the proteolytic degradation of a wide range of protein substrates by purified proteolytic enzymes, cell lysates, and cell extracts. Such ^{3}H- and ^{14}C-labeled protein substrates are also widely used to assess the effects of protein modifications, such as oxidation, denaturation, methylation, acetylation, etc., on proteolytic susceptibility and rates of turnover. In addition, the specificity of various proteolytic enzymes for putative substrates has frequently been tested using ^{3}H- and ^{14}C-labeled proteins [1–17]. The process of *in vitro* reductive methylation with ^{3}H and ^{14}C, however, has many drawbacks. The use of radioactive materials, with all the attendant exposure risks for experimenters and their colleagues, and the difficulties and ethical considerations of radioactive waste procedures rank high on the list of drawbacks. Additionally, the costs both of purchasing radionucleotides and disposing of them are extremely high. Proteolytic assays with ^{3}H- and ^{14}C-labeled protein substrates require a labor-intensive trichloroacetic acid (TCA) precipitation step, so that undegraded (TCA-insoluble) proteins can be separated from TCA-soluble degradation products; this further increases the volume of radioactive waste, limits

Abbreviations: AMC, the fluorophore 7-amino-4-methycoumarin; Hb–AMC, AMC-labeled hemoglobin; BSA–AMC, AMC-labeled bovine serum albumin; MEF, murine embryonic fibroblasts; Hb_{ox}, oxidized hemoglobin; BSA_{ox}, oxidized bovine serum albumin; TCA, trichloroacetic acid; sulfo-NHS-acetate, sulfo-*N*-hydroxysulfosuccinimide acetate.

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the number of samples that may be analyzed, increases experimental error, and forces an absolute endpoint to the assay with the result that true time courses cannot be measured.

Fluorimetric peptidase assays, in which a fluorophore covalently linked to a small peptide sequence is cleaved by a protease/proteinase, provides a solution to all the above radiolabeling problems, and fluorogenic peptides are widely used to measure peptidase activities. Such fluorogenic peptidase measurements are based on the increase in fluorescence as the fluorophore is released from the peptide by proteolytic cleavage. TCA precipitation is not required, thus enabling continuous readings to be made, as well as permitting a greater number of assays to be performed. Although this technology has been highly valuable in measuring the cleavage of short peptide sequences [6,17,18], it is only a primitive model with which to test the activities of complete proteinases, which target whole proteins rather than short peptides. Additionally many proteinases are selective for various modified (e.g., oxidized) forms of their protein substrates, and such selectivity cannot be measured by peptide hydrolysis [19].

A solution would seem to be that of adapting the fluorescence labeling technique for peptides to work with intact proteins, but there has been limited success in modifying this technology to measure the degradation of whole proteins. Two techniques have been described for attaching fluorophores onto proteins. FITC labeling has been used to label casein [20], hemoglobin (Hb) [21], and bovine serum albumin (BSA) [22]. However, FITC-labeled proteins are highly unstable and so must be precipitated and stored in 50% ammonium sulfate and then transferred out of solution, just before use. These steps are major drawbacks and present considerable contamination risks as well as limiting the time over which assays can be performed [22]. The assay is further limited by a strong dependency on pH for the sensitivity of the fluorophore, making assays of strongly acidic proteases such as pepsin, or strongly alkaline proteases such as proteinase K, impractical [23]. In addition, for measuring proteolysis, this technique is, like radiolabeling, limited by the requirement for TCA precipitation, which makes it labor intensive, error prone, and extremely limited to small-size experiments [20]. The second technique involves labeling of either casein or BSA with BODIPY [23]. This technique provides a number of advantages over both FITC labeling and radiolabeling, though it also has several drawbacks. For example, BODIPY has a very small separation between excitation and emission wavelengths (503 nm/512 nm) compared to other fluorophores such as 7-amino-4-methylcoumarin (AMC; 365 nm/444 nm), which makes it extremely difficult to detect the signal without highly specialized equipment. The label is relatively large and complex (389–634 Da, depending on type of BODIPY label) compared to the small [^{3}H]formaldehyde label (32 Da) used in radiolabeling; this raises some concerns about modification of the protein during BODIPY labeling. BODIPY is also relatively expensive for very small quantities, compared with other fluorophores. Finally, there are only a small number of assays for which BODIPY has been described. Thus, most studies of protein degradation continue to rely on in vitro radiolabeling ([^{3}H] or [^{14}C]) of purified protein substrates, using the technique of reductive methylation developed by Jentoft and Dearborn [5].

Although *in vitro* radiolabeling of protein substrates is something we would like to avoid, it occurred to us that reductive methylation remains an efficient and relatively mild procedure by which to attach a label to a protein. In addition, the careful experiments of Jentoft and Dearborn [5] demonstrated the high stability of such adducts, and thousands of studies over the past 30 years have verified the usefulness of reductively methylated protein substrates. We, therefore, set out to test whether we could take the fluorophore AMC, which is a small molecule (MW 175) that is commonly used in the substrates of peptidase activity assays (e.g., Suc-LLVY-AMC), and adduct it to protein substrates by an alternative reductive technique. We were also encouraged by preliminary experiments, which indicated that AMC should be resistant to oxidation by agents such as hydrogen peroxide and peroxynitrite, which are widely used in free radical research. Thus, we attempted to generate stable AMC-labeled proteins by a simple and rapid method that could be used to measure protein degradation by proteolytic enzymes, in diverse studies of protein modification, including exposure to oxidative stress.

