Mechanism of Inactivation of Chymotrypsin by 3-Benzyl-6-chloro-2-pyrone†

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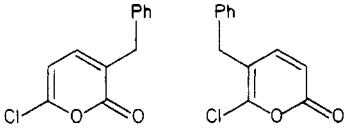
ABSTRACT: The mechanism of inactivation of chymotrypsin by 3-benzyl-6-chloro-2-pyrone has been studied. Chloride analysis of the inactivated enzyme suggests that the complex does not contain intact chloropyrone or an acid chloride. ^{13}C NMR studies of the enzyme inactivated with ^{13}C-enriched chloropyrones show that (1) the pyrone ring is no longer intact, (2) C-6 becomes a carboxylate group and C-2 becomes esterified to the enzyme, probably to serine-195, and (3) a double bond is present adjacent to the serine ester. The inactivated enzyme slowly regains catalytic activity with the concomitant release of (*E*)-4-benzyl-2-pentenedioic acid. It is concluded that double bond migration occurs during reactivation since the position of the double bond in the released diacid product is different than in the inactivator–enzyme complex. When the reactivation is carried out in $[^{18}O]H_2O$-enriched water, a single oxygen-18 is incorporated into the released product and is further evidence that the inactivator is bound to the enzyme only through a single ester linkage. A deuterium isotope effect on reactivation is observed when a chloropyrone deuterated at C-5 is used. This result demonstrates that removal of a proton from C-5 is required for reactivation and that isomerization of the double bond and not hydrolysis of the acyl-enzyme is rate determining. A variety of amines accelerate the rate of reactivation by functioning as general bases and not as nucleophiles. A reaction scheme is presented that accounts for the formation of the stable inactivator–enzyme complex as well as the production of two products derived from enzymatic hydrolysis of the chloropyrone. The importance of a C-6-derived carboxylate group in the stabilization of the acyl-enzyme is discussed.

The inactivation of chymotrypsin and other serine proteases by 3-benzyl-6-chloro-2-pyrone (**1**) and 5-benzyl-6-chloro-2-pyrone (**2**) has been recently reported (Westkaemper & Abeles, 1983).



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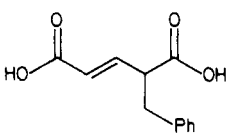
Inactivation of chymotrypsin by **2** proceeds through conjugate addition of the active-site serine oxygen (serine-195) onto the 6-position of the chloropyrone (D. Ringe, B. A. Seaton, M. H. Gelb, and R. H. Abeles, unpublished results). Loss of chloride leads to an enzyme–pyrone adduct in which the pyrone ring remains intact (Scheme I).

The mechanism of inactivation of chymotrypsin by **1** occurs by a different mechanism. The UV–vis spectrum of the inactivated enzyme does not show a band near 320 nm that is characteristic of an intact pyrone ring, suggesting that the ring has been opened during enzymatic catalysis. A previously proposed mechanism of inactivation of chymotrypsin by **1** is shown in Scheme II (Westkaemper & Abeles, 1983). In this mechanism, enzymatic hydrolysis of the haloenol lactone generates an electrophilic species, either an acid chloride or a ketene, which can react with an active-site nucleophile resulting in covalent modification of the enzyme and loss of catalytic activity.

Compound **1** is also a substrate of chymotrypsin. The products produced are primarily (*E*)-4-benzyl-2-pentenedioic acid (**3**) and the minor product 2-benzyl-2-pentenedioic anhydride (**4**) (Westkaemper & Abeles, 1983). Inactivation occurs once in every 14–40 turnovers. Inactivated enzyme slowly regains catalytic activity at pH 7.0, 25 °C ($t_{1/2} \cong 23$ h).



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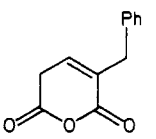

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In this report we describe a detailed investigation of the mechanism of inactivation of chymotrypsin by **1**. Our results require a modification of the mechanism shown in Scheme II.

Experimental Procedures

Materials

α-Chymotrypsin (type II) and benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Sigma Chemical Co. Gel filtration media was from Pharmacia Fine Chemicals. Barium [^{13}C]carbonate (97–98% or 90% enriched) was purchased from Mounds Facility, Monsanto, and MSD-Isotopes. [^{13}C]Methyl iodide (99% enriched) and $[^{18}O]H_2O$ (98% enriched) were from Cambridge Isotopes.

Methods

All spectrophotometric measurements were made with either a Perkin-Elmer 559 or a Lambda-3 UV–vis instrument with 1-cm quartz cells thermostated at 25 °C. Chymotrypsin concentrations were determined by measuring A_{280} nm with $\epsilon = 5.1 \times 10^4$ M^{-1} cm^{-1} (Dixon & Neurath, 1957), and enzymatic activity was determined with BTEE (Hummel, 1959). ^1H NMR spectra at 500 MHz were obtained on an instrument built in the Department of Biochemistry at Brandeis University and at 90 MHz on a Varian EM-390 spectrometer. ^{13}C NMR spectra at 100.6 MHz were obtained on a Bruker AM-400 spectrometer and at 22.6 MHz on a Bruker FT WH-90 spectrometer. All chemical shift values are reported relative to tetramethylsilane.

Syntheses. The chemical syntheses of 3-benzyl-6-chloro-2-pyrone, 3-benzyl-2-pyrone, 2-benzyl-2-pentenedioic anhydride, the dimethyl ester of (*E*)-2-benzyl-2-pentenedioic acid,

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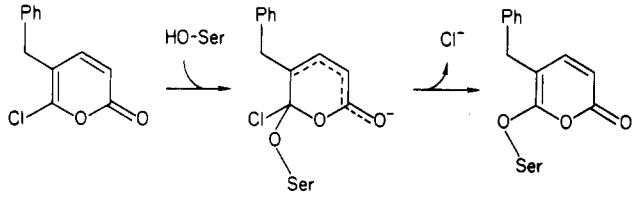
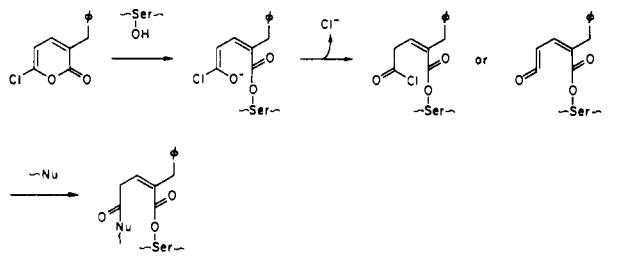
Scheme I



Scheme II



and (*E*)-4-(methoxycarbonyl)-5-phenyl-3-pentenoic acid and the enzymatic synthesis of (*E*)-4-benzyl-2-pentenedioic acid were performed as described (Westkaemper & Abeles, 1983). The ^{1}H NMR data for the above compounds and (*E*)-2-benzyl-2-pentenedioic acid, (*Z*)-2-benzyl-2-pentenedioic acid, and (*Z*)-4-benzyl-2-pentenedioic acid have been reported (Westkaemper & Abeles, 1983).

