Transglutaminase Inhibition by 2-[(2-Oxopropyl)thio]imidazolium Derivatives: Mechanism of Factor XIIIa Inactivation

Kurt F. Freund,[‡] Kundan P. Doshi,[‡] Stanley L. Gaul,[‡] David A. Claremon,[§] David C. Remy,[§] John J. Baldwin,[§] Steven M. Pitzenberger,[§] and Andrew M. Stern*[,‡]

Departments of Biological Chemistry and Medicinal Chemistry, Merck Research Laboratories, West Point, Pennsylvania 19486

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ABSTRACT: The physiologic role of several transglutaminases could be more precisely defined with the development of specific inhibitors for these enzymes. In addition, specific plasma transglutaminase (fXIIIa) inhibitors may have therapeutic utility in the treatment of thrombosis. For these purposes, the inactivation of fXIIIa and human erythrocyte transglutaminase (HET) by 2-[(2-oxopropyl)thio]imidazolium derivatives, which comprise a novel class of transglutaminase inactivators, was studied. As a specific example, 1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride (**III**) inactivated fXIIIa with an apparent second-order rate constant (specificity constant of inactivation) of 6.3×10^4 M^{-1} s^{-1}, corresponding to a rate 4×10^7 times greater than its reaction rate with glutathione (GSH). The mechanism of fXIIIa inactivation by this class of compounds was investigated utilizing two [^{14}C]-isotopic regioisomers of 1,3-dimethyl-2-[(2-oxopropyl)thio]imidazolium iodide (**II**). Structural analyses demonstrated that acetonylation of the active site cysteinyl residue of fXIIIa occurred along with the stoichiometric release of the complementary fragment of the inactivator as the corresponding thione. Kinetic analysis of the inactivation of fXIIIa by nonquarternary analogs of **II** and **III** indicated the formation of a reversible complex between the inactivator and fXIIIa prior to irreversible modification of the enzyme. At 1 mM, **III** displayed no detectable levels of inhibition or inactivation with several serine proteases and thiol reagent-sensitive enzymes. 2-[(2-Oxopropyl)thio]imidazolium derivatives and the related molecule 2-(1-acetonylthio)-5-methylthiazolo-[2,3]-1,3,4-thiadiazolium perchlorate (**I**), when present at the time of clot formation at 1–10 μM, enhanced the rates of tissue plasminogen activator catalyzed clot lysis *in vitro*. These inactivators prevented the fXIIIa-catalyzed covalent incorporation of α_2-antiplasmin into the α chain of fibrin and the formation of high molecular weight fibrin α chain polymers, providing the basis for the observed enhancements in clot lysis rates.

During the final phase of the blood clotting cascade, factor XIIIa, plasma transglutaminase (fXIIIa),[1] catalyzes covalent cross-linking reactions within fibrin clots (McKee et al., 1970; Lorand, 1972; Folk & Finlayson, 1977; McDonagh, 1987; Mosher, 1978; Sakata & Aoki, 1980; Tamaki & Aoki, 1981), increasing their mechanical strength (Shen & Lorand, 1983) and resistance to plasmin-catalyzed fibrinolysis (Gaffney & Whitaker, 1979; Sakata & Aoki, 1982; Jansen et al., 1987; Francis & Marder, 1988; Sakata et al., 1984; deFouw et al., 1988; Edwards et al., 1993). The activity of fXIIIa in plasma is determined in part by the rate of fXIIIa production from its zymogen, plasma fXIII, a reaction catalyzed by thrombin and promoted by non-cross-linked fibrin (Lewis et al., 1985; Greenberg & Miraglia, 1985). Patients who cannot generate fXIIIa because of an inherited or acquired fXIII deficiency show a delayed bleeding tendency that results from their inability to form stable clots (Duckert et al., 1960; Lorand et al., 1988). On the other hand, an inability to effect efficient fibrinolysis may be a risk factor for arterial and venous thrombosis (Juhan-Vague et al., 1987; Hamsten et al., 1985; Wiman et al., 1985; Nguyen et al., 1988). These considerations suggest that chronic modulation of fXIIIa activity with specific inhibitors might normalize low fibrinolytic rates in patients with a thrombotic tendency by ensuring production of less cross-linked clots that are more susceptible to fibrinolysis. This may allow a fine dynamic balance between the rates of clot formation and breakdown (Francis et al., 1984; Nossel, 1981; Astrup, 1958) to be maintained in these patients. The relatively rapid rate of the cross-linking reactions that determine susceptibility of fibrin clots to fibrinolysis, however, would be expected to preclude use of fXIIIa inhibitors for acute thrombolytic therapy (Bush et al., 1988; Shebuski et al., 1990; Leidy et al., 1990), where the pharmacologic agent is administered subsequent to formation of a cross-linked fibrin clot.

In transglutaminase-catalyzed reactions an acyl group is transferred from the substrate to an active site cysteinyl residue of the enzyme. The resulting active site thiol ester intermediate subsequently undergoes enzymically catalyzed aminolysis or hydrolysis (Folk & Finlayson, 1977). Like many enzymes with an active site thiol group, transglutaminases are inactivated by alkylating agents such as halomethyl carbonyl derivatives (Folk & Finlayson, 1977; Holbrook et al., 1973;

* To whom correspondence should be addressed. Phone: (215) 652-5829; FAX: (215) 652-4538.

‡ Department of Biological Chemistry.

§ Department of Medicinal Chemistry.

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[1] Abbreviations: fXIIIa, human factor XIIIa or plasma transglutaminase; HET, human erythrocyte transglutaminase; DTT, dithiothreitol; PEG, poly(ethylene glycol); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TCA, trichloroacetic acid; TFA, trifluoroacetic acid; GSH, glutathione; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; **I**, 2-(1-acetonylthio)-5-methylthiazolo[2,3]-1,3,4-thiadiazolium perchlorate; **II**, 1,3-dimethyl-2-[(2-oxopropyl)thio]imidazolium chloride; **IIa**, 1-methyl-2-[(2-oxopropyl)thio]imidazole; **III**, 1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride; **IIIa**, 1,4,5-trimethyl-2-[(2-oxopropyl)thio]imidazole; **IV**, 1,3,4,5-tetramethyl-2-[[3-[(1-methyl-1*H*-tetrazol-5-yl)thio]-2-oxopropyl]thio]-1*H*-imidazolium chloride; [^{14}C]-**II**, 1,3-dimethyl-2-[(2-oxo[2-^{14}C]propyl)thio]imidazolium iodide; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

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Curtis et al., 1974; Chung et al., 1974). The intrinsically high reactivity of these agents toward thiol groups, however, limits their therapeutic utility. The mechanism-based inactivation of transglutaminases by halodihydroisoxazoles (Castelhano et al., 1988; Killackey et al., 1989) exemplified one approach for development of more selective inactivators of transglutaminases. As an alternative approach, the present study demonstrates that selective inactivators of transglutaminases can be prepared by replacing the halo leaving group of halomethyl carbonyl compounds with a leaving group that (i) lowers the intrinsic reactivity of the inhibitor as a nonspecific alkylating agent and (ii) interacts with the active site so as to increase the reactivity of the inhibitor toward fXIIIa.

Preliminary *in vitro* and *in vivo* studies in animal models of thrombosis with one such inactivator 2-(1-acetonylthio)-5-methylthiazolo[2,3]-1,3,4-thiadiazolium perchlorate (**I**) have been presented (Bush et al., 1988; Shebuski et al., 1990; Leidy et al., 1990; Freund et al., 1988). In this study, the *in vitro* results are extended to include 2-[(2-oxopropyl)thio]-imidazolium derivatives and the mechanism by which they inactivate fXIIIa. The use of these derivatives as cellular transglutaminase inactivators in rabbit hepatocytes has been described (Barsigian et al., 1991).