Materials and methods

AMC labeling of protein substrates

The protein substrates used for AMC labeling were as follows: hemoglobin from Sigma–Aldrich (St. Louis, MO, USA; Cat. No. H-2500), superoxide dismutase from Calbiochem (San Diego, CA, USA; Cat. No. 574594), catalase from Calbiochem (Cat. No. 219001), and bovine serum albumin from Thermo Fisher (Waltham, MA, USA; Cat. No. BP1605-100). In all cases, 5 mg of protein was dissolved in 1 ml of 0.1 M Hepes buffer to which was added 500 μM AMC (Calbiochem; Cat. No. 164545), as well as 20 mM sodium cyanoborohydride (final concentration) from Sigma–Aldrich (Cat. No. S8628-25 G). Solutions were incubated at room temperature for 2 h and then extensively dialyzed though a 10,000 MWCO centrifugal filter (Millipore, Carrigtwohil, Ireland; Cat. No. 4321), and a buffer exchange was performed with proteolysis buffer (50 mM Tris–HCl, pH 7.8, 20 mM KCl, 5 mM magnesium acetate, 0.5 mM dithiothreitol). Protein content was then determined using the BCA assay kit (Thermo Scientific, Rockford, IL, USA; Cat. No. PI-23225) .

In some experiments samples were pretreated with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (Sigma–Aldrich; Cat. No. E6383-1 G) to block free protein carboxyl groups, with sulfo-*N*-hydroxysulfosuccinimide acetate (Pierce, Rockford, IL, USA; Cat. No. 26777) to block free protein amino groups, or with tryptamine (Sigma–Aldrich; Cat. No. 193747-10 G) to disrupt potential non-covalent interactions in protein hydrophobic pockets.

^{3}H labeling of protein substrates

Tritium-labeled hemoglobin ([^{3}H]Hb) and BSA ([^{3}H]BSA) were generated *in vitro* as previously described [1–4,6] using the [^{3}H]formaldehyde and sodium cyanoborohydrate method of Jentoft and Dearborn [5]. Proteins were then extensively dialyzed.

Cell culture—murine embryonic fibroblasts

Murine embryonic fibroblasts (MEF) from the ATCC (Manassas, VA, USA; Cat. No. CRL-2214) were grown in Dulbecco's modified Eagle's medium (Mediatech, Manassas, VA, USA; Cat. No. 10-013-CV) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA; Cat. No. SH30070.03). Cells were incubated at 37 °C under 5% CO_2 and ambient oxygen. To generate cell lysates, MEF were grown to confluence and then washed twice with PBS, scraped using a cell lifter, and centrifuged at 5000 *g* for 5 min. The cells were then resuspended in proteolysis buffer and subjected to three freeze–thaw cycles at −50 °C. The lysates were then centrifuged at 10,000 *g* for 10 min, after which the supernatants were retained (the pellets discarded) and protein content was determined by BCA assay.

Proteolysis assay—common procedures

Proteolysis was measured by incubation of 1 μg of AMC-labeled protein substrate or ^{3}H-labeled protein substrate in 100 μl of proteolysis buffer containing dissolved trypsin (VWR, West Chester, PA, USA; Cat. No. 100504–332), chymotrypsin (Sigma–Aldrich; Cat. No. C-7762), pepsin (Thermo Fisher; Cat. No. P53), proteinase K (Oncor, Gaithersburg, MD, USA; Cat. No. S4508), purified 20S proteasome (Biomol, Plymouth Meeting, PA, USA; Cat. No. PW8720-0050), or lysate generated from

MEF as above. In each experiment, pH was adjusted appropriately for the proteinase studied, and samples were incubated at 37 °C for 4 h.

Proteolysis of AMC-labeled proteins by fluorescence assay

This procedure was used with AMC-labeled proteins. It should be noted that free AMC is soluble in water and that it fluoresces strongly. AMC adducted to proteins, by reductive methylation, fluoresces only minimally (just enough to detect weakly in gel assays) but when liberated by proteolysis it again fluoresces strongly. During incubations described under Proteolysis assay—common procedures, fluorescence was measured every 10 min at an emission wavelength of 444 nm, with excitation at 390 nm, in a Fluoroskan Ascent microplate fluorimeter (Thermo Fisher; Cat. No. 5210480). Fluorescence emission was compared using a standard curve of the fluorescence of known concentrations of free AMC, between 5 nM and 5 mM, to quantify the moles of AMC released into solution.

Proteolysis of ^{3}H-labeled proteins by radioactive liquid scintillation assay

After incubations described under Proteolysis assay—common procedures, the remaining intact protein was precipitated by addition of 20% trichloroacetic acid and 3% BSA (as carrier) as previously described [2,17,18,24,25]. The percentage protein degraded was estimated by the release of acid-soluble counts into the TCA supernatants, measured by liquid scintillation, in which percentage protein degraded = (acid-soluble counts − background counts) × 100.

SDS and native PAGE gels

For SDS–PAGE gels, samples were mixed with 25% NuPage loading dye (Invitrogen, Carlsbad, CA, USA; Cat. No. NP0007) containing 5% 2-mercaptoethanol. Samples were boiled for 3 min and then added to a 12% Tris–glycine SDS–PAGE gel (VWR, West Chester, PA, USA; Cat. No. 12001–042) and run at 80 V for 2 h. In experiments in which gel fluorescence was analyzed, gels were placed in a chamber and exposed to an excitation wavelength of 365 nm. Silver staining was performed using a SilverSNAP Stain Kit II (Thermo Scientific; Cat. No. 24612), as described in the product manual. For Coomassie staining, gels were incubated in Coomassie stain (0.1% Coomassie Blue R350, 10% methanol, 10% acetic acid) for 30 min and then repeatedly washed in destain solution (10% methanol, 10% acetic acid) until excess stain was removed. In the case of native PAGE gels, samples were mixed with a loading buffer of 25% glycerol/Coomassie brilliant blue solution. Samples were then run on 12% native gels prepared exactly as described in the instructions for preparation of 12% SDS–PAGE gels (Bio-Rad, Hercules, CA, USA; Cat. No. 161–0154), with the exception that 10% SDS was not added to the gel.