3-Benzyl-6-chloro-2-pyrone-2,6-^{13}C. Sodium acetate-*1*-^{13}C was prepared from the reaction of methylmagnesium iodide with [^{13}]CO_2 (generated from 15.4 g of barium [^{13}C]carbonate, 97–98% or 90% enriched) as described (LeMaster, 1980; Murray & Williams, 1958). The isolation procedure avoids the use of costly silver salts used in the earlier procedures. The salt was fused, converted to the free acid by distillation from phosphoric acid, and brominated with Br_2 in trifluoroacetic anhydride containing a catalytic amount of PBr_3 (Roberts & Poulter, 1978). Bromoacetic acid-*1*-^{13}C was converted to cyanoacetic acid-*1*-^{13}C and then to diethyl malonate-*1*-^{13}C (Ott, 1981). Diethyl malonate-*1*-^{13}C (5.2 g) was converted in four steps to pure 3-benzyl-6-chloro-2-pyrone-*2,6*-^{13}C (180 mg) and 5-benzyl-6-chloro-2-pyrone-*2,6*-^{13}C (40 mg) (Westkaemper & Abeles, 1983). The ^{13}C NMR of the enriched chloropyrone in $CDCl_3$ showed two enriched resonances at 147.2 and 161.4 ppm. These resonances were assigned to C-2[1] (161.4 ppm) and C-6 (147.2 ppm) as follows: The natural abundance ^{13}C-NMR of 3-benzyl-6-chloro-2-pyrone shows four quaternary resonances at 126.8, 137.3, 147.2, and 161.4 ppm. On the basis of chemical shifts, the resonances at 126.8 and 137.3 ppm were assigned to C-3 and the quaternary phenyl carbon, respectively. The natural abundance ^{13}C NMR spectrum of 3-benzyl-2-pyrone showed a quaternary resonance at 162.6 ppm, but the 147.2-ppm peak was replaced by a new non-quaternary resonance at 149.5 ppm. Thus, the peak at 161.4 ppm must be from the carbonyl carbon (C-2) and the peak at 147.2 ppm from the haloenol carbon (C-6).

3-Benzyl-6-chloro-2-pyrone-6-^{13}C. Sodium acetate-*1*-^{13}C was prepared as described above and converted to the free acid by distillation from polyphosphoric acid (Fitzell, 1975). The acetic acid was brominated, converted to cyanoacetic acid, and hydrolyzed to malonic acid-*1*-^{13}C as described previously except that the final sublimination step was omitted. Instead, the malonic acid was recrystallized from ether–petroleum ether (bp 20–40 °C). A mixture of 1.06 g (10.1 mmol) of malonic acid-*1*-^{13}C, 1.98 g (9.6 mmol) of ethyl α-formylhydrocinnamate (Goldberg, 1951), and 1 mL of pyridine (dried by filtration through grade I alumina) was heated on a steam bath for 4 h in a flask protected with $CaSO_4$ dry tube. The solution was poured onto ice (5 g) and acidified with cooling to pH 2 with concentrated sulfuric acid. The yellow oil that separated was extracted twice into ether. The ether was removed in vacuo, leaving 2.3 g of oil. To the oil was added 12 mL of 2 N NaOH and the mixture was heated at 80 °C for 1 h. The solution was cooled on ice and acidified to pH 1 with concentrated HCl. Extraction with ether (2×) followed by removal of the ether in vacuo left an oil. The oil was dissolved in a cold 20% KOH (2–3 mL) followed by acidification to pH 1 with concentrated HCl. The mixture was left for 24 h at 4 °C. The water was removed by pipet, leaving a semisolid residue. The residue was dried in vacuo over P_2O_5 and then triturated with 5 mL of CH_2Cl_2. The resulting white solid was collected by filtration and washed with 5 mL of CH_2Cl_2, giving 420 mg of (*E*)-2-benzyl-2-pentenedioic acid-*5*-^{13}C: ^{1}H NMR (CD_3CN) δ 3.53 (dd, 2 H, J_{H4-H3} = 7.2 Hz, J_{H4-C5} = 7.5 Hz, H-4), 3.65 (s, 2 H, benzylic), 7.03 (t, 1 H, J_{H3-H4} = 7.2 Hz, H-3), 7.2 (br s, 5 H, phenyl). The diacid was refluxed with acetyl chloride to give 150 mg of 3-benzyl-6-chloro-2-pyrone-*6*-^{13}C (Westkaemper & Abeles, 1983): ^{1}H NMR ($CDCl_3$) δ 3.76 (d, 2 H, $J_{benzylic-H4}$ = 1.3 Hz, benzylic), 6.14 (d, 1 H, J_{H5-H4} = 7.5 Hz, J_{H5-C6} = 0 Hz, H-5), 6.90 (ddd, 1 H, J_{H4-H5} = 7.5 Hz, $J_{H4-benzylic}$ = 1.3 Hz, J_{H4-C6} = 12 Hz, H-4) 7.28 (m, 5 H, phenyl). The ^{13}C NMR in $CDCl_3$ showed a single enriched resonance at 147.2 ppm (C-6).

3-Benzyl-6-chloro-2-pyrone-2,5-^{13}C. [^{13}C]Methyl iodide (10 g, 50% enriched) was converted to [^{13}C]methylmagnesium iodide, treated with excess CO_2, and worked up as described (Murrary & Williams, 1958) to give sodium acetate-*2*-^{13}C. The sodium acetate was used to prepare diethyl maolonate-*2*-^{13}C as described above. The diethyl malonate was converted to the chloropyrone as described above. The yield of 3-benzyl-6-chloro-2-pyrone-*2,5*-^{13}C was 200 mg from 10 g of CH_3I: ^{1}H NMR ($CDCl_3$) δ 3.76 (t, 2 H, $J_{benzylic-C2}$ = 4.0 Hz, benzylic), 6.15 (ddd, 1 H, J_{H5-H4} = 7.55 Hz, J_{H5-C2} = 8.1 Hz, J_{H5-C5} = 180 Hz, H-5), 6.94 (br, d, 1 H, J_{H4-H5} = 7.55 Hz, $J_{H4-C5} \cong$ 2 Hz, H-4), 7.33 (m, 5 H, phenyl). The ^{13}C NMR in $CDCl_3$ showed two enriched resonances at 104.0 (C-5) and 126.4 (C-3) ppm. These were assigned from the ^{1}H-coupled spectrum based on the presence of a directly bounded proton on C-5 but not on C-3.

Di-n-propylamide of (E)-2-Benzyl-2-pentenedioic Acid. (*E*)-2-Benzyl-2-pentenedioic acid (156 mg, 0.71 mmol), dicyclohexylcarbodiimide (293 mg, 1.42 mmol), and *n*-propylamine (0.117 mL, 1.42 mmol) were added to 10 mL of CH_3CN, and the mixture was vigorously shaken for 6 h at room temperature. The mixture was filtered to remove the urea, and the filtrate was concentrated in vacuo. The residue was taken up in ethyl acetate and washed with 1 N HCl (2×), 5% $NaHCO_3$ (2×), and saturated NaCl (1×). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. The residue was purified in portions by preparative thin-layer chromatography on silica (Analtech; 500 μm, 20 × 20 cm plates) using ethyl acetate as the developing solvent. The band at R_f = 0.28 was removed and the product eluted with warm CH_3CN. Concentration in vacuo left a white crystalline residue: mp 95–97 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (m, 6 H, methyls), 1.5 (m, 4 H, CH_2 β to nitrogen), 3.20 (m, 6 H, CH_2 α to nitrogen and allylic), 3.75 (s, 2 H, benzylic), 5.6–5.8 (br, 2 H, amides), 6.45 (t, 1 H, J = 6.8 Hz, olefinic), 7.23 (m, 5 H, phenyl).

[1] The chloropyrone ring is numbered according to the conventional scheme. The ring oxygen atom is position 1. The ring atoms are numbered consecutively in a counterclockwise manner around the ring.

Mixture of Half-n-propylamides of (Z)-2-Benzyl-2-pentenedioic Acid. 2-Benzyl-2-pentenedioic anhydride (108 mg, 0.53 mmol) and *n*-propylamine (0.2 mL 2.4 mmol) were dissolved in 1 mL of CH_3CN, and the mixture was refluxed for 1 h. Removal of solvent in vacuo left an oil. The oil was dissolved in ethyl acetate and extracted with 5% $NaHCO_3$ (2×). The aqueous layers were combined and acidified to pH 1 with 6 N HCl with cooling. The oil that separated was extracted into ethyl acetate (3×). The combined extracts were dried ($MgSO_4$) and concentrated in vacuo, leaving an oil. The ^1H NMR showed a mixture of half-amides of (*Z*)-2-benzyl-2-pentenedioic acid: ^1H NMR ($CDCl_3$) δ 0.9 (m, 3 H, methyl), 1.40 (m, 2 H, CH_2 β to nitrogen), 3.2 (m, 4 H, CH_2 α to nitrogen and allylic), 3.6 and 3.7 (s, 2 H, benzylic), 5.7 and 6.1 (t, 1 H, olefinic), 7.2 (m, 5 H, phenyl).

