Nucleophile Labeling of Cysteine and Serine Protease Substrates*

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Dipeptides containing fluorescein or biotin have been incorporated into proteolytic substrate cleavage products of bovine serum albumin generated by human cathepsin S or neutrophil elastase and into a fragment of the 31-kDa interleukin 1β precursor by human interleukin 1β-converting enzyme. Incorporation of the nucleophile is blocked by prior inhibition of the enzymes, and is not seen when proteolysis occurs in the absence of label, and the protease is then inhibited before the addition of label. Labeling is dependent on the pH, the time of reaction, and the concentrations of the nucleophile and substrate. Labeling of proteins can be readily detected by SDS-polyacrylamide gel electrophoresis. The pattern of elastase-labeled bovine serum albumin bands differs among P1/P2-CONH-R-P1′ and Gly, suggesting that nucleophilic attack on acyl enzyme intermediates derived from a large protein may differ from attack on small intermediates. The only observed labeled fragment catalyzed by interleukin 1β-converting enzyme is fragment 28–116 from the interleukin 1β precursor, suggesting that the cleavage between residues 27 and 28 is at least as efficient as between residues 116 and 117. This labeling method does not require organic solvent or nonphysiological pH values and thus may be useful for the discovery of novel protease substrates in cells or other in vivo systems or for diagnostic applications.

1. P2-CONH—CH(R)·P1′ + HO·Ser-protease → P2-CONH—CH(R)—NH·P1′
strate

2. Tetrahedral intermediate → P2-CONH—CH(R)—COO·Ser-protease + P1′
acyl enzyme intermediate product 1

3. Acyl enzyme intermediate + H2O → P2-CONH—CH(R)—COOH + HO·Ser-protease
nucleophile label labeled product 2

4. Acyl enzyme intermediate + H2N-label → P2-CONH—CH(R)—CONH-label + HO·Ser-protease

Scheme 1

Proteases play a central role in a variety of processes, including the degradation of cellular constituents in lysosomes (1), tumor invasion and metastasis (2, 3), prohormone or protein processing (4, 5), control of viral pathogenicity (6), shedding of receptors from cell surfaces (7), or the processing of cytokines such as interleukin 1β (8). The identification of the physiological substrates of a protease may be important for a complete understanding of the function of the protease.

The identification of small peptide substrates or protease substrate mapping has been approached by a variety of techniques. More recently, phage selection techniques have been used to screen for small peptide substrates of proteases (9, 10). The identification of proteins cleaved by proteases is more difficult. This has been approached by direct examination of an isolated substrate before and after proteolysis or by the application of analytical methods such as two-dimensional gels to more complex mixtures to follow changes in specific proteins spots due to proteolysis (see Ref. 11 or 12 for examples).

The detection of protease substrates is rendered more difficult if the substrates are unknown. A sensitive method of detection that allows direct visualization of substrates without the background of other proteins may be useful for a rapid assessment of the rate and extent of proteolysis in complex mixtures, as a diagnostic method, or for substrate identification.

Peptide nucleophiles have been used previously with proteases for peptide synthesis, which is optimal when done in high levels of organic solvent (13). Other uses include the study of the aminolysis of acyl enzyme intermediates to examine structural features important in the $S_i′$ site of human leukocyte elastase (14) and to map the $S_i′$ subsite specificity of trypsin and chymotrypsin (15).

In this paper, we examine the feasibility of the use of peptide nucleophiles for the direct visualization of protease substrates, as shown in Scheme 1.

In this scheme, the acyl enzyme intermediate (in the case of a serine protease) or thioacyl enzyme intermediate (in the case of a cysteine protease) undergoes competing reactions with 55 mM water (hydrolysis to give product 2 (Reaction 3)) or with −1 mM labeled nucleophile (in this case aminolysis to give a labeled product 2 (Reaction 4)). Hydrolysis is expected to predominate by the principle of mass action. Much lower levels of labeled substrate may not be problematic for detection and identification if the detection methodology is adequately sensitive.