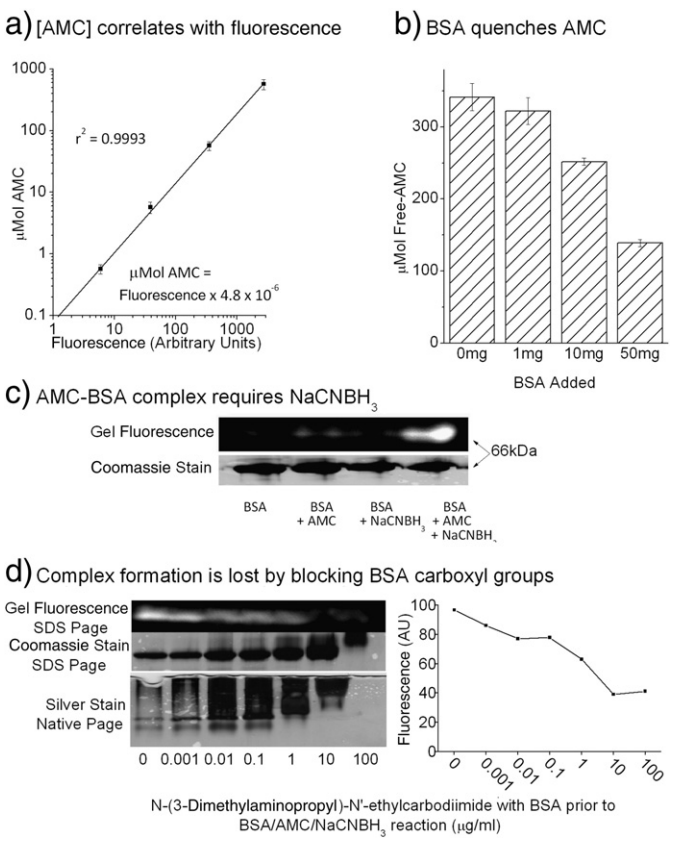

Fig. 1. AMC can be conjugated to free carboxyl groups on proteins. (a) Linear correlation between free AMC concentration, from 100 nM to 1 mM, and fluorescence. Here various concentrations of AMC, dissolved in proteolysis buffer, were incubated at 37 °C on 96-well plates. Fluorescence was analyzed at an emission wavelength of 444 nm, with excitation wavelength of 390 nm. Values are means ± SE, $n = 3$. (b) Addition of increasing amounts of BSA to AMC in the presence of $NaCNBH_3$ progressively quenches the fluorescence of AMC. Here 0–50 mg of BSA was added to 100 μM AMC and 20 mM $NaCNBH_3$ and incubated for 1 h at 37 °C. Free AMC content was determined with reference to a standard curve of known AMC concentrations. Values are means ± SE, $n = 3$. (c) Here 50 mg/ml BSA was incubated with 1 mM AMC in the presence or absence of 20 mM $NaCNBH_3$ and then run on a 12% SDS–PAGE gel. A fluorescent BSA–AMC complex was readily observed at ≈66 kDa (the approximate size of BSA), using an excitation wavelength of 365 nm and an emission wavelength of 444 nm, when all three reagents were present, but could be only faintly discerned in the absence of $NaCNBH_3$. A silver stain was later performed. (d) *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (1 ng/ml to 100 μg/ml), which blocks free carboxyl groups [26], was incubated with 50 mg of BSA for 1 h. BSA was extensively dialyzed and then prepared as for (c). Increasing concentrations of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide caused a progressive decrease in BSA's electrophoretic mobility and loss of fluorescence at 66 kDa; a representative gel is shown to the left, and fluorescence is quantified in the graph to the right.

Results

Reductively binding AMC to protein carboxyl groups

We hypothesized that sodium cyanoborohydride ($NaCNBH_3$), which is commonly used to label proteins with either [^{3}H] or [^{14}C]-linked formaldehyde, could be used to label proteins with AMC by promoting the formation of a carbon–nitrogen bond between the exposed amine group in the AMC molecule and the free carboxyl groups of target proteins (Supplementary Fig. 1).

We observed a linear correlation between the concentration of free AMC in solution and its fluorescence (Fig. 1a); this enabled us to convert fluorescence readings directly to AMC concentrations. We predicted that incubation of AMC with the protein BSA and the reducing agent $NaCNBH_3$ should result in a reductive labeling reaction, in which the AMC label becomes attached to carboxyl groups on the protein. Binding to proteins could be expected to quench AMC fluorescence. To test this we incubated AMC with increasing concentrations of BSA in the presence of $NaCNBH_3$ (Fig. 1b) and saw a BSA concentration-dependent loss of fluorescence.

