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Active Site Selective Labeling of Serine Proteases with Spectroscopic Probes Using Thioester Peptide Chloromethyl Ketones: Demonstration of Thrombin Labeling Using N^{α}-[(Acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl†

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ABSTRACT: The feasibility of a new approach to incorporation of spectroscopic probes into the active sites of certain serine proteases has been demonstrated. The method is based on inactivation of a serine protease with a thioester derivative of a peptide chloromethyl ketone. The thiol group generated by reaction of the covalent enzyme–inhibitor complex with NH_2OH provides a unique site for subsequent labeling with thiol-reactive probes. To evaluate the method, N^{α}-[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl was synthesized by reaction of the thrombin-specific tripeptide chloromethyl ketone with succinimidyl (acetylthio)acetate and purified by sulfopropyl-Sephadex and Sephadex G-10 chromatography. Reverse-phase high-performance liquid chromatography indicated that the product was 90 ± 3% pure. The compound was quantitated by using 5,5′-dithiobis(2-nitrobenzoic acid) to measure the concentration of thiol produced in the presence of NH_2OH. On this basis, titrations of the irreversible loss of human α-thrombin activity had end points of 1.1 ± 0.1 mol of inhibitor/mol of active sites, indicating a 1:1 stoichiometry for inactivation. Incubation of N^{α}-[(acetylthio)acetyl]-D-Phe-Pro-Arg–thrombin with 5-(iodoacetamido)fluorescein in the presence of NH_2OH resulted in incorporation of 0.96 mol of the fluorescence probe/mol of active sites and the appearance of fluorescein fluorescence associated with the active site containing B-chain on sodium dodecyl sulfate–polyacrylamide gels. Fluorescence labeling of thrombin required reaction of the inhibitor at the active site as well as subsequent generation of the thiol group with NH_2OH. It is concluded that active site selective labeling can be achieved by using this approach, which is likely to be applicable to other proteases, peptide chloromethyl ketones, and a wide variety of probes.

Use of covalent spectroscopic probes for studying the functions of serine proteases, such as the enzymes of the blood coagulation system, is well established. Several reagents derived from synthetic inhibitors (Powers & Harper, 1986) have been described for active site affinity labeling of serine proteases with spectroscopic probes. These include sulfonyl fluoride (Berliner & Wong, 1974; Vaz & Schoellmann, 1976) and organophosphorous (Epstein et al., 1979; Hsia et al., 1972) derivatives of fluorophores and spin labels or acylating agents (Haugland & Stryer, 1967; Moorman & Abeles, 1982) based on these probes which react with the active center serine residue of the enzymes. Irreversible inactivation of proteases by alkylation of the active site histidine residue with derivatives of amino acid or peptide chloromethyl ketones has also been

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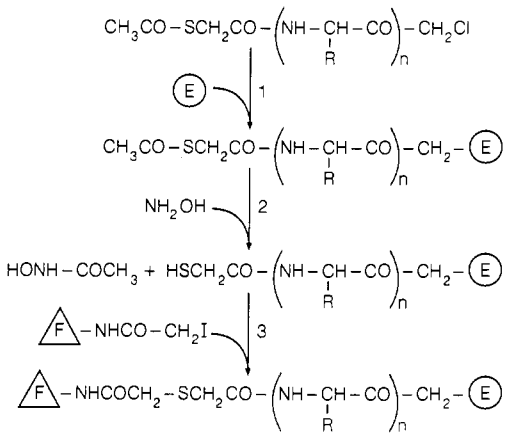
Scheme I



used to incorporate Dansyl[1] fluorophores (Schoellmann, 1972; Penny & Dyckes, 1980; Nesheim et al., 1981; Peters et al., 1984; Krishnaswamy et al., 1986), spin labels (Kosman, 1972), and NMR probes (Malthouse et al., 1983). All of these reagents are limited to incorporation of a single spectroscopic probe, requiring synthesis of a new reagent for each probe of potential interest. It would be advantageous for many studies of serine proteases to be able to readily incorporate different types of probes or spectroscopic probes which differ in their spectral properties. The latter is especially true for investigation of protein conformational changes or protein–protein interactions using fluorescence techniques, where the intrinsic properties of the probes, including overlap between absorption and emission spectra, excited-state lifetimes, and environmental sensitivities, determine their usefulness for particular applications.