Such a process would be most useful if achieved at physio-
Logical pH without the requirement of organic solvent or unusual temperatures. Using fluorophoric or biotinylated nucleophiles, we have examined a number of parameters affecting label incorporation into single protein substrates of the cysteine protease cathepsin S (EC 3.4.22.27), the serine protease human neutrophil elastase (EC 3.4.21.37), the specific cysteine protease ICE1 (EC 3.4.22.36), and into multiple protein substrates in a complex mixture. The results demonstrate the use of peptide nucleophile incorporation with large protein substrates and demonstrate the usefulness of the method under conditions suitable for in vivo labeling.

EXPERIMENTAL PROCEDURES

Materials—Electrophoretically pure BSA, leupeptin, and E-64 were obtained from Sigma. Human neutrophil elastase was obtained from Calbiochem (La Jolla, CA), and human cat S was expressed and purified as described.1 ICE was a generous gift of Dr. Nancy Thornberry (Merck). pIL-1β was obtained from Cistron, Inc. (Pinebrook, NJ), and a mouse monoclonal antibody binding mature human IL-1β was from R&D Systems Inc. (Minneapolis, MN). Biotin-NHS ester was from Sigma. Avidin-horseradish peroxidase and the LC-ROP Supersignal system were from Pierce. Cascade Blue hydrazide and lissamine rhodamine B sulfonylhydrazine were from Molecular Probes (Eugene, OR). All other chemicals were of reagent grade or better. Prepoured 4–20% gradient SDS-gels and molecular mass standards, which contained 6- and 17-kDa proteins labeled with fluorophores for visibility on fluorogram images, were from Novex. Visualization of incorporated fluorophores was with a CCD camera and software (the FACES imaging system, version 2.30) from Glyko, Inc. (Novato, CA). This method monitors fluorescent bands with excitation at 360 nm and emission at 420 nm and also records a digital image of Coomassie Blue-stained bands using a bright-field background from a fluorescent plastic sheet inserted behind the gel to be imaged. This system was also used to integrate the intensity of the gel bands. It is not optimized for fluorescein-labeled dipeptides, which have excitation and emission maxima of 490 and 515 nm, but gave adequate detection of such labeled bands. Gels used for imaging were stained with Coomassie Blue R-250 immediately after imaging so that labeling could be directly compared to total proteolysis.

Nuclearolipid Labeling of Protease Substrates

Labeling with the Cysteine Protease Cathepsin S—Fig. 1 shows a comparison of labeling of BSA proteolysis products with cat S by Ala-Ala-fluorescein and Ala-Ala-biotin. These dipeptide nucleophiles were used since cat S prefers Ala in the P1 position of small peptide substrates over 11 other tested residues (18) and since for acyl transfer the most efficient dipeptide observed by Schuster et al. (19) for papain was Ala-Ala-NH2. In Fig. 1A, the fluorescence-labeled bands (left gel) are compared to the Coomassie-stained gel (right gel). Lane 1 shows that no bands are observed when BSA is incubated with label alone for 60 min in the absence of protease. In lane 2, BSA was incubated with cat S for 30 min, IAA was added to 1 mM and reacted for 30 min to block the enzyme, and then label was added for 30 min. While the proteolysis is apparent in the right gel by Coomassie staining, no fluorescent bands were observed (left gel). In lanes 3 and 4, cat S was first blocked with 1 mM IAA and 29 mM leupentin, respectively, for 30 min, and BSA was added and incubated for 30 min, followed by addition of label for a further 30 min. No fluorescent bands were observed. Lanes 5–9 show a 1–60 min time course of labeling when BSA, cat S, and Ala-Ala-fluorescein are present. Multiple bands below BSA are labeled. Comparison of the Coomassie-stained gel (Fig. 1A, right gel) shows no evidence for proteolysis in the absence of cat S (lane 1) or in the presence of inhibitors (lanes 3 and 4), and proteolysis where expected (lanes 2 and 5–9).

In Fig. 1B, the labeling nucleophile was Ala-Ala-biotin, which was detected after blotting by avidin-horseradish peroxidase. In lane 2, 1 mM Ala-Ala-biotin was added to BSA. A small band is detected at the position of unproteolyzed BSA. In lane 3, BSA was incubated with cat S for 30 min, 1 mM of the inhibitor E-64 was added for 1 min, and Ala-Ala-biotin was then added for an additional 30 min. The same faint band at the molecular mass of intact BSA was observed. In lane 4, BSA, cat S and Ala-Ala-biotin were incubated for 30 min. A smear of bands at and below intact BSA are seen, down to an apparent molecular mass just above 30 kDa. The Coomassie-stained gel is showed on the right of Fig. 1B. As expected, proteolysis of BSA is observed in lanes 3 and 4.