EXPERIMENTAL PROCEDURES

Enzyme Purification. FXIII was purified from citrated fresh frozen (stored at −70 °C), pooled normal human plasma as previously described (Curtis & Lorand, 1976). Following DEAE-cellulose chromatography, analysis by SDS–PAGE showed two bands of apparent molecular weights 80 000 and 78 000, corresponding to the a and b subunits, respectively (Schwartz et al., 1973). This enzyme preparation showed less than 5% thrombin-independent activity. When purified fXIII was activated and treated with [^{14}C]iodoacetamide (vide infra), 1 mol of iodoacetamide was incorporated per mole of tetramer (a_2b_2 300 000 g/mol) (Curtis et al., 1974; Chung et al., 1974). Protein was determined by the method of Bradford (1976). The average yield of fXIII from 8 L of plasma was 30–40 mg. Purified fXIII at 1 mg/mL in 50 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA, pH 7.5, showed no appreciable loss of enzymatic activity when stored at 4 °C for several weeks. Human erythrocyte transglutaminase (HET) was purified as previously described (Brenner & Wold, 1978) with modifications. Erythrocytes from 740 mL of human whole blood were washed and lysed (Brenner & Wold, 1978). The supernatant fraction from the erythrocyte lysate was adsorbed to 20 g of DEAE-cellulose (DE52 Whatman) and eluted with 67 mL of 0.5 M NaCl, 5 mM Tris-HCl, pH 7.5, and 1 mM EDTA. This eluate was concentrated 5-fold using an Amicon PM 30 filter and diluted to the original volume with 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.13 mM ATP. Following concentration to 7 mL this material was applied to a MonoQ HR 5/5 column (Pharmacia) (5 × 50 mm) equilibrated with 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1 mM ATP. The transglutaminase activity was eluted with a NaCl gradient (0–0.5 M) containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1 mM ATP over 40 min at a flow rate of 1 mL/min. One-milliliter fractions were collected and monitored for transglutaminase activity (vide infra). The active fractions (5 mL) were concentrated as above to 2 mL. This enzyme preparation was stable when stored at −70 °C. Purification to apparent homogeneity was achieved by rechromatography on the MonoQ column utilizing a 0.125–0.5 M NaCl gradient followed by gel filtration on two Protein Pak 300 SW columns in series, eluting with 0.15 M NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, and 0.1 mM ATP. The pure preparation, however, was unstable as previously reported (Brenner & Wold, 1978). Thus, for most studies, the less pure preparation was used and the results were confirmed in several instances with the homogeneous preparation. With respect to our studies, no significant differences between the two preparations were detected.

Activation of fXIII and Determination of Transglutaminase Activity. fXIII (0.015–0.6 mg/mL or 0.05–2 μM) in 50 mM Tris-HCl, 100 mM NaCl, 40 mM $CaCl_2$, 1 mM EDTA, 0.1 mM dithiothrietol (DTT) (Sigma), and 0.1% poly(ethylene glycol) (PEG) (Sigma; approximate molecular weight 8000), pH 7.5, was activated by the addition of 5 NIH units/mL human thrombin (Sigma). After 10 min at 37 °C the activation was quenched by the addition of 10 NIH units/mL hirudin (Sigma). HET was prepared in the same buffer but did not require thrombin activation. Enzyme samples were diluted to a final concentration of approximately 5 nM into an assay buffer composed of 50 mM Bicine (Sigma), pH 8.0, 10 mM $CaCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.1% PEG, 20 μM monodansylcadaverine (Sigma), and 0.25% dimethylcasein (Sigma). The covalent incorporation of monodansylcadaverine into dimethylcasein was monitored by an increase in fluorescence as previously described (Curtis & Lorand, 1976) using a Perkin-Elmer LS-5 fluorescence spectrophotometer with a fixed scale set at 2.0 and 5-nm excitation and emission slit widths. Enzyme activity was a linear function of enzyme concentration from 0.5 to 10 nM. Within this enzyme concentration range the observed fluorescence change was linear with respect to time for at least 10 min. At 5 nM fXIIIa, a 20% of full-scale deflection per minute was observed.

Enzyme Inactivation Rates. In order to determine rates of inactivation, samples of activated fXIII or HET at 0.05–0.5 μM were incubated in the activation buffer described above at 37 °C for various times with at least a 5-fold excess of inactivator and then diluted (30–300-fold) into the fluorescence assay solution, and the remaining activity was measured. Control experiments indicated that upon dilution and in the presence of substrates no appreciable inhibition or inactivation occurred during the assay. Loss of activity was monitored for at least three half-lives, and in all cases pseudo-first-order kinetics were observed. The second-order rate constant was calculated as the quotient of the pseudo-first-order rate constant and the concentration of the inactivator. When DTT was not present in the activation and the assay buffer, a time-independent 20–30% diminution in enzymatic activity for both fXIIIa and HET was observed. Therefore, enzyme inactivation rates were determined in the presence and absence of DTT. For those inactivators which react slowly with DTT, the rates of enzyme inactivation were independent of DTT. For those inactivators which react relatively rapidly with DTT, the rates of enzyme inactivation reported were those determined in the absence of DTT.

Rate of Glutathione Reaction with Transglutaminase Inactivators. For those compounds detectable by UV absorbance, the rate of reaction with glutathione (GSH) was measured by following the disappearance of inactivator and the formation of products (vide infra) in the presence of excess GSH. Solutions containing N_2-purged 100 mM, Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM GSH, and 0.5 mM inactivator were incubated at 37 °C. Samples were withdrawn at various times, frozen, and stored at −70 °C until the time of analysis. The samples were then applied to a Waters μBondapak C_{18} column equilibrated with 0.1% phosphoric

acid and eluted at 1.5 mL/min with a linear gradient of 5–95% acetonitrile over 30 min. The UV absorbance at 250 nm was monitored. The disappearance of inactivator followed pseudo-first-order kinetics in all cases.

The rate of reaction of GSH with iodoacetamide was determined by two methods. In the first method, the disappearance of GSH in the presence of excess iodoacetamide was followed by thiol titration. Solutions containing 100 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM iodoacetamide, and 0.5 mM GSH were incubated at 37 °C. Samples (20 μL) were withdrawn at various times and diluted into 1 mL of 100 mM sodium phosphate, pH 8.0, containing 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Pierce). The absorbance change at 412 nm was stable for several minutes. Standards in which 0.1–0.5 mM GSH were diluted 1:50 into DTNB containing iodoacetamide were prepared. The absolute absorbance change and linearity with respect to GSH concentration were independent of iodoacetamide, indicating that DTNB reacts more rapidly with GSH than does iodoacetamide and that iodoacetamide reacts relatively slowly with 5-thio-2-nitrobenzoic acid. In the second method, the rate of reaction of iodoacetamide with excess GSH was monitored by following the disappearance of [1-^{14}C]iodoacetamide and the appearance of a radiolabeled, ninhydrin-positive product. Starting materials and products were resolved by thin-layer chromatography. Reaction mixtures containing 10 mM GSH and 0.5 mM [^{14}C]iodoacetamide (NEN; 24 Ci/mol) in the same buffer as above were quenched with glacial acetic acid (10% final concentration) and applied to silica gel plates (Eastman Chromogram sheet 6060). The plates were developed with toluene/acetic acid/water/butanol/acetonitrile (1:1:1:1:1), and the reaction products were detected with ninhydrin. The plates were cut into 0.5-cm squares, which were then immersed in water (1 mL), and the radioactivity was determined after the addition of 10 mL of Aquasol (NEN). R_f values: iodoacetamide 0.8; GSH 0.5; product 0.35. Both methods yielded pseudo-first-order rate kinetics and similar bimolecular rate constants (k = 1.6, 1.4 M^{-1} s^{-1}, first and second methods, respectively).

Incorporation of Radiolabeled Inactivators into fXIIIa. Activated fXIII [0.6 mg/mL (2 μM) in 50 mM Tris, 100 mM NaCl, 40 mM $CaCl_2$, 1 mM EDTA, pH 7.5] was incubated at 37 °C with 10 μM inactivators. Loss of activity was monitored by testing 100-μL aliquots from the incubation in the fluorescence assay. The incorporation of radiolabeled inactivator was detected by precipitating the protein in equivalent aliquots with the addition of an equal volume of ice-cold 20% trichloroacetic acid (TCA). After 1 h on ice, the precipitate was collected by centrifugation. The pellet was washed successively with 5% TCA, 1:1 ethanol/acetone, and acetone and then dissolved in Protosol (NEN). The radioactivity in the Protosol solution was determined in Econofluor-2 (NEN) after neutralizing with an equimolar amount of glacial acetic acid. Protein recovery was quantitative.