3-Benzyl-6-chloro-2-pyrone-5-d. (*E*)-2-Benzyl-2-pentenedioic acid (200 mg) was suspended in 2 mL of D_2O (99.8 atom % D) and heated with stirring at 90 °C for 1 h in a sealed vial. The D_2O was removed in vacuo and the residue dried in vacuo over P_2O_5. The ^1H NMR of the diacid in CD_3CN indicated that both allylic protons were completely replaced by deuterium. The diacid was converted to the chloropyrone by refluxing with CD_3COCl (prepared from CD_3CO_2D and PCl_3) as described above, giving the C-5-deuterated 3-benzyl-6-chloro-2-pyrone: ^1H NMR ($CDCl_3$) δ 3.76 (br s, 2 H, benzylic), 6.94 (br s, 1 H, H-4), 7.33 (m, 5 H, phenyl). The signal at 6.15 ppm from H-5 was completely absent.

p-Nitrophenyl Ester of (E)-2-Methyl-2-pentenoic Acid. (*E*)-2-Methyl-2-pentenoic acid (Chan et al., 1968) (1.0 g, 8.8 mmol) and *p*-nitrophenol (1.3 g, 9.3 mmol) were dissolved in 20 mL of ethyl acetate. A solution of dicyclohexylcarbodiimide (1.92 g, 9.3 mmol) in 5 mL of ethyl acetate was added dropwise with stirring. The mixture was stirred at room temperature overnight. The urea was removed by filtration and the filtrate washed with 5% $NaHCO_3$ (2×) and brine (1×) and dried ($MgSO_4$). The solvent was removed in vacuo, leaving an oil. The oil solidified on cooling at −20 °C. The solid was recrystallized from ethanol to give 0.8 g (39%) of pale yellow needles: mp 54–55 °C; NMR ($CDCl_3$) δ 1.1 (t, 3 H, J = 9 Hz, methyl), 1.95 [br s, 3 H, J = 1 Hz, C=C-(CH_3)], 2.30 (m, 2 H, CH_2), 7.06 (br t, 1 H, J = 8.4, 1 Hz, olefinic), 7.30 and 8.28 (m, 4 H, aromatic).

Preparation of Samples of Chloropyrone-Inactivated Chymotrypsin for ^{13}C NMR Analyses. Chymotrypsin (150 mg) was dissolved in 300 mL of 20 mM potassium phosphate, pH 7.0. A solution of chloropyrone (50 mg) in CH_3CN (1 mL) was added with stirring in three portions: 600 μL at the start, 200 μL after 1.2 h, and 200 μL after 1.6 h. After a total of 3 h at room temperature, enzymatic assay of an aliquot of the reaction mixture indicated that the enzyme was 90% inactivated. The solution was concentrated to 4–5 mL by ultrafiltration at 4 °C (Amicon PM10 membrane). The sample was chromatographed on a Sephadex G-25 column (1.8 × 30 cm) equilibrated with 20 mM potassium phosphate, pH 7.0, to remove small molecules. The protein-containing fractions were concentrated to 2–3 mL by ultrafiltration at 4 °C. D_2O (1 mL) was added and the sample concentrated to a final volume of 2 mL. The protein concentration was 1.3–1.6 mM and the enzyme was 85–90% inactivated. The samples were stored on ice overnight prior to the NMR analyses. Dioxane (1–2 μL) was added just prior to the NMR analyses as an internal reference standard.

^{13}C NMR Analyses of Chloropyrone-Inactivated Chymotrypsin. Protein samples were placed in 10-mm tubes and maintained at 10 °C during data collection. To minimize sample heating, a two-level proton decoupling scheme was used. During the data acquisition period (90 ms) the decoupler power was 2 W. The power was reduced to 0.5 W during the relaxtion period (2 s) to maintain the NOE. A 35° flip angle was used. Satisfactory spectra were obtained after 1000–2000 scans. The reported chemicals shifts values were measured from a dioxane internal standard (67.3 ppm).

pH titration experiments were carried out by adjusting the sample to the desired pH with dilute phosphoric acid and then recording the NMR spectrum. The samples were back-titrated to the desired pH by adding 1 N NaOH.

Chloride Analysis of Chloropyrone-Inactivated Chymotrypsin. Chloride analyses were performed by adapting the colorimetric procedure with $Hg(SCN)_2$ and $Fe(ClO_4)_3$ (Zall et al., 1956). Solutions of $Hg(SCN)_2$ and $Fe(ClO_4)_3$ were prepared as described (Zall et al., 1956). All aqueous solutions were prepared with quartz-distilled water. A sample of inactivated enzyme for chloride analysis was prepared as follows: Chymotrypsin (10 mg) was dissolved in 18 mL of 20 mM Tris·$HClO_4$, pH 7.4. One milliliter of a 10 mM solution of 3-benzyl-6-chloro-2-pyrone in CH_3CN was added in one portion. After 2 h (15% remaining enzymatic activity) the protein solution was concentrated to 2 mL by ultrafiltration. The solution was chromatographed on a Sephadex G-25 column (1.8 × 30 cm) equilibrated with 20 mM Tris·$HClO_4$, pH 7.4. Protein-containing fractions were pooled and concentrated to 1 mL by ultrafiltration. The final protein concentration was 60 μM and the enzyme was 85% inactivated. The enzyme solution was assayed for chloride as follows: Enzyme solution (0.5 mL) was mixed with 30% trichloroacetic acid (0.5 mL). After chilling on ice for 10 min, the precipitated protein was removed by centrifugation. The supernatant fluid (0.5 mL) was mixed with $Hg(SCN)_2$ solution (0.2 mL) followed by $Fe(ClO_4)_3$ solution (0.2 mL). The absorbance at 460 nm was immediately read. The amount of chloride was estimated from a standard curve prepared in an identical manner as above by using a standard KCl solution.

Isolation of the Product Produced during the Reactivation of Chloropyrone-Inactivated Chymotrypsin. A 2-mL solution of 1.5 mM inactivated chymotrypsin was prepared as described for the ^{13}C NMR samples. The solution was allowed to stand for 24 h at room temperature. The mixture was applied to a Sephadex G-25 column (1 × 18 cm) equilibrated with 20 mM potassium phosphate, pH 7.0. The small molecular weight fractions were pooled, acidified to pH 2.5 with 1 N HCl, and extracted 3 times with $CHCl_3$–ether (2:1). The extraction was carried out in a glass tube so that the layers could be separated by centrifugation. The solvent was removed from the extract by passing a stream of N_2 over the solution. The residue was dissolved in 0.5 mL of $CDCl_3$–acetone-d_6 (9:1) for ^1H NMR analysis (500 MHz) or dissolved in methanol and analyzed by HPLC on a μ Bondapak C-18 column (Waters) using methanol–20 mM acetic acid (1:1) as the solvent. The diacid product eluded at 9.5 min under these conditions. The time course of diacid production was determined by periodically removing aliquots from the reactivation mixture. The diacid content was determined by acidification, extraction, and HPLC analyses as described above.