If labeling involves nucleophilic attack on the acyl enzyme intermediate followed by incorporation into the resulting NH2-
terminal fragment of the substrate, this should be enhanced as the pH increases above the $pK_a$ of the NH$_2$ terminus of the label. It may also reflect the pH dependence of catalysis by cat S, which has a bell-shaped pH rate profile with apparent $pK_a$'s of 4.5 and 7.8 (21). Fig. 2 shows the pH dependence of BSA product labeling by cat S. Lanes 1, 3, 5, 7, 9, 11, and 13 contain samples from the reaction of BSA, cat S, and Ala-Ala-fluorescein for 1, 5, 10, 30, and 60 min. Right gel, lanes and experiments are identical with those of the left gel, except bands are detected by Coomassie Blue staining. B: Left gel, detection of nucleophile labeling of 1.6 $\mu$g of BSA by 1 $\mu$M Ala-Ala-biotin and cat S after electroblotting onto nitrocellulose. Detection is with avidin-horseradish peroxidase and a luminol-based substrate. Lane 1, BSA alone; lane 2, BSA mixed with Ala-Ala-biotin, showing a small amount of binding to the BSA; lane 3, BSA reacted with cat S for 30 min, blocked by 1 $\mu$M E-64 for 1 min, and then exposed to Ala-Ala-biotin for 30 min. Only a small amount of binding of Ala-Ala-biotin appears with the inhibited enzyme. Lane 4, reaction of BSA, cat S and Ala-Ala-biotin for 30 min. Label is incorporated into a number of bands by active cat S. Right gel, Coomassie Blue-stained gel with the same experiments and lane numbering except that 20 $\mu$g of BSA are digested and added to each lane. Note that the left gel is expanded relative to the right one. The molecular mass standards are shown at the right for both all gels.

Due to the limited proteolysis of BSA, a discrete number of bands are visible. If nucleophile labeling is 100% efficient, one might expect that one-half of the bands are labeled. In Fig. 2, above the free label at the bottom of the left gel, about six fluorescent bands are seen. This is one-half the number seen by Coomassie staining (right gel).

Fig. 3 shows the nucleophile concentration dependence of the labeling of BSA proteolysis products with cat S and Ala-Ala-fluorescein. In this experiment, the label, dissolved in dimethylformamide, was first dried in the Eppendorf tubes used for the reaction so that increasing levels of dimethylformamide did not affect the enzyme. In lanes 1–10 of the left gel, labeling increased from 50 $\mu$M to ~1.6 $\mu$M nucleophile and then decreased, with little labeling evident at 13 or 26 $\mu$M dipeptide. Fitting the concentration dependence of the integrated intensity of the 42- and 28-kDa bands in Fig. 3 (left gel) to a single hyperbola (not shown) suggests a $K_{1/2}$ for labeling of 0.29 and 0.37 $\mu$M, respectively. The corresponding Coomassie-stained
gel (right) shows that proteolysis was blocked by levels of Ala-Ala-fluorescein above 1.6 mM, suggesting inhibition of cathepsin S. Results in lanes 12 and 13 show that 1 mM Ala-Ala-fluorescein did not bind to either BSA (lane 12) or to its proteolysis products (lane 13). Labeling by cat S was attempted with different dipeptide nucleophiles to examine the relative importance of different P₁₉ residues for the labeling of protein substrates significantly larger than small peptides often used for such studies. Menard et al. (18) have reported that cat S prefers P₁₉ Ala over Phe by about 5-fold, as measured by the value of \( k_{cat}/K_m \) for the substrate dansyl-Phe-Arg-X-Trp-Ala. Fig. 4 (left gel) shows the results of labeling with (1 mM each) Phe-Ala-fluorescein (lanes 1–3), Ala-Ala-fluorescein (lanes 4–6), Ala-Pro-fluorescein (lanes 7–9) and Gly-Gly-fluorescein (lanes 10–12). In each experiment, the first lane contains cat S and BSA, and the second lane contains cat S and BSA reacted for 30 min, blocked by 40 \( \mu M \) E-64 for 1 min, followed by the addition of the dipeptide nucleophile. The third lane contains cat S, BSA, and label without inhibitor. The dipeptide nucleophile with P₁₉ Phe gives more intense labeling than P₁₉ Ala, which is better than P₁₉ Gly. Ala-Pro-fluorescein gives only a single faint band.