Isolation of Labeled Active Site Peptide of fXIIIa. Thirty milligrams of activated fXIII [0.6 mg/mL (2 μM) in 50 mM Tris, 100 mM NaCl, 40 mM $CaCl_2$, 1 mM EDTA, pH 7.5] was incubated with 10 μM (10.6 Ci/mol) 1,3-dimethyl-2-[(2-oxo[2-^{14}C]propyl)thio]imidazolium iodide (vide infra) at 37 °C for 5 min. Loss of activity was monitored by testing samples from the incubation in the fluorescence assay ($T_{1/2}$ = 20 s). The inactivated enzyme was tested for incorporation of radiolabeled inactivator by precipitating an aliquot of the protein in TCA as previously described. The range of inactivator incorporated per mole of fXIIIa was 0.7–0.9 mol. Inactivated fXIIIa was then dialyzed extensively against 50 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, and 1 mM EDTA, pH 7.0, to remove excess inactivator. The enzyme was treated with chymotrypsin A_4 (Boehringer Mannheim) (1:100 chymotrypsin/fXIIIa) for 4 h at 37 °C. The digest was lyophilized and redissolved in water. Approximately 77% of the lyophilized material was recovered as determined by radioactivity. The solution was applied to a Sephadex G-25 column (1 × 120 cm) equilibrated in 10% acetic acid and eluted with a flow rate of 20 mL/h. All of the radioactivity applied to the column eluted in a single peak. Fractions containing the radioactivity were pooled and lyophilized (100% recovery). The labeled peptide was further purified on reversed-phase HPLC. The sample was applied to a Waters μBondapak C_{18} column (0.39 × 30 cm) equilibrated with 0.1% TFA and eluted with a flow rate of 1.5 mL/min using a linear gradient of 0–50% acetonitrile over 30 min. All of the radioactivity applied to the column eluted in a single peak and was analyzed by rechromatographing an aliquot using a shallower gradient (60 min). This analysis showed two peaks of absorbance at 214 nm. Therefore, the remaining sample was lyophilized, redissolved (75% recovery), and rechromatographed using the 60-min gradient. The two absorbance peaks were resolved, and the peak which contained all of the applied radioactivity was collected. The structure of this material was determined by proton NMR, UV analysis, and amino acid sequence analysis as described in the Results and Discussion section. An authentic sample of this material was prepared by the following procedure. A solution containing 10 mM Gly-Gln-Cys-Trp-OH (kindly provided by Dr. Victor Garsky, Merck), 100 mM 1,3-dimethyl-2-[(2-oxopropyl)thio]imidazolium chloride (vide infra), 100 mM Tris-HCl, and 5 mM EDTA, pH 7.4 (N_2 purged), was incubated at 37 °C for 24 h. Analysis by HPLC as described above demonstrated the presence of a reaction product which cochromatographed with the radiolabeled peptide derived from inactivated fXIIIa and another reaction product identified as 1,3-dimethylimidazole-2-thione. The peptidyl reaction product was isolated and its structure determined to be *S*-acetonyl-Gly-Gln-Cys-Trp-OH as described in the Results and Discussion section.

Proton NMR. Spectra were recorded with a Varian VXR-400S spectrometer at 400 MH_z and a sample temperature of 25 °C. D_2O (100 atom % D; Merck Isotopes) was used as a solvent in all cases. Chemical shifts are referenced to internal sodium 3-(trimethylsilyl)proprionate-*2,2,3,3-d*$_4$. The HOD resonance from residual water in the sample of isolated chymotryptic peptide was suppressed by presaturation. pH* values refer to direct readings with a pH meter and are uncorrected for the deuterium isotope effect.

Covalent Cross-Linking of ^{125}I-α_2-Antiplasmin into Plasmin Clots. Human α_2-antiplasmin (American Diagnostica) was radioiodinated either by using Pierce Iodo-Beads following the manufacturer's specifications with a 25-min reaction time at 0 °C or by the lactoperoxidase method (Marchalonis, 1969) employing Bio-Rad Enzymo-Beads with a 30-min reaction time at 20 °C. The radiospecific activities were 1.8 × 10^6 and 3.3 × 10^5 dpm/μg of protein, respectively. Both preparations had at least 60% of the specific activity of the unlabeled α_2-antiplasmin as determined by a plasmin inhibition assay (Naito & Aoki, 1978). To 0.3 mL of human citrated plasma was added approximately 2.5 × 10^5 dpm ^{125}I-α_2-antiplasmin followed by the addition of various concentrations of fXIIIa inactivators ~5 s before clot formation was initiated with human thrombin (Sigma) (5 units/mL) and $CaCl_2$ (40 mM)

(final concentrations). Clots were aged up to 2 h at 37 °C. To some samples was added 5 mM EDTA in place of $CaCl_2$ to prevent fXIIIa-catalyzed cross-linking without appreciably affecting clotting under these conditions. ^{125}I-fibrinogen (vide infra) was used as an indicator of clotability. Similar controls for fXIIIa inhibited samples were prepared using fXIII-deficient plasma (G. F. King). The clots were harvested and washed to remove noncovalently bound α_2-antiplasmin, and ^{125}I-radioactivity incorporated was determined as previously described (Sakata & Aoki, 1982). Approximately 15–20% of the α_2-antiplasmin was covalently bound. Sixty percent of this total was found within 3 min after clot formation was initiated. Less than 2% of the α_2-antiplasmin was covalently bound in those samples which contained EDTA or fXIII-deficient plasma. The labeled clots were solubilized in 8 M urea, 10% sodium dodecyl sulfate (SDS), and 10% mercaptoethanol at 37 °C for 16 h prior to 100 °C for 5 min and then applied to 7.5% polyacrylamide gel electrophoresis (SDS–PAGE) for further analysis.

Measurement of Clot Lysis Rates in an in Vitro Plasma Assay. Clots were formed (Gaffney & Whitaker, 1979) from 0.3 mL of citrated normal human plasma containing 5–10 μg/mL ^{125}I-fibrinogen [labeled by the Iodogen method (Fraker & Speak, 1978) to a radiospecific activity of 25 μCi/mg and exhibiting 90–95% clotability] by the addition of 40 mM $CaCl_2$ and 5 units/mL thrombin final concentration. Addition of fXIIIa inactivators was made ~5 s prior to the addition of thrombin and $CaCl_2$. The clots were incubated at 37 °C for 30 min, harvested, and washed as previously described (Gaffney & Whitaker, 1979). The clots were transferred to lysis buffer (2 mL) containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 300 IU/mL tissue plasminogen activator (two-chain form, Bioresponse). The clots were incubated at 37 °C, and 100-μL aliquots of the lysis buffer (supernatant fluid above the clot) were withdrawn at various times. Solubilized ^{125}I-fibrin was determined and the rate of clot lysis calculated, correcting for subsampling.

Generation of High Molecular Weight Fibrin α Chain Cross-Linked Polymers from Platelet-Enriched Whole Blood (Francis & Marder, 1987). To 300-μL aliquots of human or canine (mongrel) whole blood drawn without anticoagulants and enriched with autologous platelets 6 times the normal concentration (Derian & Friedman, 1988) was added, respectively, 180×10^3 dpm ^{125}I-human or canine fibrinogen (Birken et al., 1975) (Fraker & Speak, 1978) (1×10^5 dpm/μg of protein; 88% clotability). Clot formation was initiated by the addition of 5 units/mL human thrombin (Sigma) and 40 mM $CaCl_2$ final concentrations. The clots were aged for 2 h at 37 °C in a water bath with gentle shaking. Polypropylene tubes were used throughout this procedure. fXIIIa inactivators were added to the whole blood before the initiation of clot formation or to the serum of the retracted clot at various times during the aging process. The clots were then washed in 100 mM EDTA, 0.05M Tris-HCl, pH 7.5, and 0.1 M NaCl to prevent further cross-linking and immediately placed in a solubilizing buffer consisting of 8 M urea, 10% SDS, and 10% 2-mercaptoethanol for 48–72 h at 56 °C. The cross-linked high molecular weight fibrin α chain polymers were resolved as previously described (Francis & Marder, 1987) using 2% agarose, 0.1% SDS gels with a 0.8% agarose stacking gel. The air-dried gels were autoradiographed using SB5 X-ray film for 3 days at −70 °C.

Assay of Other Enzymes. The following enzymes were assayed using published procedures: papain (Sigma, type IV) (Thompson et al., 1986), fatty acid synthetase (Alberts et al., 1974), 2-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthetase (Greenspan et al., 1987), and HMG-CoA reductase (Alberts et al., 1980).

Syntheses of Transglutaminase Inactivators. 2-(1-Acetonylthio)-5-methylthiazolo[2,3]-1,3,4-thiadiazolium Perchlorate (**I**). The title compound was prepared as described by Jones (1966).

1,3-Dimethyl-2-[(2-oxopropyl)thio]imidazolium Chloride (**II**) (*Kister et al., 1979*). To a solution of 0.41 g (0.018 mol) of sodium in 20 mL of absolute ethanol was added 2.0 g (0.0175 mol) of 1-methyl-2-mercaptoimidazole (Aldrich). The solution was stirred at room temperature, and 1.62 g (0.0175 mol) of chloroacetone was added. After 2 h, the ethanol was removed under reduced pressure, and the residue was partitioned between ethyl acetate and water. The organic phase was washed with water, 2–10-mL portions of 5% sodium hydroxide solution, and 2–10-mL portions of water and dried over $MgSO_4$. After filtration, evaporation of the solvent gave 1.34 g of 1-methyl-2-[(2-oxopropyl)thio]imidazole (**IIa**) as a tan oil. The methiodide salt of this compound was prepared and crystallized from ethanol; mp 180–184° (darkens). Anal. Calcd for $C_8H_{13}IN_2OS$: C, 30.78; H, 4.20; N, 8.98. Found: C, 30.79; H, 4.23; N, 8.84.

