Photoreactivation of Irreversibly Inhibited Serine Proteinases

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Abstract: The *p*-amidinophenyl ester of *o*-hydroxy-α-methylcinnamic acid has been synthesized and characterized. This compound irreversibly inhibits thrombin, Factor Xa, and trypsin. Inhibition by this derivative is, however, reversible (80–100%) upon irradiation of the enzyme in the presence of excess compound. Photolysis of the inactive 1:1 acylenzyme complexes of all three enzymes (purified to homogeneity) also regenerated full enzymatic activity in 15 min or less. Thrombin clotting times for α-thrombin inactivated with this inhibitor were >120 s (essentially incapable of clotting) prior to irradiation and 20 s (identical with controls) after complete photoreactivation. This derivative is the first example of a photosensitive inhibitor of the vitamin K dependent proteinases.

Numerous approaches have been taken to photosensitize enzymatic processes. Photochemical reductions have generated light-sensitive reversible inhibitors[1] and active heme-containing enzymes.[2] Light-induced bond homolysis has produced active papain molecules,[3,4] and photoautomerization reactions which produce strong acids or bases have regulated enzymatic activity with light-induced "pH jumps".[5,6] Another approach to enzyme photoregulation involves the trans/cis photoisomerization of substituted alkenes. Irradiation of synthetic inhibitors with suitably substituted double bonds has generated photoisomeric inhibitors which display different inhibitory capacities. Under conditions in which one photoisomer is extremely labile, the photolytic interconversion of isomeric states has produced marked changes in enzyme activity.

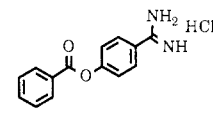
One of the earliest reports of cis/trans photosensitization of enzyme activity involved *p*-azophenyldiphenylcarbamyl chloride (PADPCC). Kaufman and co-workers defined conditions in which illumination of the *trans*-PADPCC–chymotrypsin complex by 320-nm light increased the rate of inactivation approximately fivefold, while irradiation of the *cis*-PADPCC–chymotrypsin adduct at 420 nm decreased inhibition by roughly the same factor.[7] Specific photochromic azobenzene inhibitors for acetylcholinesterase were also developed to photoregulate levels of esterase activity, and these derivatives were used to control ion flux and electric potential across the electrogenic membrane of the electric fish (electrophorus electricus).[8-11]

In addition to azobenzene compounds, cinnamoyl derivatives have also been studied as light-sensitive chymotrypsin substrates. Martinek and co-workers found that the *trans*-cinnamoyl-chymotrypsin complex (acylated on the active serine hydroxyl) deacylated more than 100 times faster than the photoisomeric cis adduct (9×10^{-3} vs 6×10^{-6} s^{-1}, pH 7.3, ambient temperature).[12] However, since the chromophore of the unsubstituted cinnamoyl group (λ_{max} = 307 nm) and the protein overlap considerably, the efficiency of the photoisomerization reaction was very low. Introduction of a *p*-NO_2 group sufficiently resolved substrate and enzyme absorption bands to produce a higher quantum efficiency.[13,14] These cinnamoyl derivatives also gave similar results with bovine trypsin and bacterial subtilisin.[15-17]

All of these approaches rely solely on steric effects to differentiate photoisomers. Generally the *cis*-cinnamoyl adduct is more stable than the trans complex, but in some cases this difference is as low as fivefold (vide supra). Irradiation of the *trans*- or *cis*-acylenzyme complex produces a photostationary mixture of isomers which must be separated. Usually differentiation is obtained under conditions in which one adduct is more labile than the other, but both trans and *cis*-cinnamoyl complexes generally have measurable deacylation rates at ambient temperature and moderate pH.

Ideally a photosensitive inhibitor should be indefinitely stable in the absence of light and reactivate quickly (μs) upon absorption of a photon. Rather than rely solely on steric factors to distinguish photoisomeric states, we sought to develop a more active chemical system. Our approach is to use photochemistry to present a nucleophilic center to the enzyme active site. This nucleophile is strategically located to internally deacylate the active serine hydroxyl of a serine protease.

The compounds **1–4** were prepared and studied as inhibitors of several serine proteases. Our initial studies with **1–3** were



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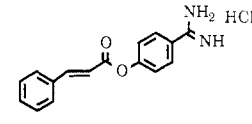

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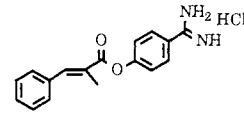

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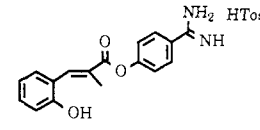

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carried out to determine the structural requirements of the enzyme active site.[18] When tested with factor Xa, the long term stability

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of the 1:1 acylenzyme complexes were **2** < **1** < **3**. This ranking also reflects the order of increasing steric bulk at the carbon α to the carbonyl (site of acylation). Drawing on the stability data from the series of model compounds studied, the *p*-amidinophenyl ester of *o*-hydroxy-α-methylcinnamic acid **4** was chosen as an initial target for photoreactivation studies. We report here the results of those studies.

Materials and Methods

Reagents. Russell's viper venom (RVV), *p*-nitrophenyl-*p*-guanidinobenzoate hydrochloride (*p*N*p*GB), bovine serum albumin (BSA), and Sepharose 4BCL, were purchased from the Sigma Chemical Co., St. Louis, MO. ^{125}Iodine, carrier free, and iodo beads were obtained from New England Nuclear, Boston, MA and Pierce Chemical Co., Rockford IL, respectively. Sephadex G-25 and G-100 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. The factor Xa substrate, *N*-benzoyl-Ileu-Glu-Gly-Arg-*p*-nitroanilide hydrochloride (and its methyl ester), S-2222, the thrombin substrate H-D-Phe-L-pipecolyl-Arg-*p*-nitroanilide dihydrochloride, S-2238, and the chymotrypsin substrate 3-carbomethoxy propionyl-Arg-Pro-Tyr-*p*-nitroanilide hydrochloride, S-2586, were purchased from Helena Laboratories, Beaumont, TX. The trypsin substrate benzoyl-Arg-*p*-nitroaniline, BAPNA, was purchased from Sigma. The Centricon 10 microconcentrators were purchased from Amicon Corp., Danvers, MA. Salicylaldehyde, (carbethoxyethylidene)triphenylphosphorane, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate, and *p*-cyanophenol were obtained from Aldrich Chemical Co., Milwaukee, WI. Benzene was distilled from sodium prior to use. All other reagents were of the best commercial grade available.

Proteins. Human α-thrombin was purified and characterized as detailed elsewhere.[19] Bovine α-chymotrypsin was purchased from Worthington Biochemical Corp., Freehold, NJ. Bovine factor X (Stuart) and trypsin (type XIII) were purchased from Sigma Chemical Co., St. Louis, MO. These preparations appeared homogeneous as determined by SDS-polyacrylamide gel electrophoresis (data not shown).

