Synthesis and Characterization of a Selenium-Containing Substrate of α-Chymotrypsin. Selenium-77 Nuclear Magnetic Resonance Observation of an Acyl-α-chymotrypsin Intermediate†

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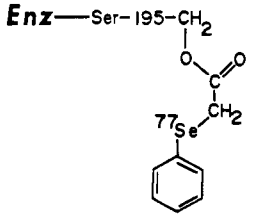
ABSTRACT: The selenium-containing ester *p*-nitrophenyl (phenylselenyl)acetate, $C_6H_5SeCH_2C(O)OC_6H_4$-*p*-(NO_2), has been synthesized, characterized as a substrate for α-chymotrypsin (k_2/K_M = 15.2 × 10^3 M^{-1} s^{-1}, K_M^{app} = 5.16 × 10^{-6} M, pH 7.77, 33% CH_3CN, 25 °C), and shown to be an active-site titrant for the enzyme. A synthesis of the selenium-77 enriched *p*-nitrophenyl (phenylselenyl)acetate in 53% yield from 94.4% elemental selenium-77, followed by its reaction with α-chymotrypsin (pH 5.0, 0–3 °C), permitted the observation of the (phenylselenyl)acetyl-α-chymotrypsin reaction intermediate by selenium-77 NMR spectroscopy. This acyl-enzyme species had a chemical shift of 275.1 ppm relative to dimethyl selenide. Accompanying this resonance was a lower intensity, pH-dependent resonance that is assigned to (phenylselenyl)acetate on the basis of a pH titration of the model compound. Deacylation in the presence of hydrazine sulfate produced a resonance at 332.3 ppm in addition to the 302.2 ppm resonance of (phenylselenyl)acetate at pH 7.85. Denaturation of the acyl-enzyme resulted in a shift of the 275.1 ppm resonance to 334.6 ppm at pH 4.90, in good agreement with the selenium-77 chemical shift of the model compound, methyl (phenylselenyl)acetate, in $CDCl_3$ (333.3 ppm). The large shielding observed for the native acyl-enzyme in comparison to the denatured species can be attributed to (1) a resonance-perturbed ester linkage and/or (2) steric compression at a nonbonding orbital of the selenium nucleus.

Selenium-containing biomolecules that have been synthesized by in vivo and chemical methods include naturally occurring selenocysteine-containing proteins (Stadtman, 1980a,b; Odom, 1983), selenomethionine-containing proteins (Huber & Criddle, 1967; Coch & Greene, 1971; Frank et al., 1985), selenium-containing t-RNAs (Stadtman, 1980b; Wittwer, 1983), and a wide range of cofactors and substrates with varying degrees of enzymatic activity (Stadtman, 1979; Odom, 1983; Klayman & Günther, 1973). In connection with the last area, we have recently communicated results on the observation of a protein–selenoligand complex by selenium-77 NMR[1] spectroscopy utilizing the selenium-77 isotope at natural abundance (7.58%) (Mullen et al., 1985). Previously, we have demonstrated the feasibility of observing selenium-77 resonances in selenenyl sulfide derivatives of proteins chemically modified with the sulfhydryl reagent 6,6′-diselenobis(3-nitrobenzoic acid), DSNB (Luthra et al., 1982). In this paper we report the use of selenium-77 NMR spectroscopy for investigating a (phenylselenyl)acetyl-α-chymotrypsin reaction intermediate (**1**) in the α-chymotrypsin-catalyzed hydrolysis of the substrate *p*-nitrophenyl (phenylselenyl)acetate.