The imidazolium chloride salt could be prepared from the methiodide salt by the use of Dowex-1 (Cl^-) ion-exchange chromatography. The product was recrystallized from isopropyl alcohol; mp 174–177 °C. Anal. Calcd for $C_8H_{13}ClN_2OS$: C, 43.53; H, 5.94; N, 12.69. Found: C, 43.60; H, 5.96; N, 12.90.

1,3,4,5-Tetramethyl-2-[(2-oxopropyl)thio]imidazolium Chloride (**III**) (*Kister et al., 1979*). To a solution of 3.55 g (0.025 mol) of 1,4,5-trimethyl-2-mercaptoimidazole (Kjellin & Sandstrom, 1969) and 3.15 g (0.031 mol) of triethylamine in 250 mL of acetone was added 2.55 g (0.28 mol) of chloroacetone in 250 mL of acetone. The solution was stirred at room temperature for 16 h. The acetone was removed by evaporation, and the residue was partitioned between ethyl acetate and water. The ethyl acetate phase was washed with water and brine and dried over magnesium sulfate. Evaporation of the solvent gave 3.39 g of 1,4,5-trimethyl-2-[(2-oxopropyl)thio]imidazole (**IIIa**) that was purified by flash chromatography on silica gel using 1% CH_3OH in $CHCl_3$ as an eluant to afford 3.0 g of pure **IIIa**.

To a solution of 3.0 g (0.0151 mol) of **IIIa** in 20 mL of CH_2Cl_2 was added 2.48 g (0.0151 mol) of methyl (trifluoromethyl)sulfonate, and the solution was stirred overnight at room temperature. The solvent was evaporated and the residue was converted to the chloride ion form by use of Dowex-1 (Cl^-) ion-exchange chromatography using 20% methanol in water as a solvent. Evaporation of the solvent and crystallization of the residue from isopropyl alcohol/hexane gave the title compound; mp 172–175 °C. Anal. Calcd for $C_{10}H_{17}ClN_2OS$: C, 48.28; H, 6.89; N, 11.26. Found: C, 48.33; H, 7.02; N, 10.82.

1,3,4,5-Tetramethyl-2-[[3-[(1-methyl-1H-tetrazol-5-yl)thio]-2-oxopropyl]thio]-1H-imidazolium Chloride (**IV**). To a solution of 6.5 g (0.05 mol) of 1,3-dichloroacetone in 15 mL of ether and 10 mL of absolute ethanol was added dropwise a solution of 1.38 g (0.01 mol) of 5-mercapto-1-methyltetrazole (Aldrich) sodium salt hydrate in 10 mL of ethanol. After stirring at room temperature for 3 h, the solution was filtered and then was cooled. The precipitate that formed was removed by filtration, triturated with ether, and collected to give 0.33 g of 1-chloro-3-[(1-methyltetrazol-5-yl)thio]-2-propanone. A solution of 0.33 g (0.00159 mol) of this compound and 0.249

g (0.00159 mol) of 1,3,4,5-tetramethylimidazoline-2-thione (Kjellin & Sandstrom, 1969) in 10 mL of acetone was stirred overnight at room temperature. The product that precipitated was collected by filtration and was recrystallized from isopropyl alcohol/acetone/hexane to give 0.27 g of the title compound; mp 115–117 °C. Anal. Calcd for $C_{12}H_{19}ClN_6OS_2$: C, 39.71; H, 5.28; N, 23.16. Found: C, 39.54; H, 5.11; N, 23.12.

1,3-Dimethyl-2-[(2-oxo[2-^{14}C]propyl)thio]imidazolium Iodide ([^{14}C]-**II**). Sodium [1-^{14}C]acetate (87 mg, 1.06 mmol, 28.2 mCi, 27 mCi/mmol) was treated with 0.1 equiv of oxalyl chloride in methylene chloride, and the reaction mixture was stirred at room temperature for 48 h. Filtration of the reaction mixture provided a solution of acetyl chloride in methylene chloride (23.96 mCi, 85%). This solution was treated with 7.5 mL of an ethereal solution of diazomethane (0.48 M, 4 equiv) at 0 °C. The reaction mixture was allowed to warm to room temperature gradually over a period of 4–5 h, treated with ethereal HCl (1.7 mL, 2.96 mmol/mL, 5.03 equiv), and then stirred overnight. GC analysis indicated the formation of chloroacetone (Carbowax column, 95 °C). This solution of chloroacetone in ether/methylene chloride was reacted with excess sodium imidazole thiolate in ethanol at room temperature. Workup after overnight stirring provided crude 1-methyl-2-[(2-oxo[2-^{14}C]propyl)thio]imidazole (10 mCi). Preparative silica HPLC purification (Whatman M9, 1% MeOH in CH_2Cl_2) of the crude product yielded two fractions of 1-methyl-2-[(2-oxo[2-^{14}C]propyl)thio]imidazole (6.7 mCi, radiochemical purity 92%, and 1.2 mCi, radiochemical purity 80%). A portion of the higher purity 1-methyl-2-[(2-oxo-[2-^{14}C]propyl)thio]imidazole (18 mg, 2.5 mCi) was treated with 1 equiv of methyl iodide in acetone. Filtration of the reaction mixture after overnight stirring provided 16 mg of the title compound (radiochemical purity 89%). This material was swished three times in 2-propanol to furnish 8 mg of 96% radiochemically pure title compound. Dilution of this product with carrier title compound (13 mg) in acetonitrile followed by swishing in 2-propanol gave pure title compound (17.7 mg, 33.9 μCi/mg, 600 μCi, radiochemical purity 98%).

[N-methyl-^{14}C]1,3-Dimethyl-2-[(2-oxopropyl)thio]imidazolium Iodide. A solution of 1 mmol of 1-methyl-2-[(2-oxopropyl)thio]imidazole in 0.5 mL of acetone was added to 1.5 mL of acetone containing 1 mmol of [^{14}C]methyl iodide (28 mCi/mmol). After stirring at room temperature for 15 h, the solid which precipitated was collected by filtration, washed with cold acetone, and dried *in vacuo* to yield 12.6 mg of the title compound; radioactive purity >95%; specific activity 95.1 μCi/mg.

[2] The apparent second-order rate constants (Table 1) were calculated as the quotient of the observed pseudo-first-order rate constant and the concentration of the inactivator, $k_{obs}/[I]$. For all cases in Table 1, k_{obs} values were directly proportional to [I], behavior consistent with a simple second-order irreversible reaction between enzyme and I. However, for compounds **I–IV**, the following pathway for enzyme inactivation is likely to be operative (please see text):

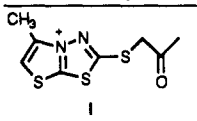
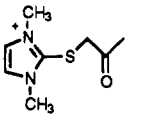
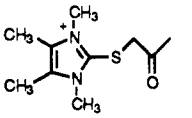
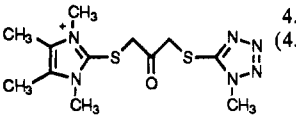
$$E + I \underset{K_i}{\rightleftarrows} (E\cdot I) \xrightarrow{k} EI'$$

Under conditions where formation of the initial complex (E·I) is rapid relative to irreversible conversion of (E·I) to EI′ and [I] ≫ [E], the dependence of k_{obs} on inhibitor concentration is (Kitz & Wilson, 1962):

$$k_{obs} = k[I]/([I] + K_i)$$

When [I] ≪ K_i (see text), $k_{obs} = k[I]/K_i$ and $k_{obs}/[I] = k/K_i$. Thus, for this mechanism the apparent second-order rate constants for compounds **I–IV** are equal to k/K_i. The latter is analogous to k_{cat}/K_m (see text) and has been referred to as a specificity constant of inactivation.

Table 1: Comparison of Apparent Second-Order Rate Constants for fXIIIa and HET Inactivation and Glutathione Reaction

compound	k_{fXIIIa}	k_{HET} (M^{-1} s^{-1})	k_{GSH}
 I	2.3×10^4 [a] (1.9×10^5)[b]	1.9×10^4 (1.6×10^5)	120×10^{-3}
 II	0.35×10^4 (1.9×10^6)	0.53×10^4 (2.9×10^6)	1.8×10^{-3}
 III	6.3×10^4 (3.9×10^7)	2.6×10^4 (1.6×10^7)	1.6×10^{-3}
 IV	4.9×10^4 (4.1×10^6)	0.69×10^4 (5.8×10^5)	12×10^{-3}
iodoacetamide	0.8×10^4 (5.0×10^3)	12×10^4 (7.5×10^4)	1600×10^{-3}

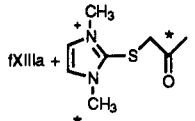
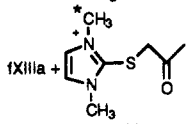
[a] The range of these values was ±15%. [b] The numbers in parentheses represent the ratio of the rate constant for fXIIIa or HET inactivation to the rate constant for glutathione reaction.