Protein Concentrations. The activities of purified human α-thrombin and bovine factor Xa, trypsin, and chymotrypsin were determined by active site titration as previously described.[20] The molecular weights of human thrombin, bovine factor Xa, chymotrypsin, and trypsin are 39 000, 51 000, 23 800, and 25 000, respectively.

Protein Radiolabeling. Human α-thrombin, bovine factor Xa, trypsin, and chymotrypsin were radiolabeled with ^{125}I by using iodo beads as previously described.[19,21]

Thrombin Clotting Time Assays. Thrombin clotting time (TCT) data were collected in the Duke University Medical Center Clinical Coagulation Laboratory. The TCT studies were performed in a fibrometer with bovine thrombin (Parke-Davis, Inc.) as a standard. The thrombin concentration was adjusted so that the TCT of platelet poor pooled plasma was 17–20 s. For in vitro experiments, different concentrations of the three *p*-amidinophenyl esters (dissolved in 10 μL of phosphate buffered saline (PBS) containing 15% ethanol) were added to 990 μL plasma. In control experiments, the same volumes of PBS and ethanol were added to plasma in the absence of the test compounds. The TCT was then measured.

In Vitro Studies. Time courses of inhibition were conducted for the reaction of various concentrations of compound **4** with thrombin, factor Xa, trypsin, and chymotrypsin. All experiments were conducted at room temperature, pH 7.4 (0.05 M Tris), except for the chymotrypsin assays which were conducted at pH 8.3 (0.1 M Tris). Serial dilutions of **4** were made just prior to each experiment to maintain a constant volume of ethanol at each inhibitor concentration (10%). Enzyme controls were treated with identical volumes of ethanol alone. Unless undergoing photolysis, solutions containing derivative **4** were protected from light. Percent enzyme activity in the presence of inhibitor **4** was determined by chromogenic assay with S-2222 (factor Xa), S-2238 (thrombin), BAPNA (trypsin), or S-2586 (chymotrypsin). The absorbance of 405 nm of each sample was recorded, and the average of duplicates was graphed as a percentage of an average control value (pooled over the first 4–6 h).

At designated times, aliquots of certain inhibitor concentrations were subjected to photolysis by a medium pressure mercury xenon source. Test tubes (plastic or Pyrex) were placed in a Pyrex, water-jacketed flask maintained at 25 °C. Aliquots of these samples were removed and assayed with S-2222 during the experiment to determine a time course of enzyme reactivation (usually 10–75 min). Aliquots of unphotolyzed inhibited enzyme were also assayed immediately before and after each photolysis as dark controls. Ethanol treated enzyme was also photolyzed and assayed to control for the effect of irradiation alone. Usually no diminution of enzyme activity was noted for photolysis periods of 1 h or less. The absorbance of 405 nm of reactivated enzyme samples was reported as percentages of A_{405} for photolyzed controls.

To follow reactivation of inactivated enzyme in the absence of light, thrombin, factor Xa, trypsin, and chymotrypsin (with the appropriate radiolabeled enzyme added in trace amounts) were incubated at room temperature (12–24 h) with a 1000-fold excess of compound **4**. In the case of trypsin and chymotrypsin, two aliquots of inhibitor (each at 1000-fold excess) were added over a 12-h period. The inhibited enzyme was then purified at room temperature on BSA treated Sephadex G-25 or in Centricon 10 concentrators in the ultracentrifuge (4 °C). The gel filtration procedure was performed on a 20 × 0.5 cm column. For purification of centrifugation, 2-mL aliquots of enzyme solutions containing the excess inhibitor were spun at 5500 rpm (4600 g) until the volume above the membrane was decreased to approximately 100 μL. This solution was then diluted with buffer (approximately 1 mL) and again concentrated to 100 μL. After this same dilution, the concentration step was repeated, the enzyme solution was collected by inverting the microconcentrator and spinning the sample at 2000 rpm (600 g) for 5–10 min. The entire solution containing enzyme was counted in the γ counter to determine protein recovery (recovered counts expressed as a percentage of initial counts; typically 50%).

Portions of the 1:1 acylenzyme complex purified by either method were subjected to photolysis, and both photolyzed and unphotolyzed solutions were subsequently monitored by chromogenic assay.

Photolysis Studies. Photolysis experiments were conducted in a water-jacketed Pyrex cell with use of a medium pressure mercury xenon lamp. Samples were maintained at 25 °C with an LKB 2219 Multi Temp II thermostatic circulator.

X-ray Determination. All measurements were performed on an Enraf-Nonius CAD-4 diffractometer (Cu Kα radiation, λ = 1.5418 Å; incident-beam graphite monochromator; ω-$^2\theta$ scans, θ_{max} = 67°). Unit cell parameters were derived by least-squares treatment of the diffractometer setting angles for 25 reflections (42° < θ < 57°) widely separated in reciprocal space. Crystallographic calculations were performed on a PDP11/44 computer by use of the Enraf-Nonius SDP suite of programs in the full-matrix least-squares refinement of atomic positional and thermal parameters.

***p*-Amidinophenol (4-Hydroxybenzenecarboximidamide, Monohydrochloride).** *p*-Cyanophenol (3 g, 25 mmol) and methanol (1.6 g, 50 mmol) in 11 mL of *p*-dioxane were treated under argon at room temperature with excess HCl gas (generated in situ from ammonium chloride and H_2SO_4) until a yellow color persisted. The reaction mixture was then cooled to 5 °C for 48 h. The resulting yellow solid was filtered and dried in vacuo. This precipitate (1.9 g) was dissolved in 60 mL of dry ethanol and treated with excess anhydrous ammonia (doubly distilled) until a pink color persisted. This pink solution was warmed to 60 °C for 3 h, its color quickly fading with elevated temperature. After removal of solvent under reduced pressure, the product was purified from methanol by forced precipitation with anhydrous ether in an overall yield of 96%: mp 222 °C (22), IR (KBr) 3000–3400 (H-bonded OH, amidine NH, aromatic C–H), 1680 (amidine C=N), 1610, 1600 cm^{-1} (amidine N–H); 300 MHz ^{1}H NMR (CD_3OD) δ 7.75 (d, 2 H, *J* = 7.8 Hz), 6.95 (d, 2 H, *J* = 7.8 Hz); ^{13}C NMR (CD_3OD) δ 167.3, 164.4 (phenolic C and aromatic amidine C), 119.0 (amidine C), 131.1, 117.1; MS (C.I.), 137 (100, M – HCl), 120 (21.21, M – HCl, OH); UV λ_{max} (H_2O) 305, 237 nm.