An alternative strategy for incorporation of spectroscopic probes into the active sites of certain serine proteases, which overcomes the limitation of a single choice for the probe, is summarized by the reactions shown in Scheme I. This approach depends on synthesis of a derivative of a peptide chloromethyl ketone containing a thioester at the amino terminus. Reaction of a susceptible serine protease with the inhibitor will result in complete inactivation of the enzyme and stable, covalent incorporation of the thioester peptide (reaction 1). Subsequent reaction of the covalent complex with hydroxylamine, under mild conditions, will generate a thiol group (reaction 2). For proteases that do not contain essential thiols, this group can be selectively modified with a thiol-reactive spectroscopic probe, such as a fluorophore iodoacetamide (reaction 3). The principal advantage of this approach is that synthesis of a single reagent should enable any protease, which does not contain essential thiols and undergoes active site directed inactivation by the inhibitor at a practical rate, to be specifically labeled with any one of a wide variety of probes.

The feasibility of active site selective labeling according to the reactions of Scheme I is demonstrated in this report by the covalent labeling of human α-thrombin with 5-(iodoacetamido)fluorescein after stoichiometric inactivation of the blood-clotting protease with N^{α}-[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl. Relatively simple methods for synthesis and quantitation of the new derivative of the thrombin-specific inhibitor (Kettner & Shaw, 1979) are described.

Experimental Procedures

Synthesis of N^{α}-[*(Acetylthio)acetyl*]-D-*Phe-Pro-Arg-*CH_2Cl (*ATA-FPR-CK*). Three preparations of ATA-FPR-CK used in this study were synthesized by mixing 5–25 mg of D-Phe-Pro-Arg-CH_2Cl (FPR-CK, Calbiochem) in 50 mM sodium phosphate buffer, pH 7.0, with 0.5–1 volume of succinimidyl (acetylthio)acetate (SATA, Molecular Probes) in methanol to give a final concentration of 3–6 mM FPR-CK and a 4–5-fold molar SATA excess. After 20–30 min at room temperature, the reaction mixture was diluted 5-fold with 25 mM sodium phosphate–H_3PO_4 buffer, pH 3.0, and the pH adjusted to 3.0 with 1 N H_3PO_4. It was immediately chromatographed on a 1.5 or 2.5 × 95–190 cm column of sulfopropyl- (SP-) Sephadex C-25 equilibrated at room temperature with 50 mM sodium phosphate–H_3PO_4, pH 3.0, and eluted with the same buffer. The separation was monitored by the absorbance at 210 nm, concentration of thioester using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and thrombin inhibitory activity as described below. Fractions containing the product were pooled and lyophilized. The product was extracted with three to five 5-mL aliquots of methanol, and the combined extracts were evaporated under a stream of nitrogen. The residue was dissolved in 2–3 mL of 1 mM HCl and chromatographed on a 1.5 × 97 cm column of Sephadex G-10 in 1 mM HCl at room temperature. Desalted fractions of constant (±5%) inhibitor/thioester concentration were pooled, lyophilized, dissolved in a small volume of 1 mM HCl, and stored at −70 °C. Solutions of the compound (typically ≤5 mM) were stable under these conditions for several months.

Amino acid compositions of Pro (1.00) and Phe (0.84), determined as described in Shishikura et al. (1987), were obtained for both ATA-FPR-CK and FPR-CK. No other amino acids were detected.

^{1}H NMR (300-MHz) spectra of FPR-CK (16 mM) and ATA-FPR-CK (26 mM) in 1 mM DCl/D_2O were collected with a General Electric QE-300 instrument. The HDO resonance, assigned a chemical shift of 4.80 ppm relative to tetramethylsilane, was used as an internal reference. The spectrum of ATA-FPR-CK compared with that of FPR-CK showed a new singlet at 2.41 ppm (3 H) and ~2 H in the 3.5–3.8 ppm region, unresolved from other resonances, consistent with the presence of one (acetylthio)acetyl group. Other differences noted in the NMR spectra, which may be due to a different distribution of proline isomers, have not been further investigated.

HPLC. Reverse-phase HPLC was performed with a Varian 5500 instrument using a Varian MicroPak MCH-N-Cap-5 C_{18} silica column (4 × 150 mm) equilibrated at room temperature with 1 mM H_3PO_4 and eluted at 1.5 mL/min with 15 mL of this solvent followed by a linear gradient of CH_3CN. Twenty-microliter samples of 0.5–2 mM ATA-FPR-CK were injected and the results compared to those obtained by injection of the solvent alone. Relative areas of absorbance (210-nm) peaks were obtained by weighing cut out tracings of the peaks.