RESULTS AND DISCUSSION

The thioacetonyl heterocycles (**I–IV**) and iodoacetamide inactivated the transglutaminases fXIIIa and HET with apparent second-order rate constants[2] that ranged from 0.3 × 10^4 to 12 × 10^4 M^{-1} s^{-1} (Table 1). The ratio of the rate constants for enzyme inactivation and for the reaction with GSH provides a measure of the selectivity of each inactivator. These values are also listed in Table 1 (in parentheses) to underscore the marked dependence of selectivity on the structure of the inactivator. For example, the reactivity of **III** toward fXIIIa was 8-fold greater than that of iodoacetamide even though the reactivity of **III** toward GSH was 1000-fold lower than that of iodoacetamide. Additionally, **III** inactivated fXIIIa 18 times faster than **II** even though both compounds exhibited similar reactivity toward GSH; **III** and **IV** inactivated fXIIIa at similar rates even though the reactivity of **IV** toward GSH was more than 7-fold greater than that of **III**. A comparison of the rates of inactivation of fXIIIa to those of HET by the compounds listed in Table 1 suggested that the structure of the inactivator, and not any difference in intrinsic nucleophilicity that may exist between the two enzymes, was the primary determinant of selectivity. For example, iodoacetamide preferentially inactivated HET while **IV** preferentially inactivated fXIIIa.

On the basis of these results the mechanism of fXIIIa inactivation by the thioacetonyl heterocycles was studied. For this purpose, the reaction of fXIIIa with radiochemically labeled **II** was investigated. Examination of the data in Table 2 revealed that, like iodoacetamide, the thioacetonyl heterocycle **II** formed a covalent complex with fXIIIa with a 1:1 stoichiometry as judged by the amount of radioactivity incorporated into fXIIIa during its inactivation. The observed stoichiometry of labeling with iodoacetamide or **II** of only one group per dimer of activated fIIIa is consistent with several studies that have shown "half-of-the-sites" reactivity for this

Table 2: Incorporation of Radioactivity Derived from [1-^{14}C]Iodoacetamide and [^{14}C]-Isotopic Regioisomers of **II** into fXIIIa

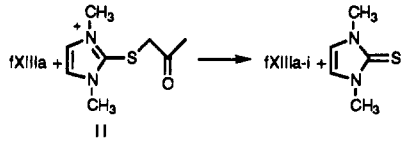
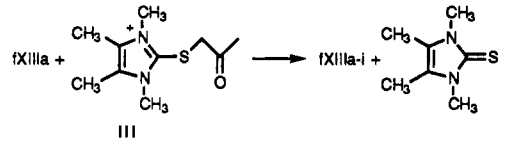
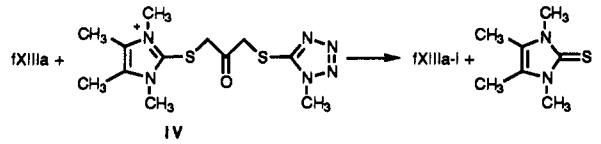
	mol of [^{14}C]/mol of fXIIIa
fXIIIa + [^{14}C]iodoacetamide	1.0 ± 0.1[b]
fXIIIa + 	0.9 ± 0.2[b]
fXIIIa + 	<0.01[c]
fXIIIa-i[a] + [^{14}C]iodoacetamide	<0.01[c]

[a] fXIIIa-i represents fXIIIa pretreated (inactivated) with nonradiolabeled **I**, **II**, **III**, or **IV** (5 μM) prior to treatment with [^{14}C]iodoacetamide. [b] Values represent the average of duplicate determinations ± range. [c] The limit of detectability.

enzyme (Chung et al., 1974; Seelig & Folk, 1980; Hornyak et al., 1989; Bishop et al., 1990; Hornyak & Shafer, 1991). The incorporation of radioactivity into fXIIIa, however, depended upon which isotopic regioisomer of **II** was employed. The failure to observe radioactive labeling of fXIIIa that was inactivated with **II** containing ^{14}C at the *N*-methyl carbon atoms (Table 2) indicated that inactivation does not involve direct methylation of fXIIIa or irreversible addition of an enzyme nucleophile to the heterocyclic ring. Furthermore, these results implied that *N*,*N*′-dimethyl-2-thioimidazole might be eliminated during inactivation via the scheme depicted in Table 3 (row 1). This conclusion was supported by HPLC analysis of reaction mixtures that corresponded to fXIIIa inactivation by **II**–**IV** (Table 3). Stoichiometric with inactivation of fXIIIa, inactivators **II**–**IV** were depleted and a product was generated that cochromatographed with the indicated thione derivative. In the case of **IV**, two different thiones might be generated. Exclusive release of the imidazole-containing thione was obtained in the reaction of **IV** with fXIIIa. In the reaction of **IV** with GSH, however, a 15% yield of the tetrazole-containing thione was observed (data not shown).

Prior inactivation of fXIIIa with **I**–**IV** blocked the reaction of fXIIIa with [^{14}C]iodoacetamide (Table 2). Additionally, the inactivation of fXIIIa by **I**–**IV** showed a dependence upon Ca^{2+} which was similar to the Ca^{2+} dependence for inactivation by iodoacetamide (data not shown). These observations are consistent with the hypothesis that, like iodoacetamide, compounds **I**–**IV** react with the active site thiol (Folk & Finlayson, 1977; Holbrook et al., 1973; Curtis et al., 1974; Chung et al., 1974). Verification of this mode of inactivation of fXIIIa by **II** was obtained by characterizing the radiochemically labeled chymotryptic peptide obtained from fXIIIa that had been inactivated by [^{14}C]-**II**. Amino-terminal sequence analysis of a sample of this radiochemically labeled peptide indicated Gly (0.94 nmol)–Gln (1.28 nmol) in cycles one and two, respectively (data not shown). This analysis is in good quantitative agreement with the expected yield of 1.1 nmol based on the specific activity of the radiolabel and a 1 mol/mol incorporation of inactivator to peptide. UV analysis indicated the presence of a stoichiometric amount of tryptophan. These data, along with the known specificity of chymotrypsin and the primary structure of fXIII (Takahashi et al., 1986), are sufficient to define the structure of the radiolabeled peptide as Gly-Gln-X-Trp, where X is the modified active site cysteine containing the radiolabel.[3] Proton NMR (Figure 1) confirmed the composition of the peptide and provided the structure of this modified cysteine. The resonances which are attributable to the tetrapeptide derivative are characteristic of Gly, Glx, Cys, and Trp residues. Signals from the Trp aromatic protons are found in the 7.1–7.7 ppm region. Similarly, characteristic Gly-α proton resonances are found centered at 3.8 ppm and Glx-β and γ proton resonances

Table 3: HPLC Identification of Low Molecular Weight Products Released from **II**–**IV** during fXIIIa Inactivation[a]

	mol of inactivator consumed per mole of fXIIIa	mol of LMW product released per mole of fXIIIa
 **II**	1.18 ± 0.19[b]	1.08 ± 0.12
 **III**	0.93 ± 0.16	1.01 ± 0.18
 **IV**	0.73 ± 0.36	0.89 ± 0.15

[a] fXIIIa (250 μL of 0.6 mg/mL, 2 μM, in 50 mM Tris, 100 mM NaCl, 40 mM $CaCl_2$, and 1 mM EDTA, pH 7.5) was incubated at 37 °C with 10 μM inactivator (**II**, **III**, or **IV**). Loss of activity was monitored by fluorescence assay. The inactivated fXIIIa solution was ultrafiltered (10K MW cutoff, Millipore), and the filtrate was analyzed by HPLC. The sample was applied to a Waters μBondapak C_{18} column (0.39 × 30 cm) equilibrated with 0.1% phosphoric acid and eluted at 1.5 mL/min with a linear gradient of 5–95% acetonitrile over 30 min. The UV absorbance at 270 nm was monitored. The chromatograms were compared with those of filtrates each containing either fXIIIa, 10 μM inactivator, or 2 μM putative product standard (1,3-dihydro-1,3-dimethyl-2*H*-imidazole-2-thione, 1,3-dihydro-1,3,4,5-tetramethyl-2*H*-imidazole-2-thione, or 5-mercapto-1-methyltetrazole) prepared in the same manner. To confirm identity, in some samples putative product standard was added to the inactivated enzyme solution before ultrafiltration. [b] Values represent the mean ± SD (n = 4).