Ethyl *o*-Hydroxy-α-methylcinnamate ((*E*)-Ethyl 2-Methyl-3-(2-hydroxyphenyl)-2-propenoate). Freshly distilled salicylaldehyde (2.5 g, 1 equiv) was added to a dry round-bottomed flask containing 35 mL of dry benzene and 8.25 g (1.1 equiv) of (carboethoxyethylidene)triphenylphosphorane. The mixture was allowed to stir at room temperature under argon for 1–2 h until the reaction was complete by TLC (R_f values for the ethyl ester and methylcoumarin (90/10 hexane, ethyl acetate) were approximately 0.2 and 0.4, respectively). The crude product was chromatographed on silica (80/20 hexane, ethyl acetate) to yield 4.18 g of the *E* isomer (98% mp 52–53 °C) and 0.19 g of the methyl coumarin (mp 88–89 °C). Spectral data for ethyl *o*-hydroxy-α-methylcinnamate: IR (KBr) 3250–3400 (OH), 2900–3100 (C–H), 1680 (C=O), 1610–1620 (C=C), 1200–1300 cm^{-1} (C–O): 300 MHz ^{1}H NMR ($CDCl_3$) δ 1.5–1.6 (t, 3 H), 2.2 (s, 3 H), 4.4–4.5 (q, 2 H), 5.7 (br s, 1 H), 7.05–7.15 (m, 2 H), 7.35–7.45 (m, 2 H), 7.9 (s, 1 H); ^{13}C ($CDCl_3$) δ 169.3 (C=O), 154.3 (Ar C–OH), 61.1 (O–CH2), 14.4 and 13.7 (CH_3's), 135.1, 135.0, 129.7, 128.1, 122.5, 119.5, 115.5. MS (C.I.),

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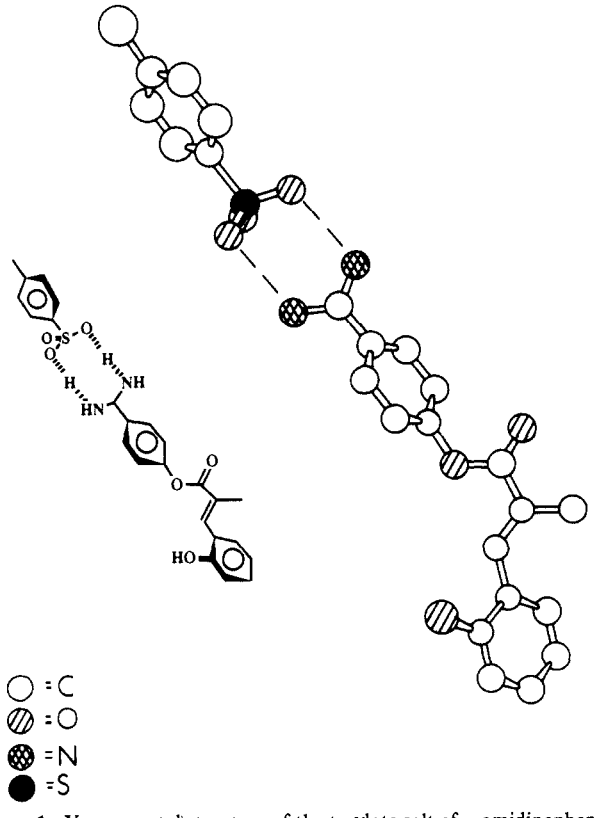


Figure 1. X-ray crystal structure of the tosylate salt of *p*-amidinophenyl *o*-hydroxy-α-methylcinnamate **4**. The insert displays a different representation of the X-ray data.

207 (100, M^{1+}), 189 (17.4, M - OH), 161 (96.2 M - OEt), 133 (13.6, M - COOEt).

Anal. Calcd for $C_{12}H_{14}O_3$: C, 69.88; H, 6.84. Found: C, 70.04; 69.88; H, 6.83, 6.95. UV λ_{max} (EtOH) 312, 267, 216 nm. Spectral data for 3-methyl coumarin (2*H*-1-benzopyran-3-methyl-2-one).

IR (KBr) 1710 (C=O), 1610 cm^{-1} (C=C); 300 MHz ^1H NMR (CD_3OD) δ 2.2 (s, 3 H), 7.25-7.35 (m, 2 H), 7.45-7.6 (m, 2 H), 7.8 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 162.1 (C=O), 153.1, (ArC-O), 138.2 (C = CCH_3), 17.1 (CH_3), 130.4, 126.9, 125.7, 124.2, 119.4, 116.3; MS (C.I.), 161 (100, M^{1+}), 97 (6.2), 79 (12).

Anal. Calcd for $C_{10}H_8O_2$: C, 74.99; H, 5.03. Found: C, 75.04; 75.09; H, 5.20, 5.08. UV λ_{max} (EtOH) 307, 275 nm.

***o*-Hydroxy-α-methylcinnamic Acid ((*E*)-2-Methyl-3-(2-hydroxyphenyl)-2-propenoic Acid).** Ethyl *o*-hydroxy-α-methylcinnamate (300 mg) was added to 7.5 mL of ethanol, 7.5 mL of water, and 30 mg of excess potassium hydroxide. The yellow solution was refluxed vigorously for 5-10 min (until complete reaction by TLC). The crude product was acidified, extracted with ether, and chromatographed on silica gel (70/30 hexane, ethyl acetate) to yield 160 mg (73%) of the pure acid: mp 137-138 °C; IR (KBr) 3400-3500 (OH), 2500-3100 (C-H), 1660 (C=O), 1600 (C=C), 1200-1300 cm^{-1} (C-O); 300 MHz ^1H NMR (CD_3OD) δ 2.0 (s, 3 H), 6.8-6.9 (m, 2 H), 7.1-7.2 (m, 1 H), 7.25-7.35 (m, 1 H), 7.85 (s, 1 H); ^{13}C NMR (CD_3OD) δ 172.4 (C=O), 156.8 (Ar C-OH), 136.7 (C=CCH3), 14.4 (CH_3), 131.1, 130.9, 128.6, 124.2, 120.2, 116.3; MS (C.I.), 179 (51.8, M^{1+}), 161 (100, M-OH), 132 (11, M-COOH). Anal. Calcd for $C_{10}H_{10}O_3$: C, 67.41; H, 5.66. Found: C, 67.63, 67.42; H, 5.78, 5.84.