Quantitation of ATA-FPR-CK with DTNB. DTNB reactions were monitored by the increase in absorbance at 412 nm using a Cary 219 spectrophotometer, at 25 °C. Routinely, 0.1 M HEPES, 0.3 M NaCl, 1 mM EDTA, pH 7.0 (pH 7 buffer), DTNB, and 1 M NH_2OH in the same buffer were mixed to give 1.0 mL of 500 μM DTNB and 0.1 M NH_2OH

[1] Abbreviations: ATA-FPR-CK, N^{α}-[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl; FPR-CK, D-Phe-Pro-Arg-CH_2Cl; SATA, succinimidyl (acetylthio)acetate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoic acid; IAF, 5-(iodoacetamido)fluorescein; Dansyl, 5-(dimethylamino)naphthalenesulfonyl; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PEG, poly(ethylene glycol) 8000; S2238, H-D-Phe-Pip-Arg-*p*-nitroanilide; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography.

in a polystyrene cuvette. The background rate of DTNB breakdown was recorded for a short time before addition of a small volume of ATA-FPR-CK and continuous recording of the subsequent reaction. The amplitude of the exponential phase was measured from the difference in absorbance between the initial and final linear rates, extrapolated to the time of ATA-FPR-CK addition and corrected for dilution. Indistinguishable amplitudes were obtained from reactions initiated with NH_2OH. The total absorbance change corresponded to <10% consumption of DTNB for all of the reactions. An extinction coefficient of 14150 M^{-1} cm^{-1} was used for 2-nitro-5-thiobenzoic acid (TNB) (Riddles et al., 1979).

Measurement of Acetohydroxamate. Acetohydroxamate was quantitated by its formation of a purple-colored complex with ferric ion at acidic pH (Balls & Wood, 1956). ATA-FPR-CK (~0.1 μmol) was incubated in 400 μL of 10 mM HEPES and 0.5 M NH_2OH, pH 7.0, for 30 min at 25 °C. An equal volume of 0.23 M HCl was added, followed immediately by 200 μL of 10% (w/v) ferric chloride in 0.1 M HCl. The absorbance at 540 nm was measured after 45–60 min against a blank from which ATA-FPR-CK was omitted. Acetohydroxamate produced by reaction of 2–4 mM *p*-nitrophenyl acetate with NH_2OH under the same conditions and quantitated by the release of *p*-nitrophenol by using an extinction coefficient at 16600 M^{-1} cm^{-1} at 410 nm (Chase & Shaw, 1969) in 0.1 M HEPES, 0.1 M NaCl, and 0.1 mM EDTA, pH 8.3 (pH 8.3 buffer), was used as a standard. The absorbance at 540 nm was linear in acetohydroxamate concentration up to at least 400 μM, corresponding to an absorbance of about 0.4. No significant reaction (≤6%) was obtained with ATA-FPR-CK incubated in the absence of NH_2OH or with FPR-CK.

Inactivation of Thrombin by ATA-FPR-CK. Human α-thrombin was generously supplied by Dr. John Fenton of the New York State Department of Health, Albany, NY. The enzyme was dialyzed against 5 mM MES/0.3 M NaCl, pH 6.0, centrifuged in a Beckman Microfuge for 2 min or Millipore filtered (0.45 μm), quick frozen, and stored at −70 °C. A molecular weight of 36000 and 280-nm extinction coefficients of 1.83 $(mg/mL)^{-1}$ cm^{-1} in 0.1 M NaOH or 1.74 $(mg/mL)^{-1}$ cm^{-1} in pH 7 buffer were used to determine protein concentration (Fenton et al., 1977). The active enzyme concentration was determined from the average of three active site titration reactions at thrombin concentrations of 5–10 μM with 100–230 μM *p*-nitrophenyl *p*-guanidinobenzoate in pH 8.3 buffer, with corrections (<6%) for turbidity (Chase & Shaw, 1969; Lottenberg et al., 1982). Preparations of thrombin used in this study were 85–98% active.

Rates of hydrolysis of H-D-Phe-Pip-Arg-pNA (S2238, Helena Laboratories) by thrombin were measured by using a Cary 219 spectrophotometer, at 25 °C. Initial velocities (<10% substrate consumed) with 90–100 μM S2238 in 0.1 M Tris-HCl, 0.1 M HEPES, 0.1 M NaCl, and 1 mg/mL PEG, pH 7.8, were calculated from the linear increase in absorbance at 405 nm with time. S2238 concentration was determined from the 342-nm absorbance by using 8266 M^{-1} cm^{-1} for the extinction coefficient (Lottenberg & Jackson, 1983). Graphical analysis of progress curves (Orsi & Tipton, 1979) for 3.5–47 μM S2238 gave an estimate of K_m = 1.4 μM, K_i(product) = 12 μM, and k_{cat} = 105 s^{-1}, in reasonable agreement with previously published values determined under slightly different conditions (Lottenberg et al., 1982).

Titrations of thrombin with ATA-FPR-CK were measured by the residual S2238 activity in mixtures containing 1 μM thrombin and ATA-FPR-CK in pH 7 buffer containing 1 mg/mL PEG, after incubation at 25 °C for 15–60 min, more than sufficient time for completion of the reaction. PEG-coated polypropylene tubes were used to prevent losses of enzyme due to adsorption (Latallo & Hall, 1986). During purification of ATA-FPR-CK, concentrations of inhibitor were calculated from the residual thrombin activity by equating the concentration of thrombin inactivated with the concentration of inhibitor present. For assaying fractions from chromatographic experiments, an initial estimate of the ATA-FPR-CK concentration was calculated from the absorbance by using a rough value of 20000 M^{-1} cm^{-1} at 210 nm. Measurement of the inhibitor activity in fractions from the HPLC separation was performed similarly, except that 10 nM thrombin was used and the incubations were for ≥3.5 h.