To examine labeling in a complex mixture of proteins, cat S was added to E. coli proteins. Fig. 5 (left gel) shows the time course resulting from incubation of 0.5 \( \mu g \) of cat S with 54 \( \mu g \) of E. coli proteins and 1 mM Phe-Ala-fluorescein in pH 8.0, 0.1 M borate buffer. Lanes 1–10 show the time course for 1 to 30 min, with the label intensity increasing in a number of bands, especially those in the 6–10-kDa range, with time. Lane 13 shows the results of incubation of E. coli proteins with 1 mM Phe-Ala-fluorescein without cat S. No bands of intensity comparable with that occurring in the presence of protease were seen. Lanes 10–12 show the effect of buffer concentration, with a little less labeling in 20 mM borate than in 60 or 100 mM borate. In lane 14, the E. coli proteins were added to cat S in the absence of Phe-Ala-fluorescein.

If only some of the E. coli proteins are substrates for cat S, it might be expected that label would be incorporated into only...
some of the gel bands. The time course observed by Coomassie staining would also be less obvious than that seen by fluorescence, and the pattern of fluorescent gel bands might differ from the Coomassie staining pattern. Comparison of the fluorescent-labeled gel before (Fig. 5, left gel) and after Coomassie staining (Fig. 5, right gel) is consistent with cat S substrate selectivity.

Labeling with Human Neutrophil Elastase—Labeling of BSA proteolysis products was also examined using human neutrophil elastase. Studies on the aminolysis of acyl enzymes by peptide nucleophiles have suggested that side chain hydrophobicity of the P1' residue of the nucleophile may be more important than specific structural features and that there may be no important binding interactions beyond the P1' residue (14). Thus the dipeptide nucleophile Phe-Ala-fluorescein was used for a number of preliminary labeling experiments.

Fig. 6 shows substrate labeling catalyzed by elastase with a number of different dipeptide nucleophiles. Lanes 1–3 show labeling of 20 μg of BSA by 0.1 or 1 μg of elastase. After digestion of BSA by elastase was finished, the enzyme was blocked by 1 mM phenylmethanesulfonyl fluoride for 20 min, and different dipeptide-fluorescein labels were subsequently added. No labeled bands were seen (lanes 3, 6, 9, and 12). Higher molecular mass bands were seen with 0.1 μg of enzyme (lanes 2, 5, 8, and 11), and lower bands seen with 1 μg of elastase (lanes 1, 4, 7, and 10). In lanes 1–3, 4–6, 7–9, and 10–12, this experiment was repeated with 1 mM Phe-Ala-, Ala-Ala-, Ala-Pro-, and Gly-Gly-fluorescein, giving defined bands but a slightly lower amount of label incorporation. In lanes 7–9, 1 mM Ala-Pro-fluorescein was used, giving fewer discrete bands. The dye appears to be smeared toward the bottom of the gel in each lane. The presence of Pro in the P2' position thus appears to decrease labeling. In lanes 10–12, the use of Gly-Gly-fluorescein results in significant incorporation into several bands. In all cases, bands were not labeled when elastase was exposed to phenylmethanesulfonyl fluoride before a dipeptide nucleophile was added, the bands appear weaker than for labeling by cat S. Fig. 6 (right gel) confirms that use of a higher level of elastase results in fewer higher molecular mass bands and more lower molecular mass bands. This is consistent with increased proteolytic degradation of the larger fragments with increased amounts of elastase.

Labeling of Interleukin 1β Precursor by ICE—ICE is a cysteine protease that requires a P1 Asp for proteolysis, and it can cleave before P1' Ala or Gly in the IL-1β precursor (20). Due to its narrow substrate specificity, ICE was examined for labeling of one natural substrate, the IL-1β precursor protein. It is cleaved between Asp 116 and Ala 117 and between Asp 27 and Gly 28 (8).