To determine whether binding was actually occurring, we next ran SDS–PAGE of BSA treated with AMC ± $NaCNBH_3$ (Fig. 1c). A very weakly fluorescent band was observed at the molecular size of BSA (≈66 kDa) when AMC was incubated with BSA, but a much stronger 66-kDa fluorescent band was seen when the protein was reacted with both AMC and $NaCNBH_3$ together. This implies that the binding of fluorophore to protein requires a reductive step. It is also clear that although protein-bound AMC can be detected by fluorescence, the fluorescence yield (brightness) of protein-bound AMC is only a fraction of that seen with free AMC. To test if AMC actually binds to free carboxyl groups, as hypothesized, we incubated 50 mg of BSA with 1 ng–100 μg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide, which effectively blocks exposed carboxyl groups [26]. After 1 h of incubation we extensively dialyzed samples to remove any free *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide and then attempted to react the BSA with AMC and $NaCNBH_3$. Both SDS–PAGE and native gels of BSA showed clear

proof of dose-dependent protein carboxyl-group blocking by *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide, as evidenced by decreased electrophoretic mobility, as the protein became progressively more electropositive with treatment. The same carboxyl-blocking conditions prevented the formation of BSA–AMC adducts, as shown by gradual loss of the fluorescent band at 66 kDa (Fig. 1d and quantified in Fig. 1e).

To test whether exposed amine groups on the protein might react with the carboxyl group on the fluorophore, we used 0.5–50 mM sulfo-NHS-acetate to block exposed amine groups on BSA. Despite blocking the majority (80%) of free amine groups we saw no significant change in the fluorescence of the BSA–AMC complex (Supplementary Fig. 2a). This implies that the complex formed between AMC and BSA is independent of exposed protein amine groups.

Another possibility was that AMC might be sequestered in protein hydrophobic pockets by non-covalent interactions. To test this we performed a competition experiment with tryptamine to compete with AMC for non-covalent binding sites on the protein and measured the effect of tryptamine on quenching of AMC by BSA (Supplementary Fig. 3a). We also tested the ability of the BSA–AMC complex to function as a substrate for proteolysis (Supplementary Fig. 3b). Despite using a 100-fold excess of tryptamine (at which concentration, protein structure was probably disrupted) we were able to block only 30% of the association between AMC and BSA, and tryptamine had minimal effects on the effectiveness of BSA as a proteolytic substrate. These results imply that non-covalent interactions do not play a significant role in AMC binding to proteins.

Next, we incubated Hb with $NaCNBH_3$ alone, AMC alone, or AMC and $NaCNBH_3$ and then extensively dialyzed the samples to remove any free AMC or $NaCNBH_3$. As with BSA–AMC (above) we found that Hb formed a stable adduct with AMC (Fig. 2a). To further test the versatility of the labeling process, we repeated the above experiments using Hb, catalase, and superoxide dismutase as substrates and obtained essentially the same results, generating stable AMC–protein adducts (Fig. 2b).

Utility of AMC-labeled proteins as proteolytic substrates

We next incubated the Hb–AMC substrate with the protease trypsin to determine its usefulness as a proteolytic substrate (Fig. 2a). Trypsin released an extremely large amount of AMC fluorophore from Hb, removing any remaining doubt that the fluorophore had actually been successfully adducted to the protein. Reaction of Hb with AMC alone produced a Hb–AMC proteolytic substrate with high background release of AMC and about a 6-fold increase in AMC liberation after incubation with trypsin. In contrast, use of the full labeling procedure, with $NaCNBH_3$ to increase the strength of the adduct, produced a more stable Hb–AMC proteolytic substrate with only one-sixth the background AMC release, but with an 80-fold increase in AMC liberation after trypsin digestion (Fig. 2a). To test the broad applicability of the AMC labeling technique to measure degradation of proteins in general, we bound the AMC fluorophore to BSA, catalase, hb, and superoxide dismutase and observed that all of the AMC-labeled proteins were effective and sensitive substrates for proteolysis by trypsin, as measured by release of fluorescent AMC (Fig. 2b).

Effective and reliable proteolytic substrates exhibit linear increases in degradation when exposed to linear increases in protease concentration (at least over a fairly wide and useful range) and when substrate concentration is increased in the presence of non-limiting protease activity. To determine the usefulness and reliability of AMC-labeled protein substrates, we assayed AMC release over a wide range of trypsin concentrations and a wide range of substrate concentrations, using Hb–AMC as a model substrate. We observed a linear relationship between proteolytic activity (AMC liberation) and trypsin concentration between 320 nM and 1 mM trypsin concentrations (Fig. 3a) and 25 ng and 2.5 μg of Hb–AMC substrate (Fig. 3b), when plotted using log–log scales. With these results we were able to plot linear regression curves with correlation coefficients close to unity, indicating excellent statistical reliability.

At this point it seemed clear that free AMC is strongly fluorescent, whereas the fluorescence of protein-bound AMC is mostly (but not