Reactivation Kinetics in the Presence of Amines. A 1-mL solution of chymotrypsin (11.4 μM) in 20 mM potassium phosphate, pH 7.0, containing 700 μM 3-benzyl-6-chloro-2-pyrone was kept at room temperature for 2.5 h. The protein was purified from excess pyrone by chromatography on Sephadex G-25. A control sample without inactivator was prepared in an identical manner. The inactivated enzyme

solution was mixed with a number of different amines. Experiments were carried out at pH 7.0 and 9.50 by mixing 1 mL of inactivated enzyme solution with 1 mL of amine solution. The amine solutions at pH 9.50 were prepared by adding a known quantity of amine to 100 mM sodium borate, pH 9.5, and titrating to pH 9.5 with HCl. The amine solutions at pH 7.0 were prepared in 100 mM potassium phosphate, pH 7.0. The ionic strength was brought to $\mu = 1$ by the addition of the required amount of NaCl. The return of enzymatic activity was followed by periodically assaying 100-μL aliquots of enzyme solution with BTEE.

In all cases, the reactivation in the presence and absence of the amines followed first-order kinetics. The observed rate constant for reactivation in the presence of the amines is the sum of the rate constants for the amine-catalyzed and buffer-catalyzed reactions ($k_{observed} = k_{buffer} + k_2$[amine]). Thus, the pseudo-first-order rate constant for amine-catalyzed reactivation (k_2[amine]) was obtained by subtraction of the buffer-catalyzed rate from the rate observed in the presence of the amines. Plots of k_2[amine] vs. [amine] were linear in all cases, and the second-order rate constants (k_2) were determined from the slopes.

Reactivation of Chloropyrone-Inactivated Chymotrypsin in [^{18}O]H_2O. A solution of 90% inactivated chymotrypsin (950 μM) was prepared as described for the ^{13}C NMR samples. Enzyme solution (0.6 mL) and [^{18}O]H_2O (0.4 mL, 98% enriched) were mixed, and the solution was kept at room temperature for 24 h. The product released during reactivation was isolated by acidification and extraction (see above). The residue was dissolved in ether (0.5 mL) and excess CH_2N_2 in ether was added. After 10 min the ether and excess CH_2N_2 were removed with a stream of nitrogen. The diester was analyzed for oxygen-18 content by mass spectrometry using a Hewlett-Packard 5985b mass spectrometer in the electron impact ionization mode (20 eV). Samples were inserted directly into the source with a DIP probe. A small aliquot of the reactivation mixture was removed at the beginning and end of the reactivation period and assayed for diacid content by HPLC as described above. A control reaction was carried out to determine if solvent exchange with the released product occurred. An anthentic sample of (*E*)-4-benzyl-2-pentenoic acid was allowed to incubate in [^{18}O]H_2O at pH 7.0 for 24 h. The diacid was isolated as above. Mass spectrometry of the diester showed no incorporation of oxygen-18.

Measurement of the Deuterium Isotope Effects on the Reactivation. One-milliliter solutions of chymotrypsin (20 μM in either 20 mM potassium phosphate, pH 7.0, or 20 mM potassium phosphate in [^2H]H_2O were inactivated by adding either the deuterated or nondeuterated chloropyrone (20 μL of a 30 mM solution in CH_3CN). After the enzyme was 90% inactivated, the solutions were chromatographed on a Sephadex G-25 column equilibrated with 20 mM potassium phosphate, pH 7.0. The protein-containing fractions were pooled and monitored for reactivation by periodically removing aliquots and assaying for catalytic activity with BTEE.

Results

Chloride Analysis of Chloropyrone-Inactivated Chymotrypsin. To determine if the enzyme–inactivator complex contains intact chloropyrone or an acid chloride, we treated inactivated enzyme with acid and assayed the mixture for chloride. Since the chloropyrone is also a substrate for the enzyme, substrate turnover will generate a large amount of unbound chloride. To remove unbound chloride, the protein was passed through a Sephadex G-25 column. Control experiments showed that a mixture of chymotrypsin and chloride separated completely by gel filtration. No chloride was detected after acid denaturation of the inactivated enzyme after passage through Sephadex G-25. When an amount of chloride equal to the molar amount of inactivated enzyme was added to a solution of inactivated enzyme, greater than 90% of the chloride was always detected. Treatment of **1** with acid yields chloride quantitatively. These results establish that the inactivator–enzyme adduct does not contain acid-labile chlorine.

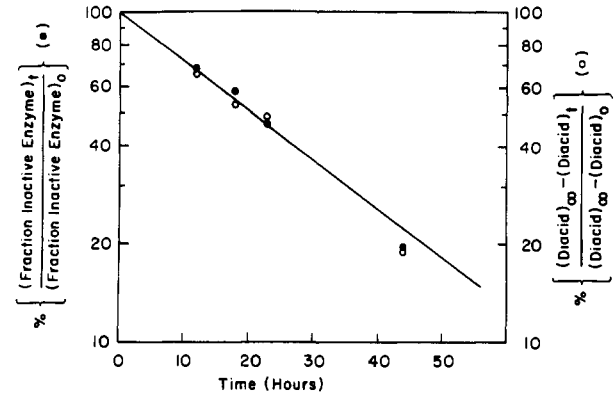


FIGURE 1: Release of **3** during reactivation of chloropyrone-inactivated chymotrypsin. The return of catalytic activity (●) and the release of **3** (○) were determined as described under Methods.

Identification of the Product Released during Reactivation of Chloropyrone-Inactivated Chymotrypsin. Inactivated chymotrypsin free of excess chloropyrone slowly regains catalytic activity upon incubation at pH 7 at room temperature with a half-life of 23 h. The product released during reactivation was isolated by acidification of the reaction mixture and extraction with organic solvent. The product was identified as (*E*)-4-benzyl-2-pentenedioic acid (**3**).

The 500-MHz ^1H NMR of the product was identical with the spectrum of an authentic sample. The four isomeric benzyl glutaconic acids are all distinguishable by ^1H NMR (Kagan et al., 1975; Westkaemper & Abeles, 1983). The diacid **3** also cochromatographed with an authentic samples on reverse-phase HPLC. It is interesting to note that the same diacid is also produced during enzymatic turnover of the chloropyrone (Westkaemper & Abeles, 1983).

Although **3** was isolated from the reactivation mixture, it may be argued that this is not the primary product released into solution but is the result of rearrangement of a precursor. However, the other three isomeric benzyl glutaconic acids and 2-benzyl-2-pentendioic anhydride are all stable to the conditions used to isolate the reactivation product (Westkaemper & Abeles, 1983). Thus, none of these compounds could have been the source of **3**.

The time course of the production of **3** was determined by periodically analyzing aliquots from a solution containing inactivated chymotrypsin for diacid by HPLC. The results are shown in Figure 1. The diacid **3** is formed concomitantly with the return of catalytic activity. A sample containing 210 nmol of 85% inactivated chymotrypsin released 160 nmol of **3** after 3 days. This result shows that **3** is the major, if not only, product released during reactivation.