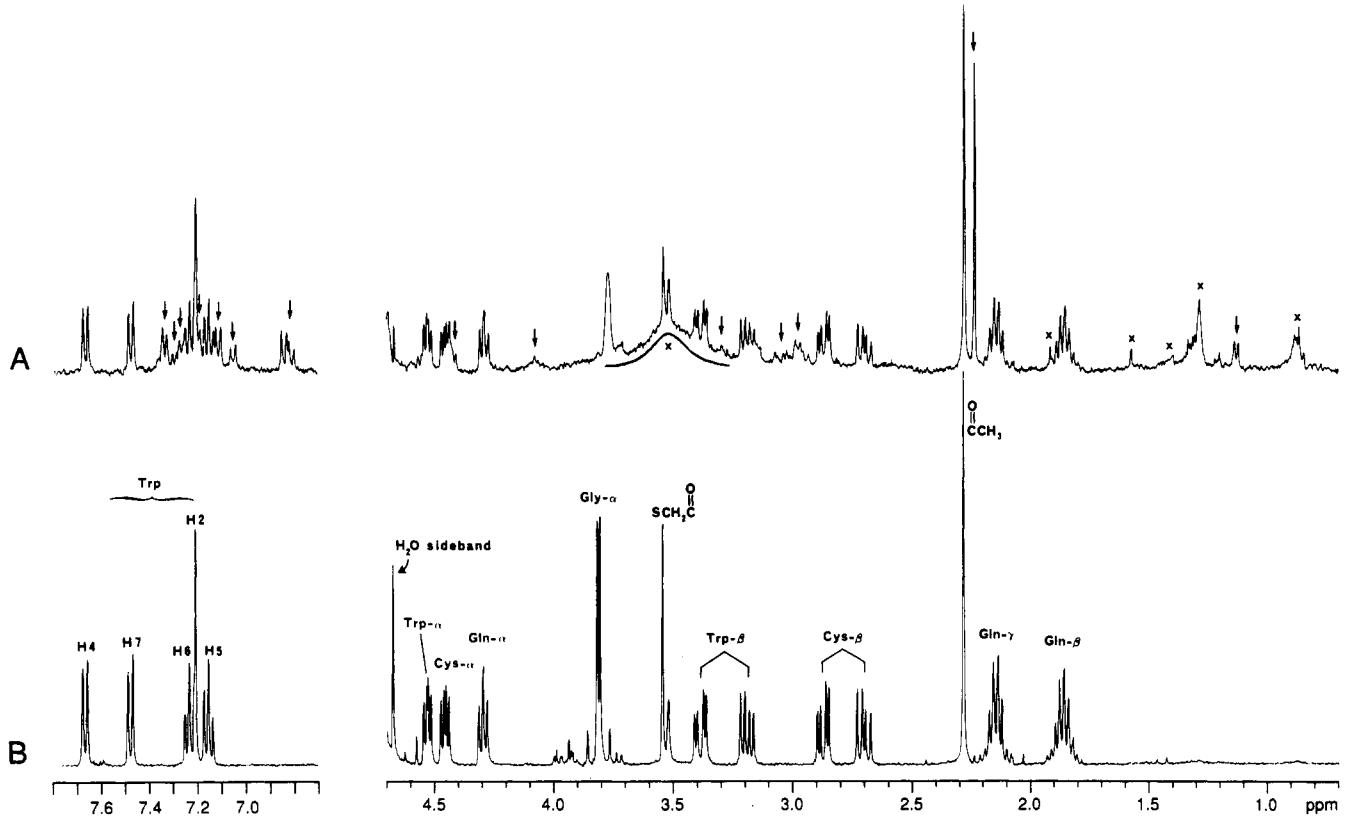
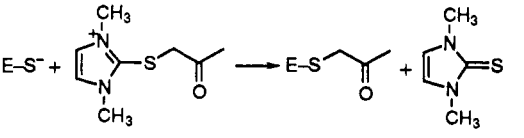


FIGURE 1: (A) Proton NMR spectrum of the radiolabeled chymotryptic peptide (30 nmol) derived from fXIIIa inactivated by [^{14}C]-**II**. Impurities which were found in spectra of HPLC cuts immediately before and after elution of the peptide are denoted with x's. Arrows denote minor, coeluting impurities. Line broadening (1.0 Hz) and a third-order polynomial base-line correction were applied to improve the appearance of the spectrum. (B) Proton NMR spectrum of synthetic Gly-Gln-(acetonyl)Cys-Trp (250 nmol) dissolved in D_2O.

are found at 1.86 and 2.15 ppm. Decoupling experiments show that the α proton resonance at 4.30 ppm belongs to the Glx residue. The AMX patterns of resonances at 4.53, 3.39, and 3.19 ppm are appropriate for the Trp-α and two Trp-β protons, respectively. The AMX system at 4.46, 2.87, and 2.70 ppm can be attributed to an S-substituted Cys residue where the substituent has shifted the α and β resonances upfield [nominal chemical shifts (Bundi & Wuthrich, 1979) are 4.69, 3.28, and 2.96 ppm for the α and β resonances, respectively].

Consistent with the conclusion that the substituent on the Cys sulfur is an acetonyl group, proton NMR spectra of a freshly prepared sample of the chymotryptic tetrapeptide show resonances for the acetonyl group at 3.55 ppm (SCH_2CO) and at 2.28 ppm ($COCH_3$). Diagnostic of this functional group is an exchange process where the five acetonyl protons exchange with deuterons of the D_2O solvent. The SCH_2CO protons exchange over a period of hours, and the $COCH_3$ protons exchange over a period of days. For an unbuffered D_2O solution, the relative rates of exchange are approximately 1:100 (rate constants are statistically corrected for the number of exchangeable hydrogens at the site being observed). The ratio compares favorably with a 1:137 ratio reported for the corresponding sites of 1-(methylthio)-2-propanone (Guth et al., 1982).

Although peptide sequences can often be determined by nuclear Overhauser enhancement NMR experiments, the limited sample size (~30 nmol) prevented these experiments. Conclusive evidence for the Gly-Gln-(acetonyl)Cys-Trp sequence was obtained by spectroscopic comparison of synthetically prepared peptide with the chymotryptic peptide (Figure 1). The only difference between the proton resonances of the two peptides is that Gly-α protons of the synthetic peptide resonate 0.03 ppm further downfield than those of the chymotryptic peptide. This result is consistent with the N-terminal position of the Gly residue and the fact that the synthetic peptide solution was more acidic (pH* ~5) than the solution of the chymotryptic peptide (pH* = 7.58). The nonintegral peaks seen only in the chymotryptic peptide (arrows and x's) are attributed to small amounts of coeluting peptide impurities and impurities from the HPLC solvents. Thus, these results, in conjunction with those discussed above, demonstrate that inactivation results from the acetonylation of the active site cysteine residue with concomitant release of the complementary thione:



The high reactivity of the alkylating agents with the enzymes relative to GSH probably reflects (i) a higher intrinsic reactivity of the active site thiol group relative to that of the thiol group in GSH and (ii) orientational effects that can facilitate or inhibit a reaction with the active site thiol group. An ion pair interaction similar to the one that exists in the thiol proteinase papain (Drenth et al., 1976) might serve to ensure that the active site thiol group exists primarily as a thiolate anion at the pH where reaction with the enzymes was studied. Since only 0.038 of the thiol group of GSH (pK 8.9) would be present in the reactive thiolate form at the pH of 7.5 where the GSH reaction was studied, an environment that

[3] During sequence analysis, 20% of the applied radioactivity was released at cycle three; the remainder of the radioactivity was retained on the sequencing disc.

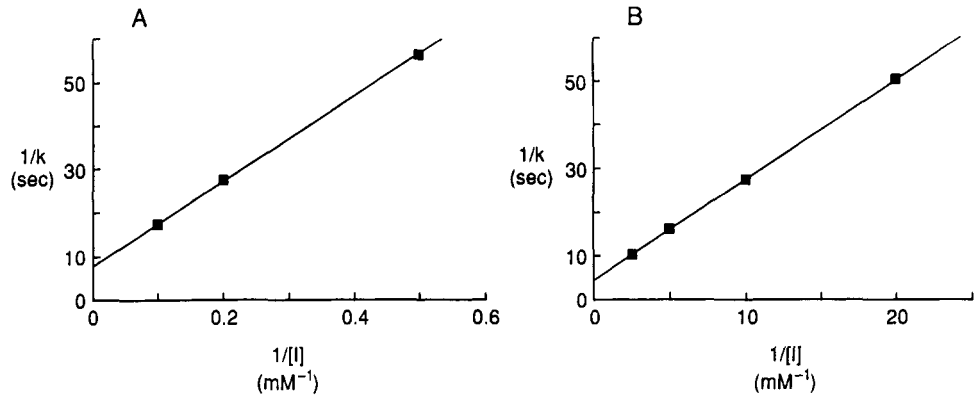
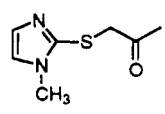
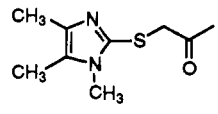