Fluorescence Labeling Experiments. ATA-FPR–thrombin (<0.01% active) and FPR–thrombin (<0.01% active) were prepared by mixing 46 μM thrombin in 5 mM MES, 0.3 M NaCl, and 0.1 mM EDTA, pH 6.0, with 0.5 volume of pH 7 buffer containing a final 2.4-fold molar excess of inhibitor and incubating at 25 °C for 1 h. Excess inhibitor was removed by overnight dialysis against 1000 volumes of 50 mM HEPES, 0.3 M NaCl, and 1 mM EDTA, pH 7.0. FPR–thrombin was subsequently incubated with a 2.5-fold excess of ATA-FPR-CK and dialyzed as described above. IAF (Molecular Probes) was suspended in pH 7 buffer and centrifuged for 2–5 min in a Beckman Microfuge. IAF concentration was determined from the 491-nm absorbance in 0.1 M Tris-HCl and 1 mM EDTA, pH 8.0, by using 85000 M^{-1} cm^{-1} for the extinction coefficient (Mercola et al., 1972). ATA-FPR–thrombin was incubated in the dark at a final concentration of 14 μM with a 5-fold molar excess of IAF in the absence or presence of 0.1 M NH_2OH in degassed pH 7 buffer at 25 °C for 1 h. Excess IAF was removed by chromatography of the reaction mixture on a 4-mL column of Sephadex G-25 (superfine) in 10 mM HEPES, 0.3 M NaCl, and 1 mM EDTA, pH 7.0 at room temperature, and dialysis against the same buffer overnight at 4 °C. Reactions of active thrombin and FPR–thrombin with NH_2OH and IAF were performed in the same way. SDS-denatured thrombin was prepared by boiling a 25 μM solution containing 2% SDS for 3 min. It was incubated with a 2.5-fold excess of ATA-FPR-CK, dialyzed, and reacted with NH_2OH and IAF as described above except in buffers containing 0.1% SDS.

Incorporation of IAF was quantitated by dilution of 200-μL aliquots of thrombin from the reactions described above with 800 μL of 0.125 M Tris-HCl buffer, 1.25 mM EDTA, and 7.5 M guanidine (Heico), pH 8.5, measurement of the absorbance at 280 nm, addition of 1 mM dithiothreitol, and measurement of the absorbance at 498 nm after a 15-min incubation. An extinction coefficient of 84000 M^{-1} cm^{-1} at 498 nm and $\epsilon_{280nm}/\epsilon_{498nm}$ = 0.19 were used to calculate the fluorescein concentration and correct the 280-nm absorbance for the contribution from the dye. These values were determined from the absorbance spectrum of 2-mercaptoethanol–IAF (IAF incubated with a 20-fold excess of 2-mercaptoethanol) relative to the spectrum obtained under the conditions used for a previously determined extinction coefficient (Eshaghpour et al., 1980). The thrombin active site concentration was calculated from the corrected 280-nm absorbance and the absorbance measured under the same conditions for the untreated, active site titrated thrombin preparation.

Samples containing equal amounts of thrombin (8 μg) were boiled in 1% SDS and 5% 2-mercaptoethanol and run on a 14% SDS–polyacrylamide minigel (AcrylAide, FMC BioProducts, was used) using the Laemmli (1970) buffer system.