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Selenium-77 (I = $^1/_2$) is a very appealing structural and electronic probe with a chemical shift range of ~2800 ppm. As a result of many investigations in this and other laboratories, selenium-77 chemical shifts are now empirically understood (Luthra & Odom, 1986). These factors, combined with the receptivitiy of selenium-77 (2.98 times that of carbon-13 for an equal number of naturally abundant nuclei), make selenium-77 NMR spectroscopy a potentially useful technique for biochemical investigations. However, observation of selenium-77 resonances for selenium covalently attached to proteins requires proper relaxation effects. We proceeded with the study reported here on the basis of a theoretical treatment of spin–lattice and transverse relaxation times for a protein molecule with a large rotational correlation time (Odom et al., 1979). For α-chymotrypsin with a rotational correlation time of 11 ns/rad at 20 °C (Kuznetsov et al., 1975), this treatment predicts T_1's of <0.25 s and minimal line widths of ~20 Hz for a chemical shift anisotropy dominated relaxation mechanism at a magnetic field of 4.7 T (200 MHz ^{1}H resonance frequency). These parameters, in principle, satisfy the requirements for observation of a selenium-containing acyl-enzyme intermediate.

Mechanistic aspects of α-chymotrypsin catalysis have been thoroughly reviewed from kinetic (Bender & Kêzdy, 1965; Bender & Killheffer, 1973) and structural perspectives (Kraut, 1977; Stein & Shulman, 1982). Particularly noteworthy in the α-chymotrypsin mechanism of catalysis is the high lability of the acyl-enzyme species, which is due in part to the location of the His-57 and Asp-102 at the active site of the enzyme. Several physical observations of acyl-enzyme intermediates

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[1] Abbreviations: NMR, nuclear magnetic resonance; FID, free induction decay; Tris, tris(hydroxymethyl)aminomethane; pH^{app}, apparent pH as measured by the electrode at the temperature of the buffer with no correction for the deuterium isotope effect; K_M^{app}, apparent Michaelis constant; k_2/K_M, second-order rate constant; k_{cat}, turnover number; SR, spin rotation; CSA, chemical shift anisotropy.

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have been directed toward elucidating structural and electronic features of these activated esters. These include an X-ray crystallographic determination of the structure of indoleacryloyl-α-chymotrypsin at 2.5 Å (Henderson, 1970), absorption spectrophotometry investigations of arylacryloyl-α-chymotrypsins (Bender et al., 1962; Bernhard et al., 1965; Charney & Bernhard, 1967; Bernhard & Malhotra, 1974), resonance Raman studies of acyl-α-chymotrypsins (MacClement et al., 1981; Phelps et al., 1981; Argade et al., 1984), carbon-13 NMR observations of acetyl-α-chymotrypsin (Nie et al., 1977; Tobias et al., 1984) and 5-*n*-propyl-2-furoyl-α-chymotrypsin (McWhirter et al., 1985), and fluorine-19 NMR observation of *p*-(trifluoromethyl)cinnamoyl- and *o*-, *m*-, and *p*-fluorocinnamoyl-α-chymotrypsins (Maddox et al., 1975; Gerig & Halley, 1981). Herein we report that selenium-77 NMR spectroscopy is particularly useful in the characterization of an appropriately substituted selenium-containing acyl-enzyme intermediate.

Experimental Procedures

Materials. α-Chymotrypsin from bovine pancreas (type II, lot 54F-8020; type I, lot 92F-8035), cinnamoylimidazole, *p*-nitrophenyl acetate, and iodoacetic acid were products of Sigma Chemical Co. Phenylselenoacetaldehyde and resublimed magnesium were purchased from Morton-Thiokol. Deuterium oxide and spectrophotometric grade acetonitrile were obtained from J. T. Baker Chemical Co. Isotopically enriched elemental ^{77}Se (94.38%) was purchased from Oak Ridge National Laboratory, Oak Ridge, TN. All other materials were of reagent quality. Cinnamoylimidazole was recrystallized twice from dry cyclohexane, mp 130–131 °C [lit. mp 133–133.5 °C (Schonbaum et al., 1961)]. Solvents were dried for the Grignard reaction by using standard procedures. All other materials were used as obtained. All buffer solutions were made from deionized water. Elemental analyses were performed by Galbraith Laboratories, Inc. Melting points were measured with a Mel-Temp apparatus and are uncorrected. Solution pH was measured with a PHM82 Radiometer pH meter calibrated to standard pH buffers.