***p*-Amidinophenyl *o*-Hydroxy-α-methylcinnamate ((*E*)-2-Methyl-3-(2-hydroxyphenyl)-2-propenoic Acid, 4-(Aminoiminomethyl)phenyl Ester, 4-Benzenesulfonate, 4).** *o*-Hydroxy-α-methylcinnamic acid (100 mg, 1 equiv) was added to pyridine (2-3 mL) containing 110 mg (1.1 equiv) of *p*-amidinophenol, 285 mg (1.2 equiv) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide, and 120 mg of *p*-toluene sulfonic acid (1.1 equiv). The reaction mixture was allowed to stir under argon at room temperature for 48 h. The crude product was filtered, and the pyridine was removed under reduced pressure. Addition of approximately 1 mL of 5% HCl eventually resulted in a white precipitate. Trituration of this solid with a second 1-2 mL of 5% HCl for at least 4 h removed urea derivatives from the desired *p*-toluenesulfonic acid salt **4**. Overall yield, approximately 15%, mp 216-217 °C, after recrystallization from methanol by slow evaporation at room temperature:[23] IR (KBr) 3350 (amidine N-H), 3000-3250 (amidine NH_2, aromatic C-H), 1725 (ester

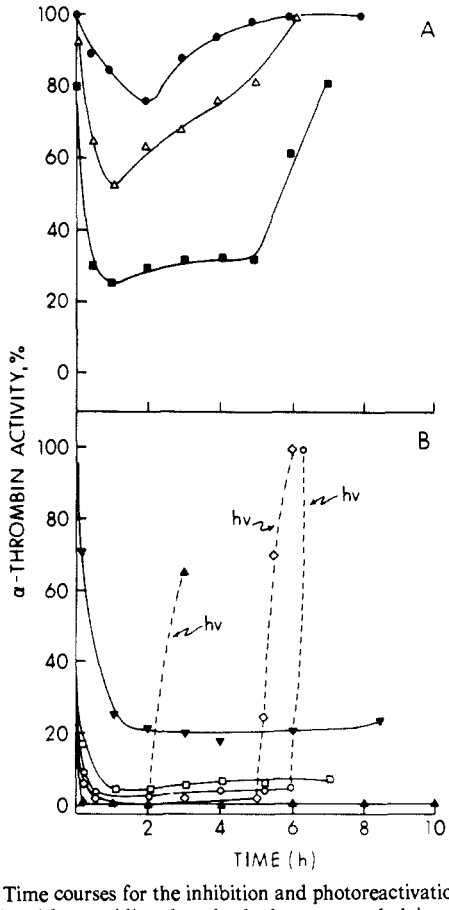


Figure 2. Time courses for the inhibition and photoreactivation of human α-thrombin with *p*-amidinophenyl *o*-hydroxy-α-methylcinnamate. The broken lines indicate reactivation of irradiated samples. Panel A, the symbols are 0.19 μM (●), 0.39 μM (△), .78 μM (■), *o*-hydroxy-α-methylcinnamate. Panel B, the symbols are 2.4 μM (□), 9.7 μM (○), 49 μM (◇), and 97 μM (▲) *o*-hydroxy-α-methylcinnamate. The concentration of human α-thrombin was 0.12 μM.

C=O), 1680 (amidine C=N), 1610 (amidine N-H), 1585, 1490 (amidine N-H), 1455 (CH_3) 1210-1000 (S=O), 760, 690 cm^{-1} (S-O); 300 MHz ^1H NMR (CD_3OD) δ 2.2 (s, 3 H), 2.4 (s, 3 H), 6.8-7.0 (m, 2 H), 7.2-7.3 (d, 3 H), 7.35-7.4 (d, 1 H, J = 10 Hz), 7.4-7.5 (d, 2 H, J = 12.5 Hz), 7.65-7.75 (d, 2 H, J = 10 Hz), 7.85-7.95 (d, 2 H, J = 12.5 Hz), 8.15 (s, 1 H); ^{13}C NMR (DMSO) δ 166.3, 165.0 (C=O, amidine C), 156.3, 155.1 (phenolic C), 137.5 (C=CCH_3), 20.8, 14.3 (CH_3's), 144.7, 138.4, 130.7, 130.0, 129.9, 128.4, 125.7, 125.5, 125.4, 122.8, 121.8, 118.9, 115.7; MS (E.I.), 296 (M - tosylate), 281 (M - tosylate, OH) 161 (100, M - tosylate, amidine).

Anal. Calcd for $C_{24}H_{24}N_2O_6S$: C, 61.52, H, 5.16. Found: C, 61.25; H, 5.23. X-ray crystal structure (Figure 2) UV λ_{max} (EtOH) 322, 274 nm. Infrared spectra were determined on a Perkin Elmer Model 297 spectrophotometer. Ultraviolet spectra were obtained on a Shimadzu UV-vis recording spectrophotometer, Model UV-240. MS data were determined on a Finnigan MAT TSK instrument (Oneida Research Service). Carbon-13 spectra were obtained on a Varian XL 300 series instrument.

Results

Synthesis. *p*-Amidinophenyl *o*-hydroxy-α-methylcinnamate (compound **4**) was synthesized in an overall 15% yield from *o*-hydroxy-α-methyl cinnamic acid by using a water soluble carbodiimide coupling reagent in pyridine. All attempts to couple the corresponding acid chloride by the procedure used to synthesize other *p*-amidinophenyl esters failed.

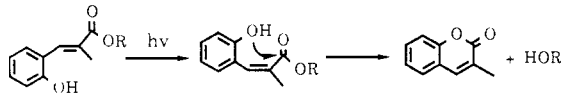
X-ray Crystal Structure of *p*-Amidinophenyl *o*-Hydroxy-α-methylcinnamate (Figure 1). X-ray analysis identified the parameters in Table I for crystals of compound **4** grown from methanol.

Photolysis of *p*-Amidinophenyl *o*-Hydroxy-α-methylcinnamate. Photolysis experiments in the absence of enzyme were carried out in ethanol on both the ethyl and *p*-amidinophenyl esters of *o*-hydroxy-α-methylcinnamate. Typically, 10 mg of ester was

Table I

molecular formula	$C_{24}H_{24}N_2O_6S$
formula wt	468.53
cryst system	triclinic
space group	$P1(C_i^1)$
a (Å)	10.508 (2)
b (Å)	12.807 (3)
c (Å)	9.108 (2)
α (deg)	98.86 (2)
β (°)	102.57 (2)
γ (°)	96.92 (2)
V (Å^3)	1166.9
Z	2
D_{calcd} (g cm^{-3})	1.333
μ (Cu Kα radtn) (cm^{-1})	15.5
cryst dimnsns (mm)	0.10 × 0.30 × 0.50
no. of nonequivalnt forms recrdd	3134
no. of reflcns retnd [$I > 3.0\sigma(I)$]	2465
R (R_w)	0.139 (0.206)

dissolved in 25 mL of ethanol and photolyzed through a water-jacketed Pyrex flask maintained at 25 °C. The course of the photolysis was monitored over a 1-h period by silica and/or cellulose TLC, and the photolysis product(s) were separated by flash chromatography and identified by 300 MHz ^{1}H NMR. No measurable lactonization or hydrolysis of either ester occurred in the absence of light over an 8-h period in ethanol or pH 7.4 Tris buffer (0.05 M). The product of photolysis of both the ethyl and *p*-amidinophenyl esters of *o*-hydroxy-α-methylcinnamate is 3-methylcoumarin and the corresponding alcohol (ethanol or *p*-amidinophenol). No other products are observed in the photolysate.