FIGURE 2: Dependence of apparent pseudo-first-order rate constant on the concentration of the nonquaternary derivatives of **II** (**IIa**, panel A) and **III** (**IIIa**, panel B). See text for structures of **IIa** and **IIIa**. Inactivation rates were determined as described in the Experimental Procedures section, and the data were analyzed by the method of Kitz and Wilson (1962). The analysis is representative of three separate experiments where the ranges of K_i and k values described in the text were <5%.

ensured complete ionization of the active site thiol group might account for a 26-fold enhancement (1/0.038) in the apparent reactivity. The observation that the active site thiol groups are 5×10^3 to 4×10^7 more reactive than the thiol group of GSH suggests that other factors must also be responsible for the increased reactivity of the active site thiol groups toward the inactivating agents. Prior to inactivation, the enzymes might interact with their inhibitors to form a reversible complex analogous to Michaelis-like complexes that form between enzyme and substrate during normal catalysis. In this regard, one possibility is that the enzyme can facilitate alkylation of the active site thiol group by catalyzing the addition of the thiol group to the carbonyl carbon atom of the inhibitor. The thiol group in the resulting thiohemiketal (reversible complex) might then be rapidly alkylated via a three-membered cyclic transition state characteristic of bivalent sulfur (Capon & McManus, 1976). Although further studies are needed to establish such a reaction pathway, it is interesting to note that precedent exists for the formation of hemiketals and hemithioketals during the inactivation of serine and thiol proteinases with ketonic reagents such as halomethyl ketones and diazomethyl ketones (Poulos et al., 1976; Brocklehurst & Malthouse, 1978; Stein & Trainor, 1986). The mechanism of fXIIIa inactivation discussed here may have several aspects in common with the mechanism of thiol protease inactivation by peptidyl sulfonium salts described by Shaw (1988). For compounds **I**–**IV** and iodoacetamide, analysis by the method of Kitz and Wilson (1962) provided no kinetic evidence for the formation of a reversible EI complex prior to fXIIIa inactivation. The observed pseudo-first-order rate constant for irreversible inactivation of fXIIIa was proportional to inhibitor concentration, behavior consistent with a simple second-order irreversible reaction between E and I. Alternatively, if the value of K_i, the dissociation constant of an (EI) complex, is significantly greater than the experimentally accessible values of [I], then similar results would obtain. The rapid rates of inactivation (e.g., $T_{1/2}$ = 0.5 s with 20 μM **III**) precluded measurements of rate constants at inhibitor concentrations higher than 20–100 μM. To assess the possibility that compounds such as **II** and **III** might form a complex with fXIIIa prior to inactivation, reactions of **IIa** and **IIIa** with fXIIIa were studied:



IIa



IIIa

These compounds inactivate fXIIIa at rates that are about 100-fold slower than the analogous quaternary compounds so that the dependence of k_{obs} on [I] could be extended to higher values of [I]. The observation that the plots (Figure 2) extrapolate to nonzero values is indicative of saturation and the formation of an EI complex. Analysis of the plots yielded values of 13 mM and 0.5 mM for K_i's of the complex between E and **IIa** and E and **IIIa**, respectively. Thus, dimethyl substitution results in a 26-fold reduction in K_i and a 2-fold increase in k (50-fold difference in k/K_i). These data provide evidence, albeit indirect, that the enhancement in rates of inactivation observed for **III** relative to **II** (Table 1) results from specific interactions between **III** and fXIIIa which can reduce K_i and perhaps increase k in much the same manner as intrinsic binding energy of substrates can be utilized to decrease K_m or increase k_{cat}.

The possible formation of EI complexes could also explain the selectivity of 2-[(2-oxopropyl)thio]imidazolium derivatives for inactivating transglutaminases. For example, **III**, which inactivates fXIIIa 4×10^7 times more rapidly than it reacts with GSH (Table 1), was studied with five other thiol reagent-sensitive enzymes: papain, calpain, fatty acid synthetase, HMG CoA synthetase, and HMG CoA reductase. At 1 mM, **III** displayed no detectable levels of inhibition or inactivation of these enzymes (data not shown). **III** was also negative in the microbial mutagenesis assay (Ames test) and at 10^{-4} M (the highest concentration tested) had no effect on prothrombin time or partial thromboplastin times, indicative of its relatively weak potency for serine proteases.

The ability of the fXIIIa inactivators **I**–**IV** to inhibit fXIIIa-catalyzed cross-linking reactions within whole blood and plasma clots was studied. Since one of the goals of this research is to determine the effects of pharmacologically inhibiting fXIIIa in animal models of thrombosis, human and canine whole blood was utilized. In the experiments discussed below no species-related differences were observed; fXIIIa activity assayed directly from human and canine plasma is equally sensitive to inhibition by **I**–**IV**. The formation of high molecular weight cross-linked α chain fibrin polymers (some unable to enter 2% SDS agarose gels) has been observed and characterized in platelet-enriched whole blood clots (Francis & Marder, 1987) (Figure 3). The progress of α chain polymer formation can be arrested by fXIIIa inactivators such as **I** and **III** (Figure 3). This is evident from the molecular weight distribution of ^{125}I-fibrin from a clot aged for 30 or 60 min in the absence of an inactivator (Figure 3A) to the molecular weight distribution of ^{125}I-fibrin from a clot to which was

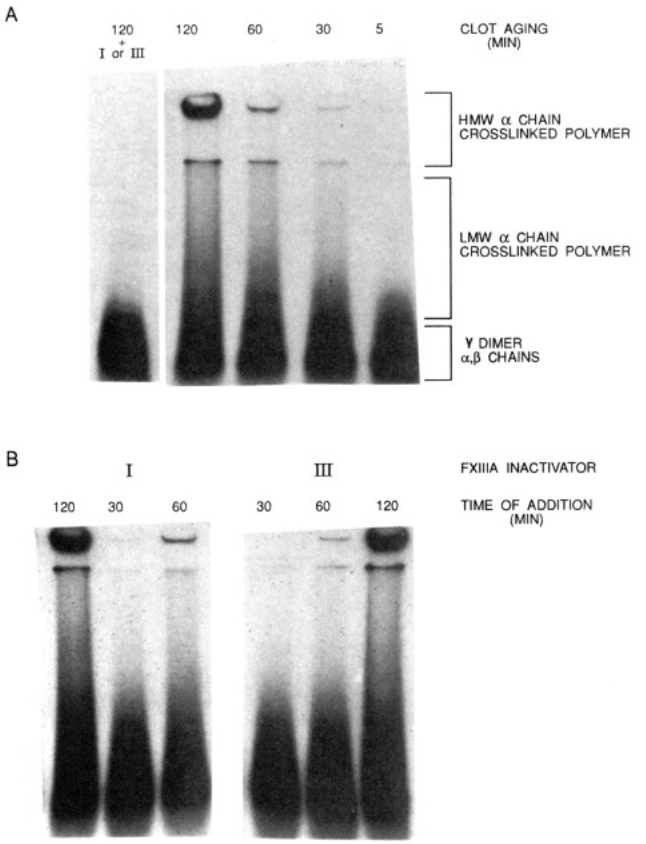


FIGURE 3: Effects of the fXIIIa inactivators **I** and **III** on the generation of high molecular weight α chain cross-linked polymers from platelet-enriched whole blood. Conditions for clot formation and analyses are described in the Experimental Procedures section. Panel A: Clots aged for 5, 30, 60, and 120 min (right to left) in the absence of a fXIIIa inactivator. **I** or **III** at 20 μM was added prior to clot formation and the resulting clot (most left) aged for 120 min (results were identical with **I** or **III** at 20 μM). Panel B: All clots were aged for 120 min. The fXIIIa inactivators **I** or **III** were added to a final concentration of 20 μM at 30, 60, and 120 min during the time of clot aging.

added either **I** or **III** at 30 or 60 min (Figure 3B) during a 120-min aging period. (In control experiments at 10^{-4} M, **I** and **III** do not inhibit platelet aggregation.) These same clots were analyzed for lower molecular weight fXIIIa-catalyzed cross-linked products on 7% SDS–PAGE. γ-γ chain dimer formation, a reaction which occurs much more rapidly than α chain polymer formation (Tamaki & Aoki, 1981), was inhibited 30% by **I** and 50–60% by **III** (data not shown). The covalent incorporation (fXIIIa-catalyzed) of the potent plasmin inhibitor, α_2-antiplasmin, into the α chain of fibrin can also be inhibited by fXIIIa inactivators. As a specific example, **I** at 20 μM prevents 93% of the incorporation of ^{125}I-α_2-antiplasmin into the α chain of fibrin (Figure 4, lane 1). **I** at 7 μM prevented 85% incorporation, while **II**, a less potent inactivator (Table 1), at 100 and 30 μM prevented 95% and 80% incorporation, respectively (data not shown). Clots aged for 5 min in untreated controls contained a significant amount of ^{125}I-α_2-antiplasmin (Figure 4, lane 2) incorporated into the fibrin chain. Reflecting the ongoing polymerization in untreated controls of the fibrin chains to which the ^{125}I-α_2-antiplasmin was cross-linked, the molecular weight distribution of the radioactivity favored higher molecular weight species as the time of clot aging was increased to 30 min (Figure 4, lanes 2–4). The distribution of radioactivity derived from the relatively low amounts of ^{125}I-α_2-antiplasmin that were cross-linked to fibrin in clots treated with **I** and aged for 30 min (Figure 4, lane 1) indicated that