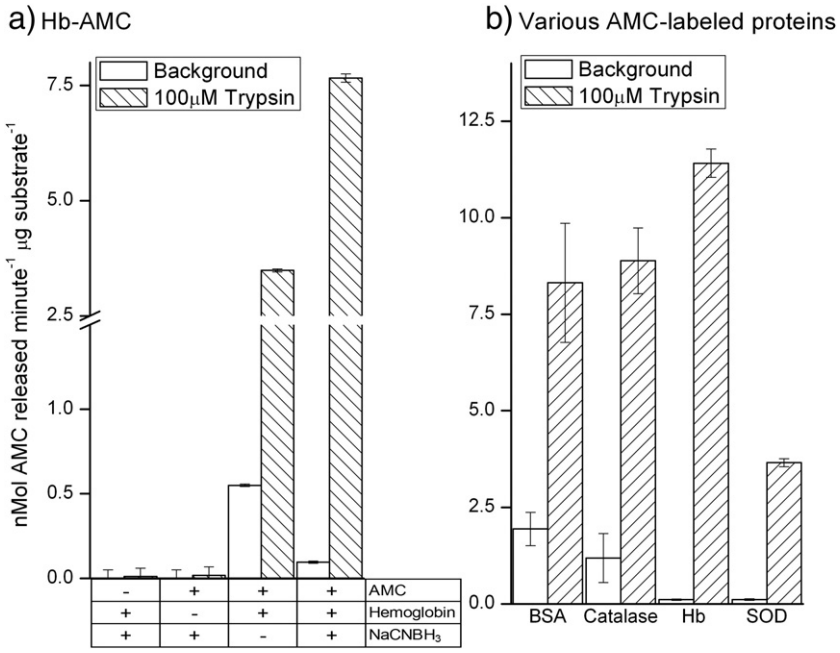

Fig. 2. Proteolysis of AMC-labeled proteins by trypsin. (a) Incubation of 1 mg/ml hemoglobin with 100 μM AMC and 20 mM $NaCNBH_3$ followed by extensive dialysis produced a stable and sensitive substrate for measuring protease activity, in which 10 μg/ml Hb–AMC was combined with 10 μM trypsin. Free AMC content was determined with reference to a standard curve of known AMC concentrations. Values are means ± SE, $n = 3$. (b) AMC labeling of BSA, catalase, Hb, or superoxide dismutase (SOD) generates valid substrates for trypsin digestion, as measured by liberation of fluorescent AMC. All assay conditions (including trypsin concentration) were identical to those in (a), and each substrate protein was used at a final concentration of 10 μg/ml. Free AMC content was determined with reference to a standard curve of known AMC concentrations. Values are means ± SE, $n = 3$.

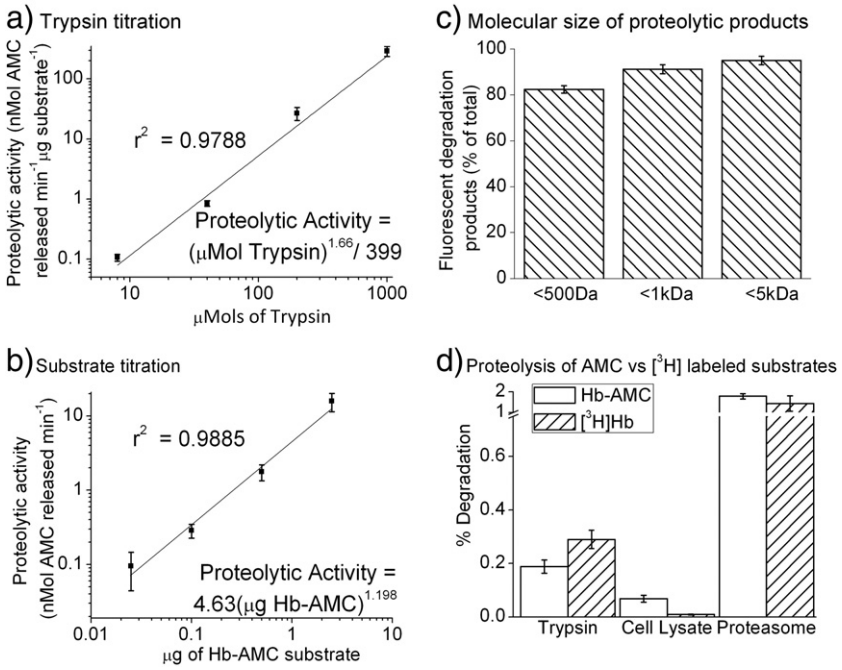

Fig. 3. Protease and substrate titration and particle size of proteolytic degradation products. (a) A linear relationship between the concentration of protease and AMC release is seen at trypsin concentrations between 320 nM and 1 mM, using an Hb–AMC protein concentration of 10 µg/ml. (b) A linear relationship between the concentration of Hb–AMC substrate and proteolytic activity (AMC release) is seen between 25 ng and 2.5 µg of Hb–AMC. All other conditions in both (a) and (b) were as described in the legend to Fig. 2 and, in both, values are means ± SE, $n = 3$. (c) Dialysis of partially digested Hb–AMC substrate shows that the majority of liberated fluorescent AMC products consist of particles smaller than 500 Da. For this experiment, Hb–AMC (10 µg/ml) was incubated with 10 µM trypsin at 4 °C for 24 h in dialysis tubing, to generate sufficient fluorescent products to measure but also to preclude complete digestion of the substrate. Values are means ± SE, $n = 4$, for which the fluorescence of controls was subtracted. (d) Hb was labeled with AMC, or with tritium, by reductive labeling in both cases, as described under Materials and methods. Protein degradation was measured in (a) by AMC fluorescence and in (b) by release of acid-soluble ^{3}H counts by liquid scintillation, as described under Materials and methods. Background fluorescence or radioactivity was measured in the absence of protease (proteolysis buffer alone), and proteolysis was measured by increased fluorescence or acid-soluble radioactivity after incubation with 10 µM trypsin, 1 µg/ml purified 20S proteasome, or 150 µg/ml MEF cell lysate. Percentage degradation of Hb–AMC is reported as the percentage of total fluorescence that could be released from Hb–AMC after exhaustive proteolytic digestion (not shown), whereas percentage degradation of [^{3}H]Hb is reported as the percentage of total (initial) radioactive counts released into TCA-soluble form by proteolysis. All values are means ± SE, $n = 3$.

completely) quenched, and that trypsin-mediated AMC release from AMC-labeled proteins reflects protein degradation. We next wanted to determine the size(s) of protein–AMC degradation products that actually produce fluorescent signals. To study this we partially digested a sample of Hb–AMC. We then dialyzed the sample through <5-kDa, <1-kDa, and <500-Da size-exclusion membranes into a 500× volume of proteolysis buffer. Dialysis through a 500-Da filter caused an ≈80% reduction in signal, compared to an ≈90% reduction with a 1-kDa filter and an ≈95% reduction with a 5-kDa filter (Fig. 3c). From this we concluded that the majority (80%) of fluorescent products are smaller than 500 Da, whereas another 15% are particles between 500 Da and 5 kDa, and only some 5% of the signal comes from peptides larger than 5 kDa. These results seem quite consistent with proteolysis assays using radiolabeled protein substrates, in which a TCA precipitation step is routinely used to precipitate remaining intact protein, and peptides larger than about 5 kDa, so that soluble radioactivity reflects free amino acids and only very small peptides [25].