Thrombin (Figure 2). The reaction of α-thrombin (0.12 μM, pH 7.4) with **4** present at 1.5, 3.2, and 6.5-fold excess (0.19–0.78 μM) resulted in virtually complete enzyme reactivation in less than 8 h (no photolysis). At higher molar excesses of inhibitor, percent activity remained under 5% for 80, 400, and 800-fold excess of derivative **4** over the same period.[23] Photolysis of enzyme aliquots containing 80- and 400-fold excess inhibitor resulted in 100% and 40% reactivation in 12 and 24 min, respectively. Photolysis for 1 h of a thrombin sample containing 800-fold excess of **4** (97 μM) regenerated approximately 65% of the original activity. In each case, unphotolyzed solutions of enzyme inactivated with these inhibitor concentrations demonstrated less than 5% activity. Percent reactivation was based on the average of five controls, A_{405nm} = 2.846 ± 0.025. Control enzyme solutions were treated with identical concentrations of ethanol containing no inhibitor; these solutions were also subjected to identical periods of photolysis when used as controls for reactivation experiments. For short periods of photolysis (30 min or less), no difference was detected between photolyzed and unphotolyzed controls.

Photolysis of the purified inactive 1:1 acylenzyme complex resulted in fully reactivated thrombin in approximately 15 min (Figure 6).

Since thrombin is an extremely important blood coagulation proteinase, standard clinical assays, in addition to the S-2238 chromogenic assay mentioned above, have also been developed to monitor thrombin activity. Typically, a thrombin clotting time (TCT) is measured to determine the time required for a given thrombin sample to form a fibrin clot. When this classic assay was employed to study the inhibitory capacity of compound **4**, the results correlated well with data from the chromogenic assays. Unphotolyzed samples containing 80-fold excess inhibitor never clotted (TCT > 120 s), and fully photoreactivated samples clotted in 20 s (identical with controls). One sample which had been 40% reactivated (as determined by S-2238 assay) clotted in 38 s. Thus, in agreement with the chromogenic assay, the TCT data indicated that some but not all of the thrombin present in this solution was capable of cleaving fibrinogen to fibrin, resulting in a clot.

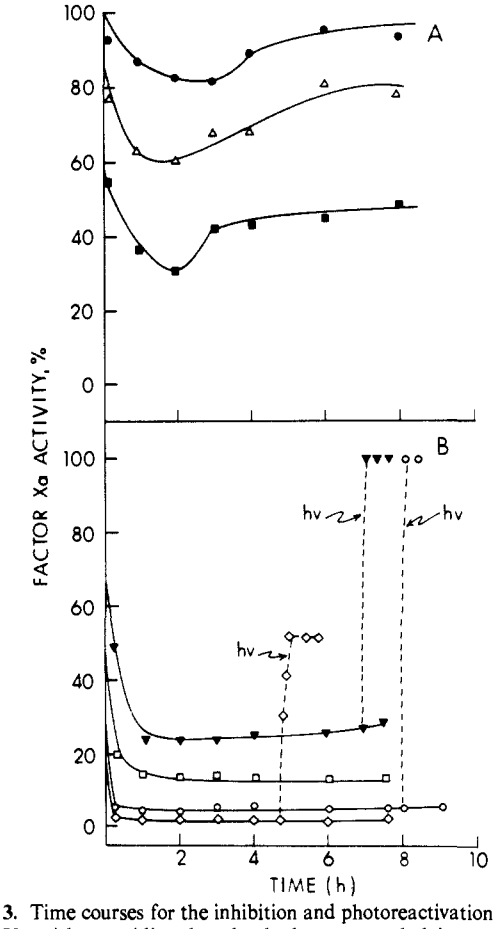


Figure 3. Time courses for the inhibition and photoreactivation of bovine factor Xa with *p*-amidinophenyl *o*-hydroxy-α-methylcinnamate. The broken lines indicate reactivation of irradiated samples. Panel A, the symbols are 0.12 μM (●), 0.24 μM (△), 0.48 μM (■) *o*-hydroxy-α-methylcinnamate. Panel B, the symbols are 0.6 μM (▼), 1.5 μM (□), 6 μM (○), 30 μM (◇) *o*-hydroxy-α-methylcinnamate. The concentration of bovine factor Xa was 0.057 μM.

Factor Xa (Figure 3). At low inhibitor concentrations (two-, four-, and eight-fold excess of derivative **4**), factor Xa (0.057 μM, pH 7.4) activity diminished somewhat over the first 2 h, but increased slowly over the next 6 h. As in the case of thrombin, higher concentrations of **4** resulted in enzyme activities below 5% (100- and 500-fold excess inhibitor). Factor Xa aliquots containing 10- and 100-fold excess of compound **4** (0.6 and 6.0 μM, respectively) were fully reactivated by irradiation in less than 10 min, while 1 h of photolysis of factor Xa containing 500-fold excess inhibitor (30 μM) regenerated 52% activity. In every case, solutions of enzyme inactivated with these inhibitor concentrations demonstrated virtually unchanged activity over the same period. Percent reactivation was based on the average of 10 controls, A_{405nm} = 2.635 ± 0.041. Control enzyme solutions were treated with identical concentrations of ethanol containing no inhibitor; these solutions were also subjected to identical periods of photolysis when used as controls for reactivation experiments. No difference was detected between unphotolyzed controls and controls irradiated for 1 h.

Photolysis of the purified **4**:Xa complex resulted in complete reactivation in less than 10 min (Figure 6).