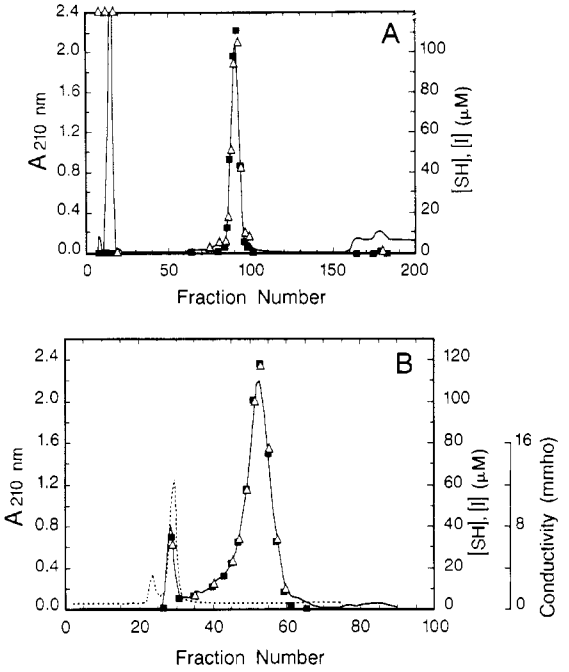


FIGURE 1: Purification of ATA-FPR-CK. (A) Chromatography of a reaction mixture containing 5 mg of FPR-CK and a 5-fold excess of SATA on SP-Sephadex (1.5 × 95 cm) as described under Experimental Procedures. A_{210nm} (—), thioester concentration ([SH], Δ), and inhibitor concentration ([I], ■) were measured in 10-mL fractions. Elution of unreacted FPR-CK with buffer containing 0.5 M NaCl was started at fraction 150. Fractions 87–94 were pooled for the next step. (B) Chromatography of the ATA-FPR-CK pool from the separation in (A) on Sephadex G-10 as described under Experimental Procedures. A_{210nm} (—), conductivity (- - -), thioester concentration (Δ), and inhibitor concentration (■) were measured in 3.6-mL fractions. Some of the inhibitor eluted with the salt peak at fraction 30 while the majority was adsorbed to the column and eluted subsequently. Fractions 43–57 were pooled as the final product. The yield could be increased slightly by rechromatography of the remaining active fractions, which resulted in a similar elution profile.

Samples run under nonreducing conditions were prepared by boiling in 1% SDS and 1 mM iodoacetamide. The fluorescence was photographed by using a Fotodyne 300-nm transilluminator.

RESULTS

N^{α}-[(Acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl (ATA-FPR-CK) was synthesized by reaction of the single N-terminal amino group of the peptide chloromethyl ketone (FPR-CK) with the *N*-hydroxysuccinimide ester of (acetylthio)acetate (SATA). Under the conditions used for the reaction less than 15% of the activity as a thrombin inhibitor was lost and the generation of side products was minimized. ATA-FPR-CK and unidentified minor reaction products were separated from both reactants by cation-exchange chromatography at pH 3.0 as shown in Figure 1A. Elution behavior of the reactants was established by independent chromatography of control reaction mixtures in which either SATA or FPR-CK was incubated under the conditions used for synthesis. SATA eluted with 1 column volume of buffer as a peak of absorbance and thioester concentration. FPR-CK, identified by absorbance and inhibitor activity, remained quantitatively bound to the column and could be eluted with buffer containing 0.5 M NaCl. Chromatography of the synthetic reaction mixture resulted in elution of ATA-FPR-CK with 5.5-column volumes of buffer as a peak of absorbance, coincident thioester concentration, and thrombin inhibitory activity, preceded by one or more minor, inactive products (Figure 1A). Negligible residual FPR-CK was eluted with buffer containing 0.5 M NaCl, indicating that the synthetic reaction had gone to completion. ATA-FPR-CK was desalted and further purified by adsorption chromatography on Sephadex G-10 as shown in Figure 1B. Fractions containing a constant (±5%) ratio of thioester to inhibitor concentration were taken as the final product. The yield of ATA-FPR-CK was 29–42% for three similar preparations, calculated from the recovery of inhibitor activity.

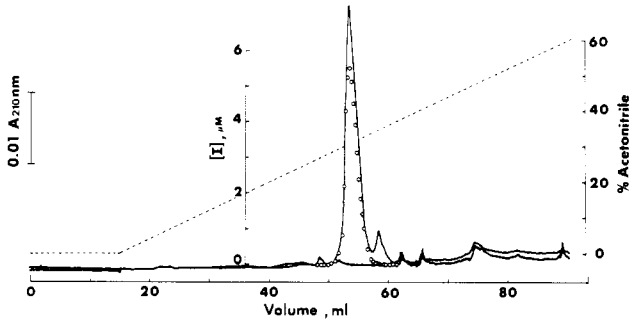


FIGURE 2: Reverse-phase HPLC of ATA-FPR-CK. A_{210nm} elution profiles are shown for 20 μL of 850 μM ATA-FPR-CK and a blank injection of solvent. Inhibitor concentration ([I], ○) was measured in 0.3-mL fractions collected during the same separation, as described under Experimental Procedures.

The purity of the product was assessed by reverse-phase HPLC, with typical results shown in Figure 2. ATA-FPR-CK from three similar preparations eluted at 31% CH_3CN as a major peak representing 90 ± 3% of the integrated 210-nm absorbance. It was frequently preceded by a small peak at 27% CH_3CN (1–6% of the total absorbance) and followed by a minor peak at 35% CH_3CN (6–9%). Measurements of thrombin inhibitory activity in fractions collected during this separation demonstrated that only material eluting in the major peak was active (Figure 2). Examination of the progress of the synthetic reaction by HPLC suggested that the major peak and 35% CH_3CN peak represented primary reaction products. Prolonged reaction generated other species including several peaks eluting at 25–30% CH_3CN. Similar species appeared on incubation of ATA-FPR-CK in 50 mM phosphate buffer and 0.5 mM EDTA, pH 7.0, for 14.5 h, accompanied by loss of 58% of the inhibitor activity. These results suggested that the 27% CH_3CN peak represented a small amount of degradation product.