^{13}C NMR of Chloropyrone-Inactivated Chymotrypsin. In order to elucidate the structure of the inactivator bound to the enzyme by nondestructive means, we synthesized chloropyrones enriched with ^{13}C and obtained the ^{13}C NMR of the inactivated enzyme. The ^{13}C NMR spectrum at 100.6 MHz of chymotrypsin inactivated with 3-benzyl-6-chloro-2-pyrone-*2,6-^{13}C* is shown in Figure 2A. The carbonyl region of the spectrum is shown. The remaining portions of the spectrum were identical with the spectrum of native enzyme. In addition

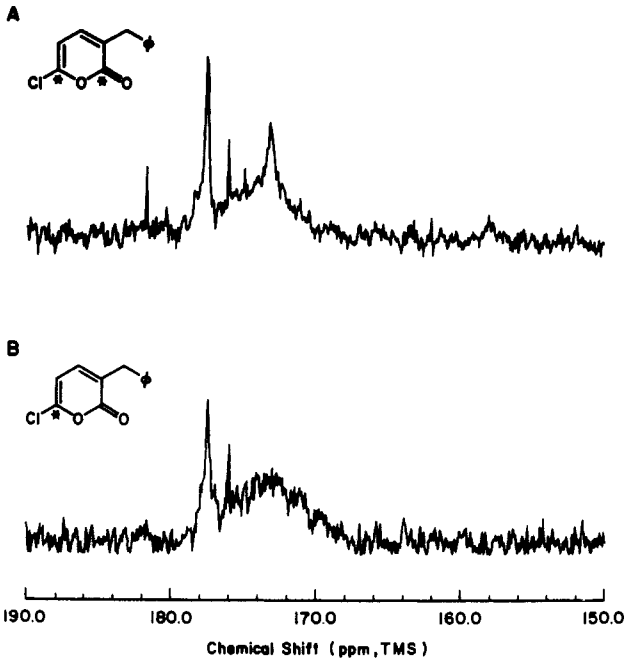


FIGURE 2: (A) 100.5-MHz ^{13}C NMR of chymotrypsin inactivated with 3-benzyl-6-chloro-2-pyrone-*2,6*-^{13}C. The conditions for obtaining the spectra are given under Methods. (B) 100.6-MHz ^{13}C NMR of chymotrypsin inactivated with 3-benzyl-6-chloro-2-pyrone-*6*-^{13}C.

to the broad envelope of carbonyl resonances (170–180 ppm), arising from the natural abundance ^{13}C content of all of the carbonyl groups of chymotrypsin, four additional peaks are seen. The two sharp lines at 181.7 and 176.0 ppm are from the product released during reactivation (**3**). These peaks represent only a small fraction of the total inactivator present in the solution. The two broad lines at 177.5 and 173.1 ppm are from the protein-bound inactivator. It is clear from this spectrum that the pyrone ring is no longer intact. The carbonyl group of the intact chloropyrone absorbs significantly upfield (161.4 ppm) from the observed resonances.

On the basis of the observed chemical shifts of the protein-bound inactivator, it seems likely that the peak at 177.5 ppm is from a carboxylate group and the peak at 173.1 ppm is due to an esterified carboxylate group. However, since the chemical shifts of esters, amides, and acids show some overlap (Pretsch et al., 1976), functional group identification based on chemical shift values alone is not conclusive. In addition, the microenvironment of the protein in the vicinity of the enriched carbon might cause a small perturbation in chemical shifts compared to the corresponding shift values observed in solution.

Evidence for the presence of a carboxylate group can be provided by measuring the chemical shift values as a function of pH. The protonation of a carboxylate group is known to produce an upfield shift of about 4 ppm in the ^{13}C NMR spectrum (Pretsch et al., 1976). The pH dependence of the chemical shifts is shown in Figure 3. Both shifts were found to move upfield as the pH was lowered from 7 to 3. The titration was reversible since titration back to pH 7 with base gave chemical shift values that followed the titration curve obtained by lowering the pH. Furthermore, the spectra before and after the titration were identical. The observed pK_a obtained from the titration experiment was ca. 4.5; the exact value cannot be determined since no shift data could be obtained below pH 3.

The pH dependence of both resonances initially suggested that both carbon atoms are carboxylate groups; however, the chemical shift value for the upfield peak at 173.1 ppm is

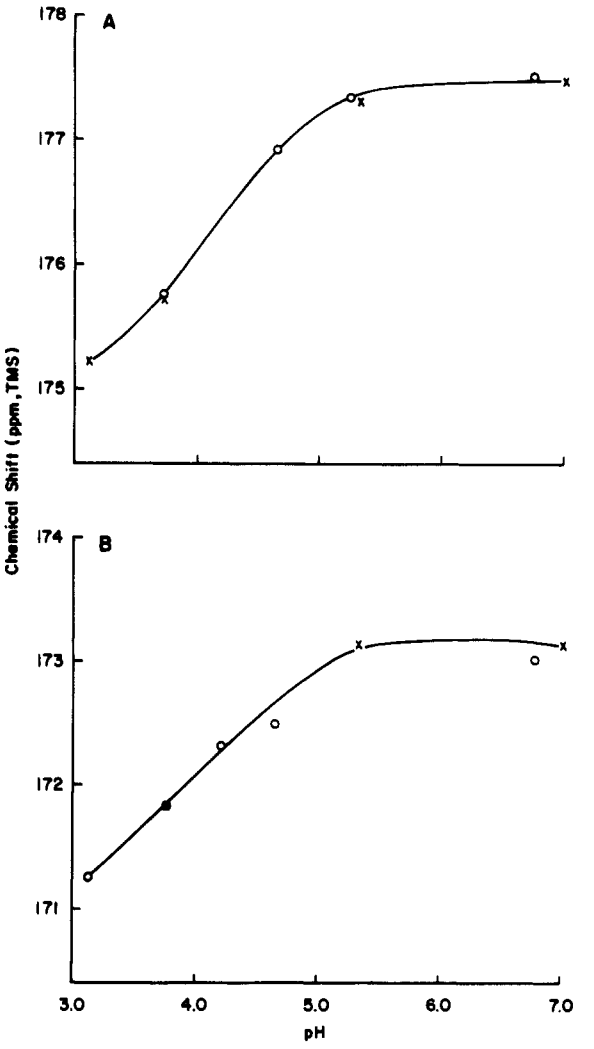
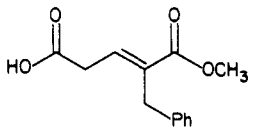


FIGURE 3: ^{13}C NMR titration of chymotrypsin inactivated with 3-benzyl-6-chloro-2-pyrone-*2,6*-^{13}C. (A) pH dependence of the chemical shift of C-6. (B) pH dependence of the chemical shift of C-2. Titration with acid is indicated by (×) and back-titration with base by (○).

significantly different from the expected chemical shift range for a carboxylate group. In order to gain some insight into this descrepancy, we studied the pH dependence of the carbonyl resonances of the model compound (*E*)-4-(methoxycarbonyl)-5-phenyl-3-pentenedioic acid in dimethyl sulfoxide–water:



As expected, the carboxylate group resonance moves upfield by 4.3 ppm upon lowering the pH. The ester carbonyl group also moves upfield during the titration; however, the upfield shift is only 1.3 ppm. Taken together, these results suggest that the resonance at 177.5 ppm in the spectrum of chloropyrone-inactivated chymotrypsin is from a carboxylate group. The resonance at 173.1 ppm may or may not be from a carboxylate group. This ambiguity was resolved by studying the oxygen-18 content of the product released during enzyme reactivation in $[^{18}O]H_2O$ (see below).