The experiments reported thus indicate that the purified **4**:thrombin and **4**:Xa enzyme complexes were inactive after isolation but were restored to 100% of their original activity (identical with photolyzed controls) in less than 15 min of irradiation. If kept

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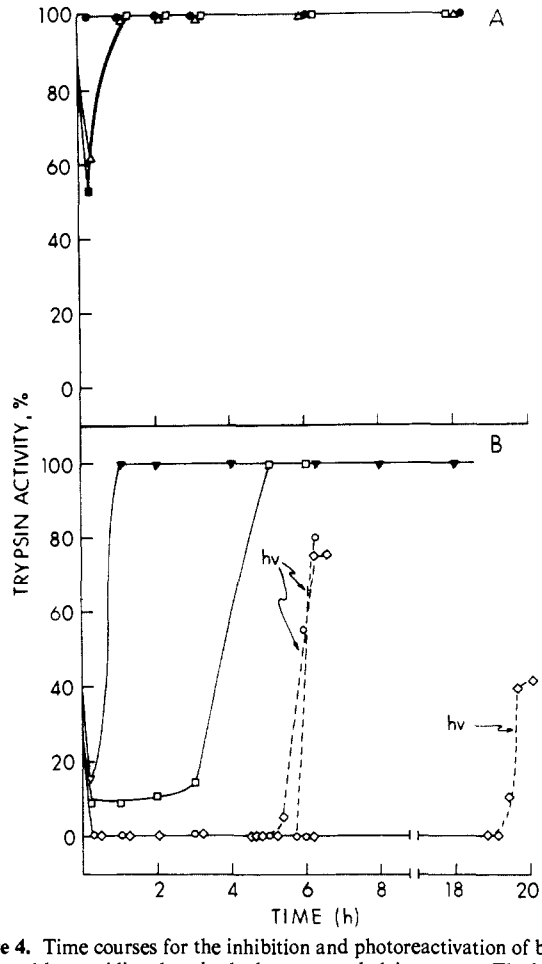


Figure 4. Time courses for the inhibition and photoreactivation of bovine trypsin with *p*-amidinophenyl *o*-hydroxy-α-methylcinnamate. The broken lines indicate reactivation of irradiated samples. Panel A, the symbols are 6 μM (●), 12 μM (△), 24 μM (□), 300 μM (○), 1500 μM (◇) *o*-hydroxy-α-methylcinnamate. The concentration of trypsin was 3 μM.

in the dark at 4 °C, the activity of the adducts remained unchanged for several hours. These results are consistent with the formation of a photoreactive acylenzyme complex. If reversible competitive inhibition had been the predominant mode of inhibition by compound **4**, then isolation of the inhibited enzyme from excess inhibitor would have resulted in highly active thrombin or factor Xa fractions.

Trypsin (Figure 4). Derivative **4** was esterified with *p*-amidinophenol to mimic the arginine specificity of thrombin cleavage. However, since trypsin also cleaves peptide bonds at arginine and lysine residues, the inhibitory capacity of **4** was also explored with this more widely studied proteinase. Compound **4** was a less effective inhibitor of bovine trypsin than was the case for thrombin and factor Xa, (3.0 μM, pH 7.4), with full enzyme activity returning within 1 h in the presence of two-, four-, eight-, and tenfold excess inhibitor (6.0–30 μM). However, virtually no residual activity was found at inhibitor to enzyme ratios of 100:1 and 500:1 (0.3 mM and 1.5 mM, respectively). Photolysis of these samples regenerated roughly 80% trypsin activity in 40 and 75 min, respectively. Percent reactivation was based on the average of eight controls, A_{405nm} = 1.078 ± 0.032. Control enzyme solutions were treated with identical concentrations of ethanol containing no inhibitor; these solutions were also subjected to identical periods of photolysis when used as controls for reactivation experiments. Irradiation (75 min of an inactive trypsin sample 19 h after the introduction of 500-fold excess of **4**) produced 42% of the original activity, based on control samples assayed after 7 h at room temperature. Note that incomplete reactivation of **4**:thrombin and **4**:Xa samples also occurred at high inhibitor concentrations (vide supra). The trypsin acylenzyme complex of **4** (1.5 mM inhibitor) could also be reactivated after 19 h at ambient temperature. However, the actual percent

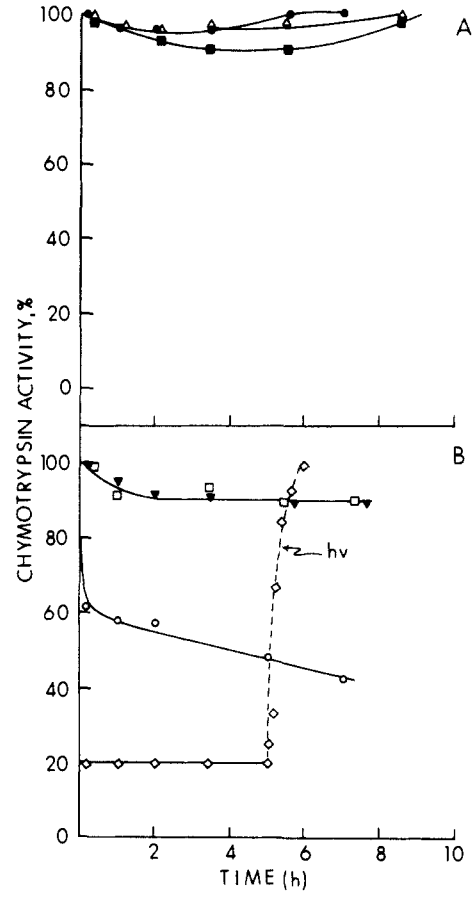


Figure 5. Time courses for the inhibition and photoreactivation of bovine trypsin α-chymotrypsin with *p*-amidinophenyl *o*-hydroxy-α-methylcinnamate. The broken lines indicate activation of irradiated samples. Panel A, the symbols are 0.42 μM (●), 0.84 μM (△), 1.7 μM (■) *o*-hydroxy-α-methylcinnamate. Panel B, the symbols are 2.1 μM (▼), 5.25 μM (□), 21 μM (○), 105 μM (◇) *o*-hydroxy-α-methylcinnamate. The concentration of α-chymotrypsin was 0.21 μM.

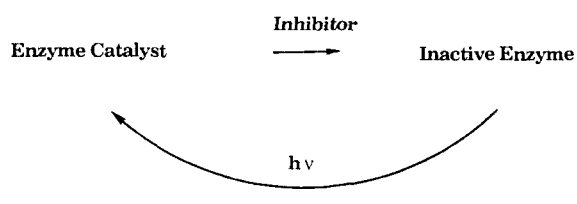
reactivation was difficult to determine, since identically treated controls autodegraded over the same period. When these results were compared to trypsin controls after 7 h at room temperature, the extent of reactivation was approximately 42%.

Purification and photolysis of the inhibited **4**:trypsin complex resulted in fully active enzyme in less than 10 min (Figure 6). This complex was fully active without photolysis after 4 h at room temperature or 17 h at 4 °C.

Chymotrypsin. (Figure 5). Compound **4** was a relatively poor inhibitor of chymotrypsin (0.21 μM, pH 8.3). Even the presence of 500-fold excess inhibitor resulted in 20% residual enzyme activity (no photolysis). Irradiation of this sample fully reactivated chymotrypsin over a period of 1 h. Percent reactivation was based on the average of seven controls, A_{405nm} = 1.479 ± 0.069. Control enzyme solutions were treated with identical concentrations of ethanol containing no inhibitor; these solutions were also subjected to identical periods of photolysis when used as controls for reactivation experiments. No conditions were found in which an inactive 1:1 acylenzyme complex could be isolated.