Fig. 7 shows the results from a labeling experiment, in which...
substrate labeling by Ala-Ala-biotin (bottom gel, lanes 5–9) was compared with detection by a monoclonal antibody against mature IL-1β (top gel). A third method of detection utilized labeling of amino groups of proteins by the addition of biotin- NHS ester (bottom gel, lanes 2–4). In lane 1 of the top gel, pIL-1β and mature IL-1β were added as standards without ICE. Lanes 2–4 show a labeling time course when ICE was added to pIL-1β with detection by biotin-NHS ester (bottom gel). Five bands (bands A, B’, C, D, and E in the bottom gel) of apparent molecular masses, based on molecular mass standards, of 31.5, 24, 17.5, 12, and 9.3 kDa, were labeled. The bands at apparent molecular masses of 24 and 9.3 kDa (bands B’ and E) appear to be contaminants of pIL-1β, since they are seen when biotin-NHS ester is used with precursor alone (data not shown). On the Western blot (top gel) in lanes 2–4, the precursor (band A) is processed to mature IL-1β (band C) and an intermediate (band B) with an apparent molecular mass of 29.6 kDa.

Besides the precursor protein (band A, top gel) and mature IL-1β (band C), possible species include fragment 28–269 (27.7 kDa), fragment 1–116 (13.4 kDa), fragment 28–116 (10.3 kDa), and fragment 1–27 (3.08 kDa). Addition of Ala-Ala-biotin to ICE and pIL-1β in lanes 7–9 (bottom gel) results in labeling of band D. Inhibition of ICE with IAA after 60 or 120 min of reaction, followed by addition of Ala-Ala-biotin to 1 μM, blocks labeling of this band (bottom gel, lanes 5 and 6). Individual bands were cut from the blots and submitted for NH2-terminal sequencing. Band C had an NH2-terminal sequence of ( )PKQM, consistent with the predicted NH2-terminal sequence of mature IL-1β of APVRSLN. Band D had an NH2-terminal sequence of ( )PKQM, consistent with the predicted sequence of the 10-kDa precursor fragment 28–116 of GPKQMK.

**DISCUSSION**

In this paper, we have examined a method to label protein proteolysis products of serine and cysteine proteases. The labeling does not require addition of organic solvent or nonphysiological pH to give detectable signals in the test systems used and is at least as sensitive as Western blotting. Labeling does not appear to represent binding to the proteolytic products, since inhibiting the enzyme after digestion of BSA, E. coli proteins, or pIL-1β prior to addition of the dipeptide nucleophile results in few or no labeled bands relative to those seen in the absence of inhibitor. Inhibiting the protease before the reaction, or omitting the protease, results in little or no labeling. Weak binding would also not be expected since free dipeptides are separated from the larger labeled peptides by SDS-PAGE preceded by boiling the substrate and products in SDS sample buffer for 5 min. Labeling catalyzed by cat S appears to be efficient under the conditions examined, resulting in labeling of about one-half of the proteolytic products seen. Labeling of products catalyzed by neutrophil elastase appears to be more selective. Labeling is seen for a variety of different dipeptide nucleophile structures and has also been observed with cat S by direct addition of hydrazide-containing fluorophoric dyes lacking an attached dipeptide, such as Cascade Blue hydrazide or lissamine rhodamine B sulfonylhydrazine (data not shown). This will allow considerable flexibility in designing the detection signal, which for example could also include radiolabeled nucleophiles.

The effects on labeling of a number of parameters that might affect attack of dipeptide nucleophiles on an acyl enzyme intermediate have been examined. Labeling of proteolysis products of cat S is dependent on the pH of the reaction, increasing as pH is increased from 6 to 8 in different nonnucleophilic buffers and diminishing as pH increases from 9 to 10. The use of a nucleophilic buffer such as Tris decreases the labeling (data not shown). This pH dependence may reflect multiple molecular events, including the pKa of the nucleophile and the pH dependence of the protease. It does give information on the allowable pH range for application of this labeling with similar proteases, and it suggests that labeling in cells at pH 7.4 may be possible.