We also considered it important to directly compare the sensitivity of proteolytic measurements using the AMC-labeled substrates we generated with that of traditional radiolabeled substrates [5]. Thus, we assessed the degradation of Hb–AMC versus [^{3}H]Hb after incubation with various, widely studied proteolytic systems. Our results reveal broadly comparable sensitivity for both substrates, with trypsin, MEF cell lysates, and purified 20S proteasome (Fig. 3d).

Stability of AMC-labeled proteins and resistance to denaturing agents

The stability of AMC-labeled substrates, the resistance of the AMC–protein linkage to various treatments, and the reproducibility of proteolytic assays after prolonged storage are important concerns in weighing the usefulness of our technique. To begin to test these matters, we stored Hb–AMC at −20 °C and then periodically thawed samples and analyzed both their background release of free AMC (representing undesirable breakdown of the complex) and their proteolytic susceptibility during incubation with trypsin. In repeated trials over 150 days, both the background AMC release and the trypsin-induced release of AMC varied by less than 15%, indicating that the substrate was quite stable and that samples can be stored for long periods of time without significant changes in proteolytic susceptibility (Fig. 4a). As a harsher test of substrate stability we subjected Hb–AMC to repeated freeze–thaw cycles and then measured background release of free AMC (Fig. 4b). This did not significantly affect the stability of the Hb–AMC complex.

We started this project because we wanted to find a new way to label proteins for studies of oxidation-induced changes in proteolytic susceptibility. In addition to oxidants, proteolytic substrates are often subjected to various other modifying or denaturing conditions, to test for effects on proteolytic susceptibility, so we considered it important to test the stability of AMC-labeled substrates over a range of harsh conditions. Hb–AMC was almost completely stable to incubation in 1 mM H_2O_2, 1 mM peroxynitrite, dilute HCl at pH 4, 10% 2-mercaptoethanol, freeze–thaw at −80 °C, or exposure to 50% methanol. Even boiling (100 °C) for 60 min caused only a 3.1% breakdown of the Hb–AMC complex (Fig. 4c).

Use of AMC-labeled protein substrates with acidic, neutral, and alkaline proteases

Whereas many proteolytic enzymes have pH optima in the neutral to slightly alkaline range, others are “designed” to function under strongly acidic or alkaline conditions. We, therefore, needed to test both the fluorescence properties of free AMC over a wide pH range and the stability of

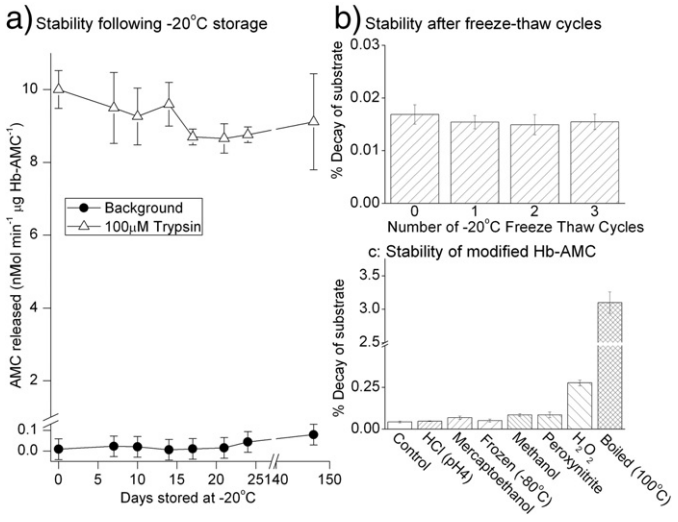

Fig. 4. Stability of AMC-labeled hemoglobin after frozen storage or denaturation. (a) Hb–AMC was stored at −20 °C for up to 21 weeks. At various time points, samples were thawed, and measurements of both background fluorescence (release of free AMC from the Hb–AMC complex) and liberation of fluorescent AMC by proteolytic digestion with trypsin were made, as described for Fig. 3a. (b) The stability of Hb–AMC was tested with repeated −50 °C freeze–thaw cycles, by measuring release of free AMC from the Hb–AMC complex (background fluorescence). (c) Hb–AMC was incubated for 60 min in dilute HCl at pH 4, 10% 2-mercaptoethanol, 70% methanol, 1 mM peroxynitrite, or 1 mM H_2O_2 or was boiled at 100 °C for 60 min or was subjected to freeze–thaw at −80 °C. Release of free AMC from the Hb–AMC complex (background fluorescence) was then measured in comparison with control (untreated Hb–AMC). Values are means ± SE, $n = 3$.

protein–AMC complexes. The fluorescence of free AMC was unaffected by mildly acidic or alkaline conditions in a broad range from pH 3 to 11; highly acidic (below pH 2) or alkaline (above pH 11) conditions, however, significantly decreased AMC fluorescence (Fig. 5a). It should be noted that the fluorescence-quenching effects of strong acid or base were completely reversed, with AMC fluorescence returning to normal levels, when pH was neutralized (not shown, but evident in the experiments of Fig. 5b below).