Having assigned the peak at 177.5 ppm to a carboxylate group, we need to establish which carbon atom of the chloropyrone (C-2 or C-6) generates this peak. Figure 2B shows the ^{13}C NMR of chymotrypsin inactivated with a chloropyrone

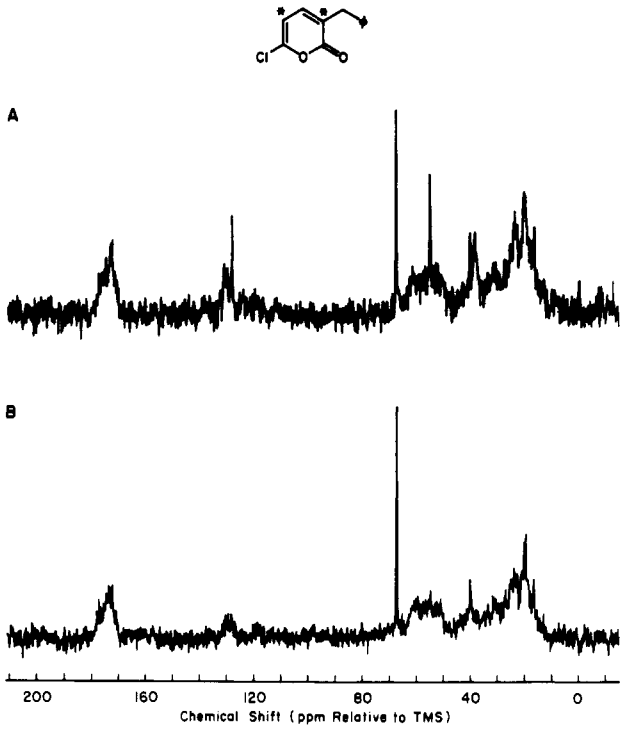
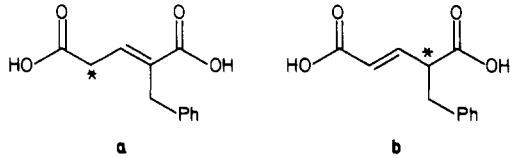


FIGURE 4: (A) 100.6-MHz ^{13}C NMR spectrum of chymotrypsin inactivated with 3-benzyl-6-chloro-2-pyrone-*3,5*-^{13}C. The conditions for obtaining the spectra are given under Methods. The sharp resonance at 67.3 ppm is from the dioxane internal reference standard. (B) 100.6-MHz ^{13}C NMR spectrum of native chymotrypsin.

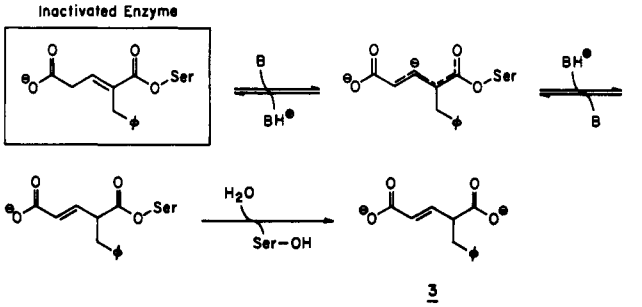
enriched solely at C-6. Only the 177.5-ppm (protein-bound) and 176.0-ppm (reactivation product) peaks are observed. This result establishes that the C-6 of the chloropyrone becomes a free carboxylate group in the inactivated-enzyme complex, thus abolishing the idea that inactivation is the result of reaction of a chloropyrone-derived electrophile at C-6 with an active-site nucleophile (Scheme II).

A direct way to determine the existence and position of a double bond in the inactivator–enzyme adduct is to observe the ^{13}C NMR spectrum of chymotrypsin inactivated with a chloropyrone enriched with ^{13}C at C-3 and C-5. Such a spectrum is shown in Figure 4A. Four additional resonances are seen, when compared to the spectrum of native enzyme (Figure 4B). The two sharp lines at 128.1 and 54.9 ppm are due to a small amount of released **3**. The two broad lines at 38.1 and 130.8 ppm are due to protein-bound inactivator. The downfield resonance demonstrates that a double bond exists in the inactivator–enzyme adduct. The position of the double bond can be easily established by comparing the observed chemical shift of the nonolefinic carbon (38.1 ppm) to that of the model compounds:



The carbon marked with an asterisk in diacid a resonates at 33.8 ppm and the carbon in diacid b resonates at 54.9 ppm. There is a 21.1-ppm difference in chemical shift between these nonolefinic carbons. The protein-bound species shows a resonance of the nonolefinic carbon at 38.1 ppm and compares well with the chemical shift of the nonolefinic carbon of diacid a. This establishes the position of the double bond between C-3 and C-4.

Scheme III



3

Reactivation in Oxygen-18-Enriched Water. Chymotrypsin was inactivated with **1** in $[^{16}O]H_2O$ and passed through a gel filtration column to remove unreacted inactivator. The inactivated enzyme was then diluted with $[^{18}O]H_2O$. The oxygen-18 content of **3** formed after reactivation was determined by mass spectrometry. The fraction of the diester molecules containing a single oxygen-18 atom, based on the ratio of ion counts at $(M + 2)^{+\cdot}$ to the sum $(M + 2)^{+\cdot} + M^{+\cdot}$, was found to be 26.5%. The amount of **3** at the beginning and end of the incubation in oxygen-18-enriched water was determined by HPLC. These results indicated that 63% of the total **3** present at the end of the incubation was released after the enzyme was added to oxygen-18-enriched water. The oxygen-18 enrichement of the water was 39.2%. Thus, the calculated value for the fractional oxygen-18 content of **3** is 24.7% assuming only *one* oxygen-18 atom per molecule of **3**. This value agrees well with the experimentally determined value. If the inactivator were covalently attached to the enzyme at both ends, one would expect 10% of **3** to contain two oxygen-18 atoms. However, none of the released **3** contained two oxygen-18 atoms (detection limit ≈2%). These results suggest that only one of the carboxyl groups of the inactivator is covalently bound to the enzyme. On the basis of the ^{13}C-NMR spectra (see above), the covalently attached end must be derived from the C-2 of the original chloropyrone. Taken together, the ^{13}C NMR results and the ^{18}O-incorporation studies suggest the structure of the bound inactivator shown in Scheme III.

Effect of Nucleophile on the Reactivation Rate. A number of nucleophiles were tested for their ability to increase the rate of reactivation of chloropyrone-inactivated chymotrypsin. Methanol (5%) or ethanol (5%) had no effect on the rate of reactivation. Amines, on the other hand, caused a dramatic increase in the reactivation rate. For example, *n*-propylamine (4.1 mM) caused a 5-fold reduction in the half-time for reactivation of chloropyrone-inactivated enzyme. This experiment was carried out at pH 9.5. The rate of reactivation in the absence of amine is 2.3 times faster at pH 9.5 than at pH 7.0. The unprotonated amine species participates in the reactivation acceleration since *n*-propylamine (pK_a = 10.6) has little effect on the reactivation rate at pH 7.0.

Reactivation kinetics were measured in the presence of amines with differing pK_a values. The second-order rate constants for the reaction of the amine with the inactivated enzyme were determined as described under Methods and were used to contract a Brønsted plot (Figure 5). The log k_2 values for most of the amines fall on a line of slope 0.45. This slope is significantly larger than the value of 0.13 reported for the aminolysis of acylchymotrypsin by amines (Inward & Jencks, 1965). In reactions in which the amine functions as a nucleophile, the log k_2 value for NH_2OH often is much larger than the values for other amines with similar pK_a values (Jencks & Carrioulo, 1960). The observation that the log k_2

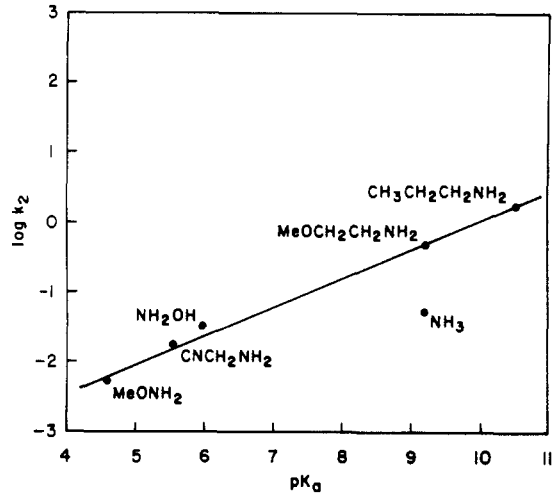


FIGURE 5: Brønsted plot for the amine-catalyzed reactivation of chymotrypsin inactivated with **1**. The logarithm of the second-order rate constant for the reaction of the amines with chloropyrone-inactivated chymotrypsin is plotted vs. the pK_a values of the conjugate acids of the amines.

value for NH_2OH falls on the Brønsted plot suggests that the amines are functioning as bases rather than as nucleophiles. The log k_2 value determined for NH_3 is seen to fall below the line determined by the other amines. This lower than predicted value of log k_2 for ammonia has been also observed in reactions catalyzed by amine bases (Walters & Long, 1969).