The stability of ATA-FPR-CK was compared with that of FPR-CK in buffers at pH 7.0 and at 25 °C by measuring the loss of thrombin inhibitory activity with time. After 9.5 h of incubation in the phosphate buffer given above, ATA-FPR-CK lost 42% of its activity while FPR-CK lost 87%. In the HEPES buffer used in subsequent experiments both compounds were more stable, losing 19% (ATA-FPR-CK) and 30% (FPR-CK) in 9.5 h. This process appeared first order, with similar losses of activity measured at 4–5 μM inhibitor and 100-fold higher concentrations. Both inhibitors were completely stable (<5% activity loss) in 1 mM HCl over the same time period.

To quantitate reactions of ATA-FPR-CK, its concentration was determined from the thioester concentration by using the spectrophotometric thiol reagent DTNB to measure the total thiol generated on reaction with NH_2OH. Preincubation of ATA-FPR-CK with NH_2OH followed by measurement of the thiol generated gave unsatisfactory results, complicated by a loss of thiols, probably due to alkylation by the chloromethyl ketone. The existence of this process necessitated measurement of the thioester concentration from the reaction in the presence of DTNB and NH_2OH as shown in Figure 3. Addition of

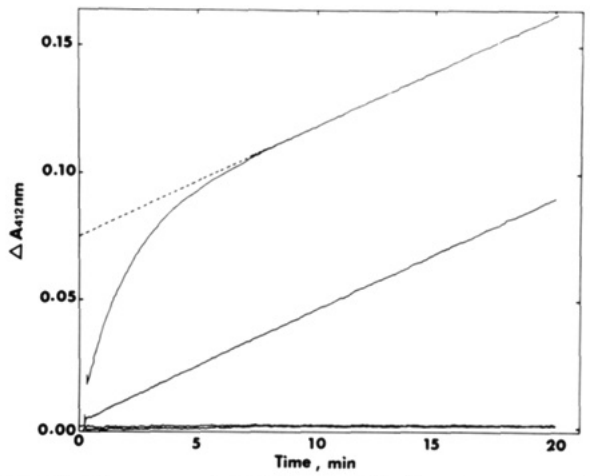


FIGURE 3: Measurement of ATA-FPR-CK thioester concentration using DTNB. Tracings of the recorded change in A_{412nm} with time are shown for reaction mixtures containing (from top to bottom) DTNB + ATA-FPR-CK + NH_2OH, DTNB + NH_2OH, DTNB + ATA-FPR-CK, and DTNB alone, at final concentrations of 490 μM DTNB, 0.09 M NH_2OH, and 5.6 μM ATA-FPR-CK, as described under Experimental Procedures.

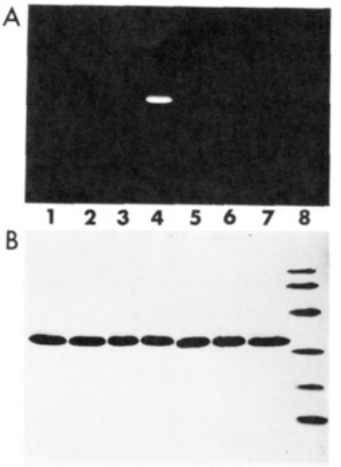


FIGURE 4: IAF labeling of α-thrombin by use of ATA-FPR-CK. Samples of thrombin, prepared as described under Experimental Procedures, were run on a 14% SDS slab gel. The fluorescence was photographed (A) before staining the same gel for protein (B). Lane 1, thrombin; lane 2, thrombin + NH_2OH + IAF; lane 3, ATA-FPR–thrombin; lane 4, ATA-FPR–thrombin + NH_2OH + IAF; lane 5, ATA-FPR–thrombin + IAF; lane 6, ATA-FPR-CK-treated FPR–thrombin + NH_2OH + IAF; lane 7, ATA-FPR-CK-treated SDS-denatured thrombin + NH_2OH + IAF; lane 8, molecular weight standards (97 000, 67 000, 45 000, 30 000, 20 000, and 14 400).