Discussion

The ideal serine protease photoactive inhibitor should form an indefinitely stable 1:1 acyl–enzyme complex in the absence of light.[24]



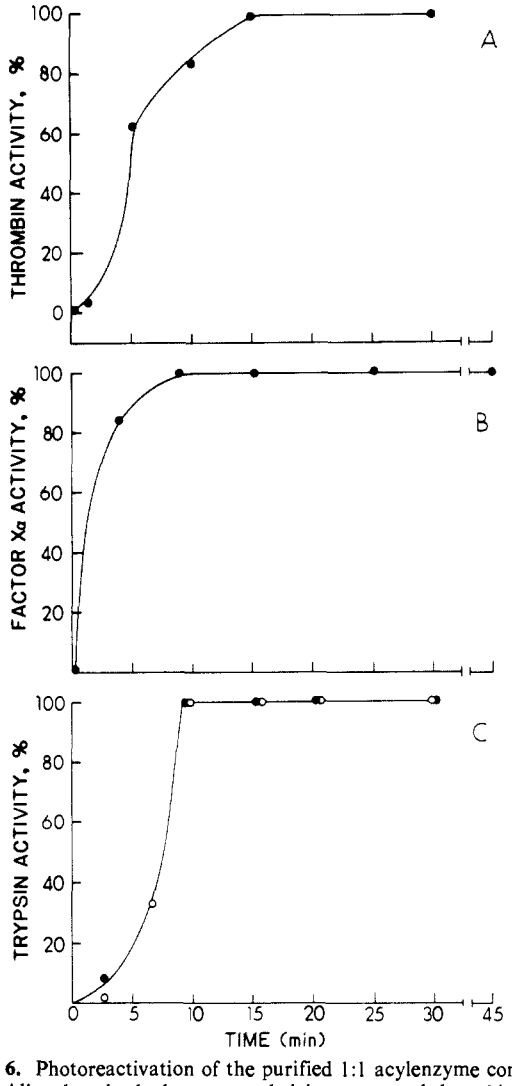
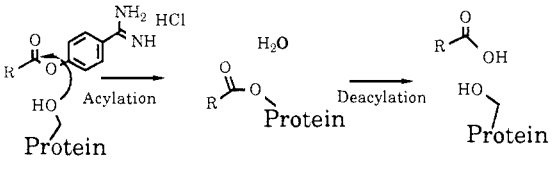


Figure 6. Photoreactivation of the purified 1:1 acylenzyme complexes of *p*-amidinophenyl *o*-hydroxy-α-methylcinnamate and thrombin, factor Xa, and trypsin: panel A, human α-thrombin; panel B, bovine factor Xa; panel C, bovine trypsin (two experiments).

In order to determine the structural requirements of such an idealized inhibitor, the *p*-amidinophenyl esters of benzoic (compound **1**), cinnamic (compound **2**), and α-methylcinnamic (compound **3**) acids were synthesized and studied as model compounds for enzyme photoreactivation.[18] Although **1** does not contain the requisite double bond for photoisomerization, its synthesis and study were undertaken to demonstrate that literature values for this known thrombin inhibitor could be reproduced. Within the series of compounds proposed for study, this derivative could also yield valuable information on the effect of steric bulk near the site of acylation (ester carbonyl). Presumably the design of any photoreactive inhibitor must incorporate the proper degree of steric hindrance to minimize hydrolysis yet maximize acylation. As



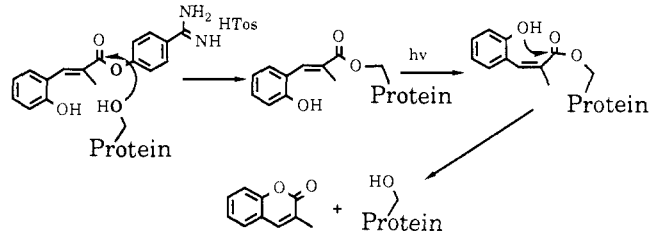
expected, **1** in a two–fourfold excess was a good inhibitor of human α-thrombin (>95% inhibiton maintained for 24 h) and bovine factor Xa (>93% inhibition maintained for 32 h). The purified **1**:Xa complex slowly reactivated over 16 h at room temperature.[18]

Incorporation of the double bond required for photoisomerization led to the synthesis and study of **2**. However, in a two–fourfold excess at 23 °C, **2** did not diminish thrombin activity even after 5 h. Under similar conditions compound **2** quickly achieved greater than 95% inhibition of bovine factor Xa, but moderately rapid reactivation resulted in factor Xa activity above 40% restored roughly half of the original factor Xa activity in less than 8 h. Chromogenic assay preformed on the purified **2**:Xa complex demonstrated complete deacylation in less than 4 h at 4 °C.[18]

Due to the rapid hydrolysis of the **2**:Xa adduct, model compound **2** was thought to be too unstable for further study. In this derivative the aromatic ring is separated from the site of reactivity by an unsubstituted carbon–carbon double bond. This structural change results in total loss of inhibitory capacity with α-thrombin, presumably because of rapid deacylation of this sterically unhindered acylenzyme intermediate.

To probe the importance of disubstitution at the carbon α to the carbonyl, the previously unreported *p*-amidinophenyl α-methylcinnamate **3** was synthesized. In this derivative, the α vinyl hydrogen of **2** has been replaced by a bulkier methyl substituent to better mimic the disubstitution of the aromatic α carbon in **1**. It was predicted that this change would result in a derivative more closely simulating the steric bulk of the α carbon of compound **1**. When tested, **3** did prove to be a thrombin inhibitor. After 4 h of incubation at two–fourfold excess, inhibitor **3** reduced α-thrombin activity by 60%. This level of inhibition remained relatively unchanged over 16 h at ambient temperature. In the presence of a 500-fold excess of **3**, bovine factor Xa and human α-thrombin (23 °C) were inactivated completely in 5 and 45 min, respectively. Chromogenic assays performed on the purified **3**:Xa complex resulted in no measurable reactivation after more than 48 h at 23 °C. Apparently, substitution of a methyl group α to the site of reactivity decreases both acylation (k_2) and hydrolysis (k_3). However, since large excesses of inhibitor can be employed to push the reaction to completion, the slower acylation rate which accompanies decreased hydrolysis can be tolerated.