To further establish the role of the amines, the product of reactivation in the presence of *n*-propylamine was identified. A sample of chymotrypsin inactivated with **1** was separated from excess inactivator and allowed to reactivate in the presence of *n*-propylamine. A reactivation mixture containing sufficient *n*-propylamine to give an ca. 10-fold increase in reactivation rate was acidified and extracted to obtain the reactivation product. The product was again **3** as shown by ^1H NMR and HPLC. If the amines were functioning as nucleophiles, the expected product of reactivation would be an amide. No amide products could be detected by HPLC. A synthetic preparation of a mixture of half-amides of (*Z*)-2-benzyl-2-pentenedioic acid and the diamide of (*E*)-2-benzyl-2-pentenedioic acid eluted on HPLC shortly after the diacid, demonstrating that any amide products could have been detected by HPLC. Taken together, these results demonstrate that the amines function as general bases and not as nucleophiles in the acceleration of reactivation of chloropyrone-inactivated chymotrypsin.

Deuterium Kinetic Isotope Effects on Reactivation. The NMR experiments cited above establish that the double bond in the enzyme–inactivator complex is in the α,β-position,[2] while the double bond in **3**, released during inactivation, is in the β,γ-position. Therefore, reactivation involves double bond migration in addition to hydrolysis of the acyl-enzyme. To determine if double bond migration is rate limiting for reactivation, we carried out the inactivation with **1** deuterated at C-5. Isomerization of the double bond requires proton abstraction from the γ-position of the enzyme-bound inactivator. If proton abstraction is rate determining, a deuterium isotope effect will be observed on the reactivation rate. Data are shown in Table I. Introduction of a deuterium into C-5 of the inactivator decreased the rate of reactivation (2.5 ± 0.2)-fold. The isotope effect on the reactivation rate in the presence of an amine was also determined. The results are summarized in Table I. The data establish that an isotope effect is observed, in the presence as well as in the absence of the amine, on the reactivation of chymotrypsin inactivated with C-5-deuterated **1**. These results show that abstraction of the C-5 hydrogen is required for reactivation and that isomerization of the double bond and not hydrolysis of the acyl-enzyme is rate determining. Scheme III shows a mechanism of reactivation that is consistent with these results. In this mechanism isomerization of the double bond (proton abstraction) precedes hydrolysis of the acyl-enzyme.

Table I: Deuterium Kinetic Isotope Effects on the Reactivation of Chymotrypsin Inactivated with **1**

source of deuterium	isotope effect[a] buffer catalyzed[b]	amine catalyzed[c]
1 deuterated at C-5	2.5 ± 0.2	3.3 ± 0.2
solvent (D_2O)	1.1 ± 0.05	1.6 ± 0.1
1 deuterated at C-5 and solvent (D_2O)	2.8 ± 0.2	4.3 ± 0.2

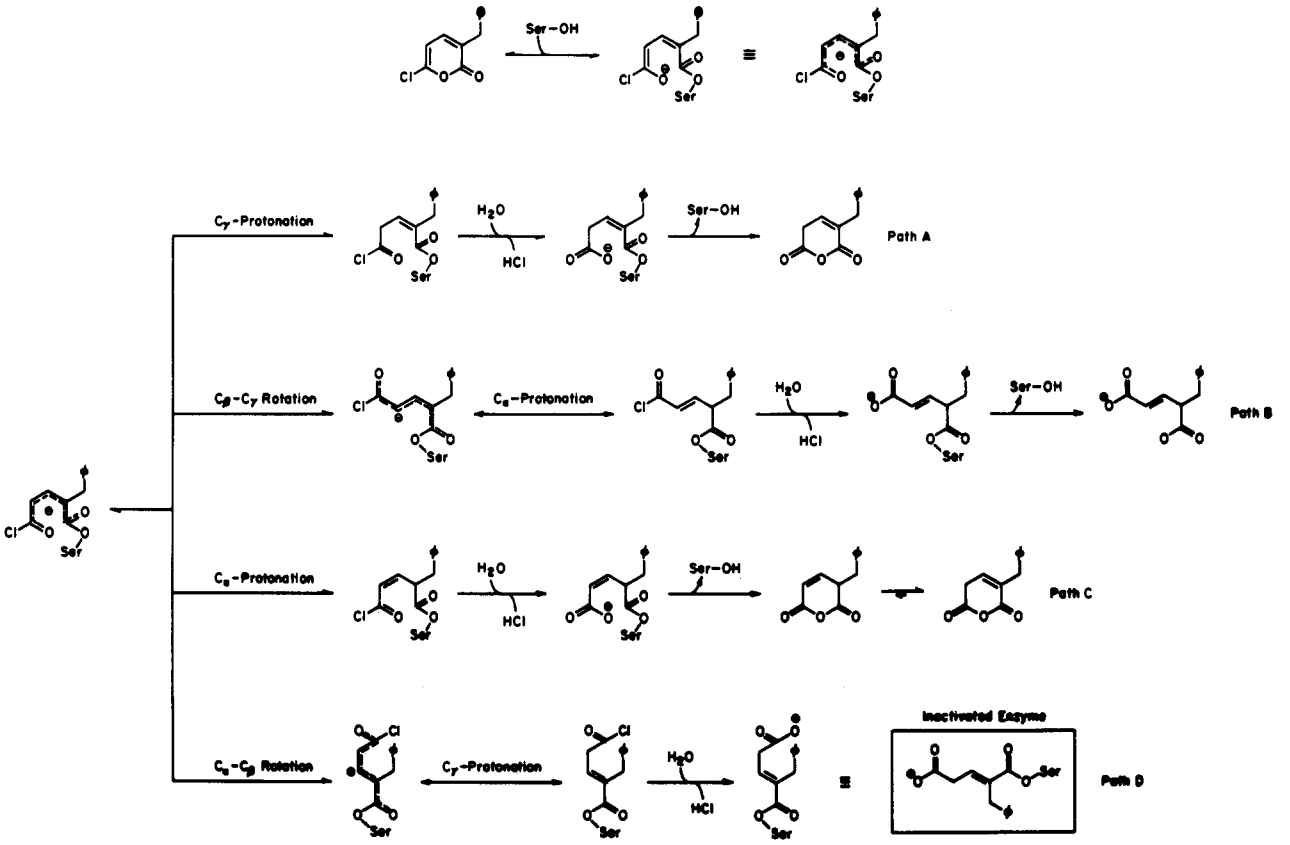
[a] All isotope effects are expressed as the ratio of the observed reactivation rate in the absence of a source of deuterium to the observed rate in the presence of a deuterium source under otherwise identical conditions. The values represent the average and standard deviation from two to three independent measurements. [b] Reactivation rates were measured in 20 mM potassium phosphate, pH 7.0, 25 °C. [c] Reactivation rates were measured in 20 mM potassium phosphate, pH 8.3, 25 °C, containing (2-methoxyethyl)amine (concentration of unprotonated amine is 49 mM).

Several other experiments were done in which the inactivation was carried out in $[^2H]H_2O$ with either deuterated or nondeuterated **1**. The data in Table I show that a small isotope effect on the reactivation rate is observed when inactivation is carried out in $[^2H]H_2O$ with nondeuterated **1** and reactivation in H_2O. This result shows that during the inactivation a proton (deuterium) from the solvent is incorporated into the inactivator. This is in accordance with the mechanism shown in Scheme III. In the formation of the inactive acyl-enzyme, the γ-carbon is converted to a methylene group with proton (deuterium) uptake from solvent. This methylene group will contain both hydrogen and deuterium. The small isotope effect observed may be a secondary isotope effect. Alternatively, the observed isotope effect may be the result of abstraction of the solvent-derived deuterium from the γ-position reflecting a lack of complete stereospecificity in the γ-carbon protonation and/or protein abstraction steps.