We next wanted to determine the stability of protein–AMC adducts over the same broad range of pH. For these experiments, Hb–AMC was incubated for 4 h, using the same pH conditions as for Fig. 5a, after which the pH of each sample was readjusted to pH 7.8 to assess the stability of the Hb–AMC complex, independent of any possible quenching effects of pH on the fluorophore. We found that the Hb–AMC complex was highly stable over the entire range from pH 1 to 12, with less than a 0.2% decrease in stability observed under any condition (Fig. 5b). We next wished to test the viability of protein–AMC complexes as substrates for proteases with widely different pH optima. As shown in Fig. 5c, Hb–AMC proved to be an excellent substrate for proteolysis with enzymes as diverse as pepsin at pH 2, proteinase K at pH 11, and trypsin or chymotrypsin at pH 7.8.

Use of AMC labeling to detect the preferential degradation of modified proteins

Whereas digestive enzymes such as trypsin, chymotrypsin, and elastase are very efficient at degrading both normal and modified proteins, major intracellular proteolytic enzymes, such as the proteasome [1,17] and the mitochondrial Lon protease [27] exhibit little activity against normal proteins, while avidly degrading their modified or damaged forms. The landmark paper of Jentoft and Dearborn [5] demonstrated that reductive methylation is a relatively mild treatment and their work, backed up by thousands of studies by other researchers in the past 30 years, has verified that radiolabeling proteins (by reductive methylation) generates protein substrates that are not extensively modified or denatured. Despite the small size of the AMC fluorophore, we had to be concerned that AMC labeling of proteins might cause a degree of denaturation that would increase the proteolytic susceptibility of normal proteins, making it harder to determine if intentional (experimental) modifications to proteins, such as oxidation, affect their degradation. For a labeling technique to be useful in this regard, one would hope to see only minor degradation of the "normal" labeled protein

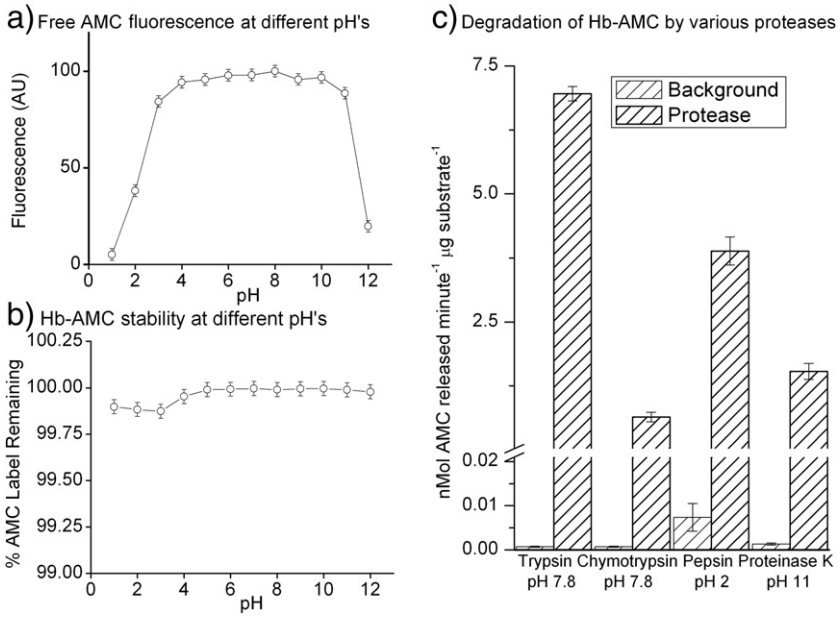

Fig. 5. pH profile of fluorescence, stability, and proteolytic susceptibility of free AMC and Hb–AMC. (a) The fluorescence of free AMC was measured in proteolysis buffer over a wide range of pH conditions. (b) Samples of Hb–AMC were incubated over a range of pH conditions for 4 h. The pH of each sample was then adjusted to pH 7.8 and AMC fluorescence was measured. Results are expressed as a percentage of total AMC label originally incorporated into the Hb–AMC complex, which was (separately) assessed by exhaustive proteolytic digestion of Hb–AMC, by incubation with 500 µM trypsin for 4 h. (c) Hb–AMC was incubated with 100 µM trypsin, 10 µM chymotrypsin, 100 µM pepsin, or 100 µM proteinase K (at the pH shown for each protease) for 4 h at 37 °C and proteolysis was measured by AMC release, as described in the legend to Fig. 3a. Values are means ± SE, $n = 3$.