0.09 M NH_2OH to a solution of ATA-FPR-CK in the presence of a large excess of DTNB resulted in an exponential increase in absorbance at 412 nm followed by a linear increase. The linear increase was completely accounted for by the slow breakdown of DTNB which occurred only in the presence of NH_2OH. This process had no significant effect on quantitation of the reaction of DTNB with a model thiol compound (2-mercaptoethanol) which was complete within the mixing time (not shown). The exponential phase of the absorbance increase observed with ATA-FPR-CK therefore represented reaction of the thioester with NH_2OH, and the amplitude could be used as a measure of the thioester concentration.[2] The amplitude was independent of DTNB (0.1–1 mM) and NH_2OH (0.02–0.4 M) concentrations and a linear function of the concentration of ATA-FPR-CK up to at least 30 μM (not shown). No reaction was obtained with ATA-FPR-CK and DTNB in the absence of NH_2OH, indicating no detectable free thiol (±3% of the total). These results indicated that consumption of the free thiol by reaction with the chloromethyl ketone did not complicate measurement of the thioester concentration by this method.[3] In addition, the amount of acetohydroxamate produced in mixtures of ATA-FPR-CK and NH_2OH was 0.94 ± 0.03 mol/mol of thioester, confirming the validity of the measurement and consistent with the structure of the compound.

By use of the thioester concentration as a measure of ATA-FPR-CK concentration, the stoichiometry of inactivation of α-thrombin by the inhibitor was determined from titrations of the loss of enzyme activity measured with a chromogenic substrate (not shown). Inactivation of thrombin was irreversible, as evidenced by the linearity of the initial velocities after extensive dilution into solutions of substrate at 60–70K_m and the unchanged residual activity of the partially or completely inactivated enzyme with time or after dialysis. The titrations were linear and resulted in complete inactivation (<0.1% active) with end points averaging 1.1 ± 0.1 mol of ATA-FPR-CK/mol of thrombin active sites, for three independent preparations of inhibitor of indistinguishable purity. The same titration end points, within experimental error, were measured at thrombin concentrations of 10 nM and 1 μM. These results, in conjunction with the estimated 90% purity of the compound by HPLC, demonstrated a 1:1 stoichiometry for the inactivation reaction.

Fluorescence labeling of thrombin by using ATA-FPR-CK according to the reactions of Scheme I was investigated with 5-(iodoacetamido)fluorescein (IAF) as a representative thiol-reactive fluorescence probe. Covalent incorporation of IAF was assessed by the appearance of fluorescein fluorescence associated with reduced and denatured thrombin on SDS–polyacrylamide gels (Figure 4A) and quantitated spectrophotometrically as described under Experimental Procedures. Electrophoresis of nonreduced samples gave indistinguishable results (not shown). Only thrombin that had been inactivated with ATA-FPR-CK and subsequently reacted with IAF in the presence of NH_2OH showed significant fluorescence labeling of the active site containing thrombin B-chain (lane 4), corresponding to incorporation of 0.96 mol of IAF/mol of active sites. The A-chain, which migrated with the dye front and is not clearly visible on the Coomassie blue stained gel (Figure 4B), was not labeled. Incorporation of IAF into thrombin was dependent on generation of the thiol group with NH_2OH, as evidenced by the incorporation of only 0.03 mol of IAF/mol of active sites in an otherwise identical reaction of ATA-FPR–thrombin with IAF (lane 5). Inactivation of the enzyme with the thioester peptide chloromethyl ketone was also required, since no significant fluorescein incorporation (0.02 mol of IAF/mol of active sites) occurred when active thrombin was reacted with IAF in the presence of NH_2OH (lane 2). Evidence that these reactions occurred at the active site was obtained from the incorporation of only 0.02 mol of IAF/mol of active sites, measured for thrombin that had been active

[2] Semilog plots of this phase of the reaction were linear for over 90% of the amplitude with an apparent rate constant of 0.011 ± 0.002 s^{-1} at 0.1 M NH_2OH, independent of DTNB and ATA-FPR-CK concentrations.

[3] Decreases in absorbance due to alkylation of TNB by the chloromethyl ketone did not complicate these measurements because of the slow rate of this reaction. Second-order rate constants of 1.3 ± 0.1 M^{-1} s^{-1} (FPR-CK) and 1.1 ± 0.1 M^{-1} s^{-1} (ATA-FPR-CK) were measured under pseudo-first-order conditions from the decrease in TNB absorbance at 412 nm. By use of this rate constant, the calculated decrease in absorbance for the full time course under typical conditions (e.g., Figure 3) was less than 2%, and that for the highest ATA-FPR-CK concentration measured (30 μM), less than 6%.

site blocked with FPR-CK before reaction with ATA-FPR-CK, NH_2OH, and IAF (lane 6). Finally, thrombin denatured in SDS did not incorporate significant IAF in the presence of NH_2OH after incubation with ATA-FPR-CK (lane 7, not quantitated), consistent with a requirement for the functional protease to incorporate the inhibitor and, subsequently, IAF.