Interaction of the p-Nitrophenyl Ester of (E)-2-Methyl-2-pentenoic Acid with Chymotrypsin. The inactivator–enzyme adduct contains an α,β double bond and a terminal carboxylate group. In order to evaluate the importance of the double bond by itself in stabilizing the acyl-enzyme, the action of chymotrypsin on the *p*-nitrophenyl ester of (*E*)-2-methyl-2-pentenoic acid was examined. Acylation of chymotrypsin with this activated ester will generate an acyl-enzyme that is structurally similar to the adduct formed with **1** but lacks the terminal carboxylate group. Incubation of chymotrypsin (23 μM) with the ester (360 μM) in phosphate buffer, pH 7, resulted in time-dependent inactivation of the enzyme. The half-time for inactivation was 0.6 min. The rate of deacylation of inactivated enzyme was measured by diluting an aliquot of completely inactivated enzyme into phosphate buffer containing an excess of BTEE. The rate of BTEE hydrolysis increased in a first-order fashion from an initial value of zero to a constant final value. This rate increase represents the time-dependent formation of active enzyme following deacylation. Analysis of

[2] The α-position of the enzyme-bound inactivator refers to the carbon atom adjacent to the carbonyl carbon of the covalent ester linkage. Thus, the α-carbon atom is derived from C-3 of the original chloropyrone. The β- and γ-carbon atoms are derived from C-4 and C-5 of the chloropyrone, respectively.

Scheme IV



the data by standard methods yields a rate constant for deacylation of $k = 0.15$ min^{-1} (half-time = 4.7 min). This half-time for deacylation is significantly faster than the half-time for reactivation of chloropyrone-inactivated enzyme (23 h).

Discussion

In a previous report (Westkaemper & Abeles, 1983), we demonstrated that **1** inactivates chymotrypsin in a time-dependent manner. In addition, **1** is also a substrate for chymotrypsin, producing **3** as the major product and **4** as the minor product. Our previous results were consistent with the mechanism of inactivation shown in Scheme II; however, the structure of the inactivator bound to the enzyme was speculative since no direct structure information was available.

In the present studies, we have used ^{13}C NMR of chymotrypsin inactivated with ^{13}C-enriched chloropyrones to determine the structure of the enzyme-bound inactivator. The results presented here make the mechanism shown in Scheme II for inactivation of chymotrypsin by **1** unlikely.

Scheme III shows the structure of the enzyme–inhibitor adduct that we now propose. The salient structure feature of this adduct, which differs from that previously proposed, is that the inactivator is attached to the enzyme at only one point, through an ester linkage between the carbonyl carbon derived from C-2 of **1** and an OH group, presumably serine-195, of chymotrypsin. C-6 of **1** becomes a free carboxylate group. We shall leave aside for the moment the question of why this adduct is stable, i.e., why it hydrolyzes with a $t_{1/2}$ of 23 h, and consider the evidence in support of the structure and the mode of hydrolysis. The ^{13}C NMR spectrum of the enzyme shows that the resonance from C-6 of the inactivator is at 177.5 ppm and the chemical shift shows a reversible pH dependence consistent with a carboxylate group. The chemical shift of C-2 is 173.1 ppm, consistent with that of an ester. We consider it likely that C-2 forms an ester with serine-195 of chymotrypsin; however, this has not been firmly established in this study. Hydrolysis of the adduct (enzyme reactivation) gives rise to the diacid **3** shown in Scheme III. When this hydrolysis is carried out in [^{18}O]H_2O, only a single ^{18}O atom is incorporated into **3**. This is consistent with the presence of single ester linkage in the adduct. The ^{13}C NMR spectrum of the enzyme inactivated with a chloropyrone enriched at C-3 and C-5 shows that a double bond is present in the α,β-position. These results taken together provide strong evidence for the structure of the enzyme–inactivator adduct shown in Scheme III.

We consider it likely that the *E* isomer of the inactivator is bound to the enzyme. The *Z* isomer apparently deacylates rapidly. This conclusion is based on the observation that the anhydride **4** is hydrolyzed by chymotrypsin and no inactivation occurs (Westkaemper & Abeles, 1983). It is likely that this reaction proceeds through an intermediate α,β-unsaturated acyl-enzyme involving the *Z* isomer.

If the structure of the adduct and the diacid **3** released upon hydrolysis is accepted, then it follows that hydrolysis must be accompanied or preceded by a shift of the double bond from the α,β- to the β,γ-position. Our data indicate that this shift is rate determining in the reactivation of the enzyme, i.e., precedes hydrolysis. When the enzyme is inactivated with C-5-deuterated **1**, an isotope effect is seen on the rate of reactivation. This establishes that dissociation of the carbon hydrogen bond from the γ-position occurs during reactivation as required by the mechanism shown in Scheme III. Furthermore, this dissociation is rate determining.

Enzyme reactivation is greatly accelerated by amines. This process does not involve nucelophylic attack on the carbonyl group, as shown by the Brønsted plot and by the fact that no amide is formed. It is probable that the amine acts as a general base in assisting proton abstraction from the γ-position in the

reactivation process shown in Scheme III.

Reaction of **1** with chymotrypsin results in hydrolysis of the chloropyrone and enzyme inactivation. From 10 to 40 chloropyrone molecules are hydrolyzed before inactivation occurs. Scheme IV shows a reaction sequence that accounts for the hydrolysis products formed (**3** and **4**) and enzyme inactivation. According to that scheme, attack of the active-site serine on the carbonyl group of the chloropyrone leads to the formation of an enolate. This enolate can then undergo several different reactions (paths A–D) that lead to either product formation or enzyme inactivation. If the enolate is protonated at the γ-position (path A) or at the α-position (path C), no inactivation occurs but hydrolysis products are formed. Alternatively, the enolate can undergo either of two rotations prior to protonation. Rotation about C_β–C_γ followed by protonation (path B) leads to formation of the major hydrolysis product (**3**). Rotation about C_α–C_β followed by protonation (path D) leads to the inactivated enzyme. This acyl-enzyme hydrolyses with $t_{1/2}$ = 23 h.

Four different acyl chymotrypsins are shown in Scheme IV. These differ in the position of the double bond and the position of the carboxyl group. Why does one of these hydrolyze much more slowly than the others? We have shown above that reactivation requires migration of the double bond from the α,β- to the β,γ-position. It is not likely that the presence of the double bond in the α,β-position is entirely responsible for the slow hydrolysis. The rate of hydrolysis of acylchymotrypsins with α,β-unsaturation has been studied (Bender et al., 1962). Their $t_{1/2}$ for hydrolysis ranges in minutes, while $t_{1/2}$ for reactivation of chymotrypsin inactivated with **1** is 23 h. We have also examined the hydrolysis of the *p*-nitrophenyl ester of (*E*)-2-methyl-2-pentenoic acid, which gives rise to an α,β-unsaturated acylchymotrypsin, structurally resembling the stable adduct formed between **1** and chymotrypsin but lacking a terminal carboxylate group. The half-time for hydrolysis was 4.7 min. We propose that the presence of the carboxylate group, specifically, the negative charge, is the major factor responsible for the slow hydrolysis. Furthermore, the location of the carboxyl group is crucial. In the reactivation process the double bond migrates and the position of the carboxylate group changes. This change in positions of the carboxyl group allows more rapid hydrolysis of the acyl-enzyme. It is premature to speculate at this time concerning the mechanism by which the negative charge of the carboxyl group retards the hydrolysis processes. We hope that further structural studies of chymotrypsin inactivation with **1** will clarify this point.

Acknowledgments

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