Having developed a model inhibitor containing the requisite photoactive double bond as well as the desired long term stability, the first target compound for photochemical studies was designated as a derivative of compound **3** substituted with an ortho hydroxyl **4**. Such a derivative would have the potential to lactonize after photoisomerization. When covalently bound to a serine proteinase (R = active serine hydroxyl), compound **4** can photoisomerize to the cis derivative. This isomeric acylenzyme should lactonize rapidly (ms) to 3-methylcoumarin, thus restoring full enzyme activity by releasing the active site serine hydroxyl.[25]



As expected, reaction of **4** in the absence of light with thrombin and factor Xa produced results similar to that of **3**. For example, an 80-fold excess (9.7 μM) of derivative **4** inhibited 95% of human α-thrombin activity within the first 2 h, and this level of enzymatic activity remained relatively unchanged for at least 6 h. Almost identical results were obtained upon reacting a similar concentration of inhibitor **4** with bovine factor Xa at ambient temperature. Presumably this inhibition results from formation of an acylenzyme complex with the active site serine, but the definitive experiment to prove or disprove this assumption (involving doubly radiolabeled **4**) has not yet been undertaken.

One major difference in the inhibitory capacity of **4** vs **3** can be seen in time course studies conducted at low molar excesses

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of inhibitor. At a fivefold excess, **3** inhibited approximately 50% of both thrombin and factor Xa activity; this level of inhibition remained relatively unchanged for more than 20 h.[18] However, under similar conditions, the inhibition curves for **4** with thrombin and factor Xa showed marked reactivation (Figures 2 and 3). These data suggest that the enzyme deacylation rate is slow but real. Experiments in progress with **4** will address the question of reversibility of enzyme acylation and will provide rates of deacylation.

Several aliquots of both α-thrombin and factor Xa inhibited with compound **4** were subjected to photolysis. Irradiation of **4** itself resulted in lactonization of the inhibitor to produce 3-methylcoumarin and *p*-amidinophenol. Since neither the coumarin nor the phenol are good inhibitors of serine proteinases,[26] photolysis of enzyme solutions containing compound **4** effectively removed inhibitor from the system. Enzyme solutions inhibited with low concentrations of **4** (0.6–10 μM) were fully reactivated in less than 1 h of irradiation, but photolysis of samples containing higher inhibitor concentrations (30–97 mM) resulted in only partial reactivation (50–65%) after the same period of irradiation. The longer photolysis periods which would be required to photoisomerize higher concentrations **4** (30–100 mM) were not feasible in presence of enzyme, since all proteinases also absorb energy within the mercury–xenon broad band emission spectrum. Control studies confirmed that prolonged irradiation of α-thrombin or factor Xa did result in decreased enzyme activity.

Although chymotrypsin preferably cleaves peptide bonds at aromatic amino acids (phenylalanine, tryosine, and tryptophan), information on its affinity for **4** was needed to better target future chymotrypsin photoactive inhibitors. The altered specificity of this proteinase can be readily accounted for by an important difference in the amino acid sequences of chymotrypsin and trypsin-like enzymes such as trypsin, thrombin, and factor Xa. In general, trypsin and chymotrypsin accommodate substrates within their active sites in exactly the same manner. However, trypsin contains a key aspartic acid residue which can form a salt bridge with the charged amino acid side chains of lysine or arginine. The formation of these ionic bonds conveys added specificity to trypsin peptide bond hydrolysis. In chymotrypsin, this residue has been replaced by Ser189.[27] Since the pK_a of the serine hydroxyl is normally around 14, this amino acid does not form ionic bonds to charged amino acid side chains. Thus, *p*-amidinophenyl esters such as **4** are not readily recognized by chymotrypsin. The speed and efficiency of all photoreactivation experiments with derivative **4** were limited by the (1) extensive overlap between enzyme and inhibitor absorbance spectra and (2) the intensity of the light source. As noted earlier, the extended periods of broadband irradiation required to lactonize high concentrations of **4** eventually degraded the enzyme. Use of a monochromator tuned to appropriate emission maxima of the source (above the Pyrex cutoff within the range of absorption by **4**) produced insufficient intensities for photoisomerization. Future photolysis experiments performed with a tunable laser should produce sufficiently high intensity light for efficient photolysis at a wavelength absorbed only by the inhibitor. Great efficiency can also be achieved by introducing appropriate substitution into the aromatic ring of **4** to red shift and intensify its absorption maxima.

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Variable Photon Energy Photoelectron Spectroscopic Studies of Copper Chlorides: An Experimental Probe of Metal–Ligand Bonding and Changes in Electronic Structure on Ionization

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Abstract: Variable photon energy photoelectron spectra (PES) are reported for the valence band region of cuprous and cupric chlorides for photon energies between 22 and 1253.6 eV. Intensity changes of the PES peaks observed with variation of photon energy are associated with (1) the photoionization cross sections of Cu 3d and Cl 3p atomic orbitals, (2) the Cooper minimum of the Cl 3p orbitals, and (3) resonance effects at the Cu 3p absorption edge. These effects allow a definitive assignment of specific PES features, an experimental estimate of covalent mixing between metal and ligand orbitals for all valence levels, and a quantitative evaluation of relaxation effects on the ionized final states. The intensity changes of the $CuCl_4^{2-}$ PES peaks are found to be dramatically different from those of $CuCl_4^{3-}$, a result of increased covalent mixing in the cupric chlorides. Quantitative analysis of the PES data indicates that in D_{4h} $CuCl_4^{2-}$, the highest energy level, $3b_{1g}$, has 65% Cu 3d character while the antibonding metal levels together have 78% d character. For D_{2d} $CuCl_4^{2-}$, the highest energy level has less covalent mixing than the D_{4h} salt (68% Cu 3d character), while the mixing averaged over all the metal levels increases (76% Cu 3d). The values for the covalent mixing of the highest energy occupied level are in good agreement with the results from other spectroscopic methods, and variable energy PES provides an additional probe of the covalent mixing averaged over all metal and ligand valence levels. The valence band PES spectra of the cupric chlorides also show satellite peaks with significant intensity out of resonance ($\sim$10% of main band intensities) and large resonance enhancement at the Cu 3p $\rightarrow$ 3d absorption edge. These data require large final-state relaxation effects which have been interpreted both in terms of SCF-Xα-SW calculations and a Configuration Interaction model. The relaxation results from a large decrease in metal-centered electron–electron repulsion on ionization which stabilizes the d^8 final state by 6.5 eV. The implications of these results with respect to the redox chemistry of Cu(II) complexes is discussed in terms of covalency effects and metal-centered electron–electron repulsion.

I. Introduction

A major goal of spectroscopic studies on transition metal complexes has been to determine experimentally the bonding interactions between metal ion d orbitals and ligand valence orbitals. One set of complexes which has served as the subject of a large number of experimental and theoretical studies of met-

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