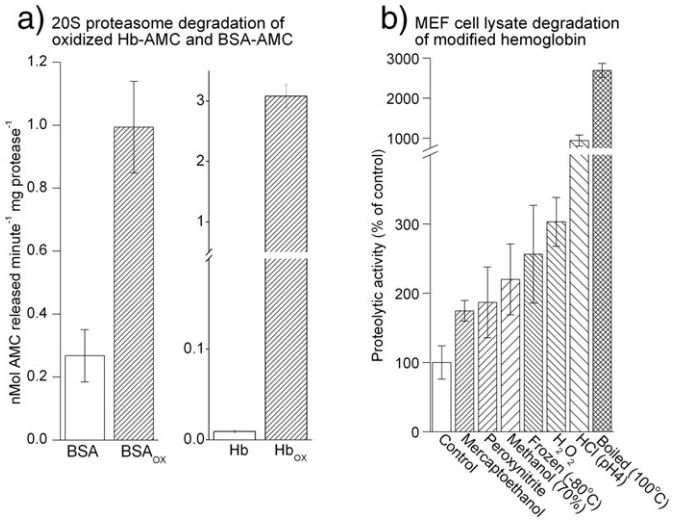

Fig. 6. Proteolytic susceptibility of modified AMC-labeled proteins. (a) The capacity of the 20S proteasome to degrade both the native and the oxidized forms of Hb–AMC and BSA–AMC was measured. For both assays, 1 μg/ml of purified 20S proteasome was combined with 10 μg/ml Hb–AMC, Hb_{ox}–AMC, BSA–AMC, or BSA_{ox}–AMC and incubated for 4 h at 37 °C. Protein degradation was then measured as per Fig. 3. Hb_{ox}–AMC and BSA_{ox}–AMC were prepared by treating Hb–AMC and BSA–AMC with 1.0 mM H_2O_2 followed by extensive dialysis. (b) The capacity of MEF cell lysates to degrade various modified forms of Hb–AMC was measured. Hb–AMC was modified by incubation with dilute HCl at pH 4, 10% 2-mercaptoethanol, 70% methanol, 1 mM peroxynitrite, or 1 mM H_2O_2 or was boiled at 100 °C for 60 min or was subjected to freeze–thaw at −80 °C. The substrates were then extensively dialyzed and incubated with 150 μg/ml MEF cell lysates for 4 h. Values are means ± SE, $n = 3$.

but significantly increased degradation of a suitably modified or denatured form by intracellular proteases.

To test this we incubated both control and oxidized forms of Hb–AMC and BSA–AMC with purified 20S proteasome, which selectively degrades oxidized proteins [1,2,19,25]. Our results show that the unoxidized forms of BSA–AMC and Hb–AMC were rather poor substrates for the purified proteasome, but BSA–AMC's susceptibility to proteasomal degradation increased some 4-fold after mild oxidation with H_2O_2, whereas that of Hb–AMC increased by more than 300-fold (Fig. 6A). We additionally tested oxidation of Hb–AMC by peroxynitrite and a number of other protein-denaturing treatments including boiling, freezing, low pH, methanol, and 2-mercaptoethanol. Both untreated (control) Hb–AMC and the variously treated Hb–AMC samples were then incubated with lysates of MEF for measurements of proteolysis. Cell lysates and extracts (which contain proteasome and many other intracellular proteolytic enzymes) are widely employed in many studies of intracellular proteolytic susceptibility [4,17,19,28]. Oxidative modification of Hb–AMC, by H_2O_2 or peroxynitrite, significantly increased its degradation during (subsequent) incubation with MEF cell extracts, in comparison with unmodified (control) Hb–AMC; similar results were also obtained with other methods of Hb–AMC modification, including boiling, freeze–thaw, or exposure to HCl, methanol, or mercaptoethanol (Fig. 6b).

Discussion

This article describes a novel technique for *in vitro* protein labeling that is free of radioisotopes. Although our technique contains a reductive step, it is quite distinct from the radiolabeling procedure originally described by Means and Feeney [29] and then subsequently adapted by Rice and Means [30] and Jentoft and Dearborn [5], in which either [^{14}C] or [^{3}H]formaldehyde forms a covalent linkage with free amino groups on target proteins, using the reducing agent $NaBH_4$ or its milder variant $NaCNBH_3$. In our method, the fluorophore AMC is reductively ($NaCNBH_3$) conjugated with free protein carboxyl groups, and no methylation step is involved.

We have described a novel technique by which an inexpensive and stable AMC fluorophore–protein complex can be formed both quickly and simply by reductively adducting AMC to free carboxyl groups. We go on to demonstrate that this technique is applicable to a wide range of protein substrates and that it can be used to measure proteolytic susceptibility with high sensitivity, comparable to that achieved with radiolabeled proteins. Finally, we show that AMC–protein adducts are stable to oxidation and various other denaturing conditions and can be used to measure the increased proteolytic susceptibility of oxidatively modified proteins, as well as proteins modified by other denaturing treatments. In addition to their utility as proteolytic substrates, AMC-labeled proteins could also be used for any other project requiring sensitive detection of stably labeled proteins.

AMC labeling seems to generate substrates that are comparable to [^{3}H] or [^{14}C]-labeled proteins in terms of versatility, stability, and reproducibility and that have several advantages over radiolabeling in terms of safety, labor, and cost. Radioisotopes can be hazardous to use and costly to store or discard and require complicated and time-consuming training and use permits. Proteolysis assays with radiolabeled substrates require an acid precipitation and centrifugation step (to precipitate undegraded proteins) before sample supernatants are transferred to scintillation vials to quantify [^{3}H] or [^{14}C] release. These steps are highly work intensive and error prone, are a limit to sample numbers, and preclude continuous monitoring of individual samples over time. In comparison, fluorescence assays with AMC-labeled proteins can be easily performed on 96-well plates, with no TCA precipitation or centrifugation and with continuous monitoring of proteolytic activity over (real) time.

AMC is relatively cheap, compared with radiolabeled formaldehyde. This makes the labeling process approximately 40 times cheaper than [^{3}H] or [^{14}C] labeling (based on label usage in Fig. 3c). The labeling procedure is also fast and easy and requires no specialized equipment or training. These factors will now make it feasible for researchers to generate, store, and study whole libraries of labeled protein substrates. Finally, AMC's fluorescence properties and the AMC–protein bond are stable to oxidation, boiling, freezing, and other modifying or denaturing conditions, whereas the protein itself can still be modified. Thus AMC-labeled proteins can be used to measure changes in proteolytic susceptibility after oxidation or any number of other protein-modifying treatments.

Supplementary materials related to this article can be found online at doi:10.1016/j.freeradbiomed.2011.08.018.

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