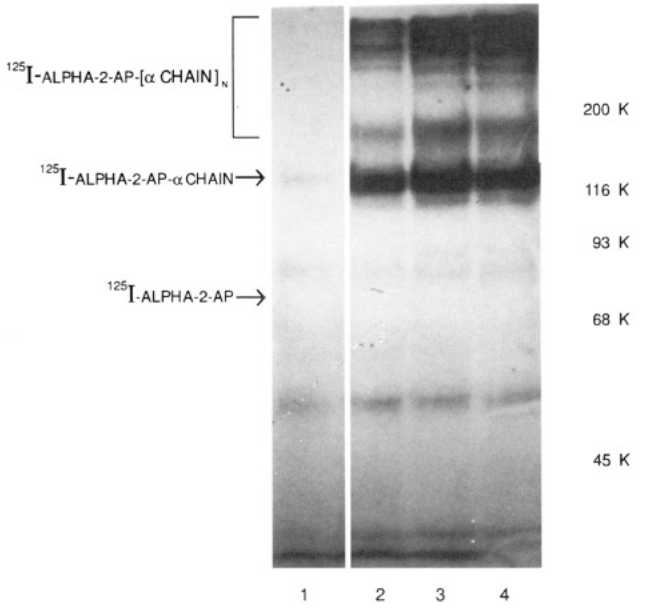


FIGURE 4: SDS–PAGE analyses of covalent incorporation of ^{125}I-α_2-antiplasmin into the α chain of fibrin in the presence of **I**. Conditions for plasma clot formation and analyses are described in the Experimental Procedures section. Lane 1: Clot formed in the presence of 20 μM **I** and aged for 30 min; lanes 2–4: clot formed in the absence of **I** and aged for 5, 10, and 30 min, respectively. ^{125}I-α_2-AP represents the migration position of ^{125}I-α_2-antiplasmin; ^{125}I-α_2-AP-α chain represents the covalent cross-linked species containing ^{125}I-α_2-antiplasmin and fibrin α chain monomer; ^{125}I-α_2-AP-[α chain]$_N$ represents ^{125}I-α_2-antiplasmin cross-linked to fibrin α chain dimers–polymers. (The band at approximately 57K is of unknown origin.)

α chain fibrin polymerization was also inhibited. This result corroborated the aforementioned data (Figure 3) and was confirmed in identical experiments to those in Figure 4 where ^{125}I-α_2-antiplasmin was replaced by ^{125}I-fibrinogen (data not shown).

Several *in vitro* studies (Gaffney & Whitaker, 1979; deFouw et al., 1988) have shown that prevention of fXIIIa-catalyzed cross-linking reactions by the use of fXIII-deficient plasma, fXIII-specific antibodies, or EDTA leads to an acceleration in clot lysis rates relative to normal or untreated controls. The magnitude of these accelerations, however, is dependent upon the specific conditions selected for the clot lysis studies. For example, under conditions where clots are lysed in the presence of limiting plasminogen activator (McDonagh et al., 1971) or limiting plasminogen[4] or where clots have undergone platelet-induced retraction (Sakata & Aoki, 1982) or mechanical compaction (Sakata & Aoki, 1982; Rampling & Flexman, 1979), accelerations in lysis rates due to an inhibition of fXIII-catalyzed cross-linking reactions appear to be the largest. Under conditions employed by Gaffney and Whitaker (1979) and where no exogenous plasminogen is used, clot lysis rates are a sensitive function of fXIII levels as determined in preliminary experiments (data not shown) where fXIII-deficient plasma with fXIII additions was utilized. The results in Figure 5 along with those in Table 1 demonstrate a correlation between the relative concentrations required to give accelerations in clot lysis rates and the apparent second-order rate constants for fXIIIa inactivation. In addition, **I** has been tested *in vivo* (Bush et al., 1988; Shebuski et al., 1990; Leidy et al., 1990), and the steady-state plasma concentration that leads to enhanced thrombolysis and fXIIIa-

[4] K. F. Freund, S. L. Gaul, and A. M. Stern, unpublished observations.

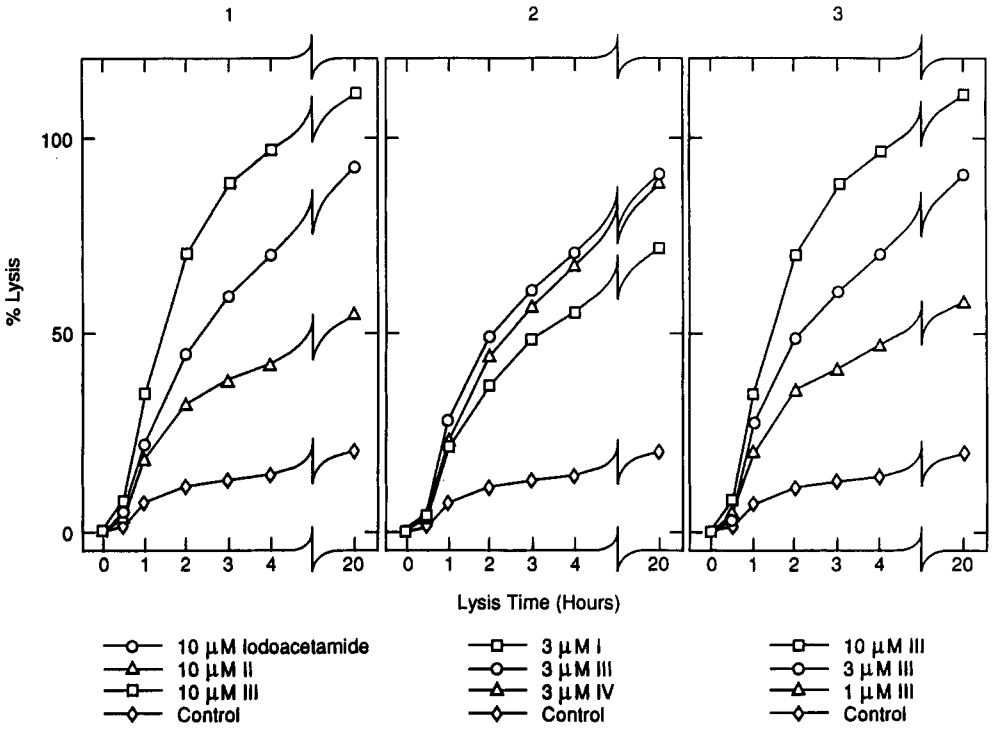


FIGURE 5: Enhancement of clot lysis rates by fXIIIa inactivators in an *in vitro* plasma assay. Conditions for clot formation and analyses are described in the Experimental Procedures section. Panels 1 and 2 show the relative effects of the fXIIIa inactivators listed in Table 1, and panel 3 is a dose response for **III**. Control values represent lysis in the absence of added fXIIIa inactivators.

catalyzed cross-linking inhibition is 5 μM. This is within the concentration range predicted by the *in vitro* clot lysis experiments (Figure 5).

Two of the fXIIIa inactivators, **I** and **III**, were tested at 100 μM and had no effect on the direct interaction of tissue plasminogen activator and of plasmin with their respective serine protease inhibitors PAI and α_2-antiplasmin. These results exclude two possible fXIIIa-independent mechanisms that could give rise to clot lysis rate accelerations.

There are three important aspects to the inactivation of transglutaminases by 2-[(2-oxopropyl)thio]imidazolium derivatives: (1) 2-[(2-oxopropyl)thio]imidazoliums have a relatively low intrinsic reactivity toward thiols at neutral pH compared to iodoacetamide, (2) substitution of the 2-thioimidazole moiety, which becomes a leaving group during the inactivation, affects potency (compare **II** and **III** in Table 1), and (3) inactivation results from modification of the active site thiol. These features suggest that affinity probes of transglutaminase active sites might be constructed from small glutamine-containing peptidyl substrates and 2-[(2-oxopropyl)thio]imidazolium derivatives[5] which can form the basis for the development of potent and selective irreversible and reversible transglutaminase inhibitors. Such inhibitors may be useful not only as a means to modulate covalent cross-links within fibrin clots but also as a way of probing the functional role of cellular transglutaminases.

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[5] 2-[(2-Oxopropyl)thio]imidazoliums have been coupled to a glutamine isostere in a peptide substrate containing the amino-terminal sequence of α_2-antiplasmin to generate a potent fXIIIa inactivator (D. A. Claremon, unpublished observations).

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