For cat S, labeling of BSA with dipeptide nucleophiles depends on the P1’ residue, with a P1’ Phe being the most preferred among those tested. This labeling preference may be a function of variables other than $k_{cat}/K_m$, such as the local structure of the large protein substrate (BSA or large fragments thereof) forming the acyl enzyme intermediate. This is not necessarily inconsistent with the observations of Menard et al. (18) that for the small substrate dansyl-Phe-Arg-X-Trp-Ala, cat S prefers P1’ Ala over Phe by ~5-fold. Labeling might also be expected to be a function of the $P_2’$ residue, which for cat S in substrates is preferably a smaller amino acid (21); thus, we have used ala-
nine as the P$_2$’ residue in most of these experiments. The experimentally defined P$_1$’ (and P$_3$’) preferences suggest that occupancy of both sites may be important for efficient labeling. This is consistent with data for aminolysis of a tripeptide acyl–papain complex from Schuster et al. (19), who found that nucleophile occupancy of the P$_1$’-P$_3$’ sites affected the rate of this reaction. An additional factor affecting the efficiency of labeling by different dipeptide nucleophiles may involve changes in the accessibility of water to the acyl enzyme intermediate, while the nucleophile is bound at the S$_1$’ binding site (16). The P$_1$’ substrate specificity of human neutrophil elastase has been reported to be more dependent on hydrophobicity than on specific structural features of the side chain (14), and the P$_2$ position was reported to have little effect. We have observed labeling of BSA products and somewhat different labeled product band patterns, with Phe-Ala, Ala-Ala-, and Gly-Gly-fluorescein. Some bands are labeled best with Gly-Gly-fluorescein, which has the least hydrophobic P$_1$’ residue. This suggests that for elastase, as well as for cat S, dipeptide nucleophile attack on acyl enzyme intermediates formed with fragments of large substrates may behave differently regarding substrate P$_1$’ preferences than with smaller substrates. Inserting Pro in the P$_1$’ position significantly diminishes labeling; thus, this position can affect nucleophile attack, perhaps by weakening the binding of the dipeptide. The differential labeling seen with different P$_1$’ residues suggests that some selectivity of labeling might be attained if these preferences are known.

The nucleophile concentration dependence for cat S shows increased labeling as concentrations approach 1.6–3.2 mM and diminished labeling at higher levels. Diminished proteolysis above 1.6 mM on the Coomassie-stained gel is also observed, suggesting that the dipeptide nucleophiles may be acting to inhibit cat S at higher concentrations. This suggests also that there may be an optimal dipeptide nucleophile concentration for the labeling by each protease.

Besides labeling of proteolytic products for less specific enzymes such as elastase or cat S, this methodology also works for more specific proteases such as ICE. This reaction can be followed by the time-dependent disappearance of the precursor protein both in a Western blot and by biotin-NHS ester detection. Three potential fragments (residues 1–27, 28–116, and 1–116) could possibly be labeled by cleavages at known ICE-susceptible sites, resulting in predicted bands at 3.1, 10.3, or 13.4 kDa. With the NH$_2$-terminal sequence to aid interpretation, nucleophile labeling gives a band at 10 kDa consistent with the pIL-1β fragment 28–116. The observed time course suggests that cleavage in pIL-1β between residues 27 and 28 may be at least as efficient as cleavage at 116–117, since no labeled 1–116-residue peptide is seen. This should result in production of fragments 1–27 and 28–116; the latter is detected as a biotinylated peptide. It would be interesting to examine the physiological activity (if any) of each of these fragments. Fragment 1–27 may have also been labeled but is not observable on this blot. Cleavage between residues 27 and 28 has been previously reported (8) and suggests that this method could be applied to such determinations for unknown substrates.

The two labeling methodologies demonstrated here (nucleophile labeling and amine labeling with biotin-NHS ester) are quite sensitive. Biotin-NHS ester labeling gives visible protein bands after electroblootling when low nanogram levels of molecular mass standards labeled by this method are loaded onto an SDS gel. This method should allow detection of any protein containing free amines and may be a convenient way to visualize most or all proteins in reactions subjected to nucleophile labeling or Western blotting. Nucleophile labeling of 3.6 pmol of pIL-1β gives a strong band at 10 kDa even after electroblootling, with about the sensitivity of detection seen with Western blots of the same sample. Combined with the lack of dependence of labeling on organic solvent and its demonstration at pH 7–8, this suggests that nucleophile labeling might be useful for the identification of natural substrates in cells or other _in vivo_ systems.

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