Discussion

The goal of this study was to determine the feasibility of using the reactions of Scheme I as a relatively general and versatile method for incorporation of spectroscopic probes into the active sites of proteases. Covalent introduction of thiol groups into proteins for subsequent labeling with probes is not a new idea, although previous methods have been restricted to reagents that react randomly with protein amino groups (Duncan et al., 1983; Klotz & Heiney, 1962; Carlsson et al., 1978; Benesch & Benesch, 1956; Perham & Thomas, 1971). Individual spectroscopic probe derivatives of peptide chloromethyl ketones have also been previously used to label proteases at their active sites (see the introduction). However, use of a thioester derivative of a peptide chloromethyl ketone to incorporate a unique thiol group for covalent probe labeling is a new approach which offers the advantages of active site selectivity as well as a broad choice in the types and properties of the probes that can be incorporated. Results obtained with ATA-FPR-CK, α-thrombin, and IAF as a model thioester peptide chloromethyl ketone, protease, and spectroscopic probe demonstrated that this approach can be used to achieve active site selective labeling.

Simple synthesis and purification procedures were used to prepare ATA-FPR-CK for evaluation of the labeling scheme. Contamination of the purified compound with either of the reactants, which would complicate subsequent labeling with probes by causing heterogeneity or nonspecific labeling, was excluded by these procedures. Reverse-phase HPLC indicated that preparations of ATA-FPR-CK consisted of 90% a single inhibitor species, with the remainder represented by two minor, inactive contaminants. One of these represents a small amount of degradation product while the identity of the other contaminant has not been established. For the purpose of labeling with probes, however, the lack of activity of these species as irreversible inhibitors makes their presence inconsequential. Addition of the thioester group to the inhibitor provided a means of quantitation of the compound based on spectrophotometric measurement, with DTNB, of the amplitude of the exponential generation of thiol in the presence of hydroxylamine. On this basis, evidence of a 1:1 stoichiometry was obtained for irreversible inactivation of thrombin by ATA-FPR-CK. Methods developed for this compound should be applicable to preparation and characterization of thioester derivatives of other peptide (arginine) chloromethyl ketones.

The minimum requirements for active site selective labeling by the approach described in this report are that the protease not contain essential thiols and that it undergo active site directed inactivation by the inhibitor at a significant rate. Many serine proteases of physiological importance, such as thrombin, do not contain thiol groups. Results of labeling experiments with IAF clearly demonstrated a dependence of its covalent incorporation into thrombin on each of the reactions shown in Scheme I. Thus, stoichiometric incorporation of IAF was dependent on irreversible inactivation of the enzyme at the active site with ATA-FPR-CK as well as on subsequent generation of the thiol group with hydroxylamine. The labeling control experiments argue against significant reaction of ATA-FPR-CK preparations outside the enzyme active site. Similarly, nonspecific labeling of thrombin by IAF did not appear to represent a significant problem under the mild conditions required for these reactions, consistent with the well-known selectivity of iodoacetamides for thiols.

Peptide inhibitors based on arginine or lysine chloromethyl ketone have been demonstrated to show great specificity in terms of their rates of inactivation of various trypsin-like proteases (Kettner & Shaw, 1981a; Lijnen et al., 1984). On the other hand, these reagents are not absolutely specific for particular proteases of this family. Only a practical rate of inactivation, while maintaining active site selectivity, is required for the purpose of incorporating probes. The Dansyl derivative of Glu-Gly-Arg-CH_2Cl, for example, inactivates blood coagulation factor Xa at the fastest reported rate (Kettner & Shaw, 1981b) but has been successfully used to inactivate and label several other proteases that react more slowly, by as much as about 1000-fold (Krishnaswamy et al., 1986; Nesheim et al., 1986; Higgins & Lamb, 1986; Lollar & Fass, 1984). Despite the slow rate, evidence of stoichiometric inactivation was obtained for the slowest of these reactions (Lollar & Fass, 1984). Similar kinetic studies have demonstrated that D-Phe-Pro-Arg-CH_2Cl is a highly specific thrombin inhibitor (Kettner & Shaw, 1979). Interestingly, no derivatives of this compound have been previously described for incorporating fluorophores or other probes into the enzyme. As shown here, ATA-FPR-CK can be used for this purpose. The previously demonstrated broad application of Dansyl-Glu-Gly-Arg-CH_2Cl and the published kinetic data for this inhibitor in comparison with that for D-Phe-Pro-Arg-CH_2Cl suggests that it will be possible to label many other trypsin-like proteases by using ATA-FPR-CK.

It is unlikely that active site selective labeling of proteases via the reactions of Scheme I will be limited to the particular reagents used in this study. The same procedure or minor variations of it should enable incorporation of various spectroscopic probes, including fluorophores, chromophores, spin labels, and NMR probes as well as other types of labels, such as radiolabels, biotin, and cross-linking reagents, all of which are available as thiol-reactive derivatives.

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