(Phenylselenyl)acetic Acid. To a 100-mL three-neck reaction vessel equipped with an addition funnel and a N_2 gas inlet and bubbler was added diphenyl diselenide (1.52 g, 4.9 mmol) and 40 mL of absolute ethanol. Dissolution was achieved by slightly heating the magnetically stirred solution. The intensely yellow diphenyl diselenide was reduced under a nitrogen atmosphere by the slow addition of an argonated water–sodium borohydride solution (2 M) (enough to produce a yellow-to-white end point), while gradually cooling the reaction mixture to 0 °C. A borate solution (0.05 M, 30 mL) containing iodoacetate (2.0 g, 10.8 mmol) was adjusted to pH 10 and added dropwise to the cooled and stirred solution of the phenylselenolate. The reaction was allowed to proceed overnight at ambient temperature. The mixture was then diluted to twice its volume with a borate buffer solution (0.05 M, pH 10), the ethanol was removed by rotary evaporation, and the remaining diphenyl diselenide was filtered. The chilled and stirred filtrate was acidified by the dropwise addition of concentrated HCl, extracted with CH_2Cl_2, dried with $MgSO_4$, and rotary evaporated. The resulting oil crystallized in vacuo over P_2O_5/NaOH in 82% yield from diphenyl diselenide consumed: mp 32.5–33 °C; ^{13}C NMR ($CDCl_3$) δ 27.1 (C-β), 128.0 (C-4), 128.8 (C-1), 129.2 (C-3,5), 133.3 (C-2,6), 177.3 (C-γ). Anal. Calcd for $C_8H_8O_2Se$: C, 44.67; H, 3.75; Se, 36.71. Found: C, 44.45; H, 3.77; Se, 36.64.

Sodium (Phenylselenyl)acetate. The synthesis of (phenylselenyl)acetic acid was scaled up 10-fold, and the resulting product was dissolved in CH_2Cl_2/ether and was shaken with 6 M NaOH at 0 °C. The precipitated sodium (phenylselenyl)acetate was filtered, dried in vacuo over P_2O_5/NaOH, and crushed to a white powder (yield 75%), mp 290–292 °C with decomposition.

Methyl (Phenylselenyl)acetate. Sodium (phenylselenyl)acetate was suspended in methanol and the mixture was saturated with dry HCl. After the mixture was refluxed with stirring for 3 h and stirred at an ambient temperature for 3 h, the reaction mixture was filtered and the resulting solution rotary evaporated. The crude product was dissolved in CH_2Cl_2, and the solution was washed twice with saturated sodium bicarbonate, dried with $MgSO_4$, and rotary evaporated to yield a light yellow oil: ^{13}C NMR ($CDCl_3$) δ 27.0 (C-β), 52.0 (–OCH_3), 127.6 (C-4), 128.9 (C-3,5, C-1), 133.0 (C-2,6), 170.9 (C-γ).

p-Nitrophenyl (Phenylselenyl)acetate. (Phenylselenyl)acetic acid (0.5 g, 2.3 mmol) was dissolved in CH_2Cl_2 (20 mL). To the chilled and stirred solution, dicyclohexylcarbodiimide (0.53 g, 2.5 mmol, 10% excess) in CH_2Cl_2 (15 mL) was added, followed immediately by *p*-nitrophenol (0.41 g, 2.9 mmol, 20% excess) in CH_2Cl_2 (15 mL) and several drops of pyridine for dissolution. After 1 h at 0 °C and 1 h at ambient temperature, glacial acetic acid (0.6 mL) was added. The dicyclohexylurea was filtered from the reaction mixture and washed with CH_2Cl_2. The organic layers were combined and washed twice with 0.1M HCl, once with saturated sodium bicarbonate solution, once with water, and dried with $MgSO_4$. After evaporation of the solvent, the oil was dried in vacuo over P_2O_5/NaOH, chilled to −10 °C, and crystallized by triturating with absolute ethanol. The solid was recrystallized from ethanol/H_2O and ethanol: mp 62–63 °C; ^{13}C NMR ($CDCl_3$) δ 26.8 (C-β), 121.9 (C-2′,6′), 124.8 (C-3′,5′), 128.0 (C-1), 128.2 (C-4), 129.1 (C-3,5), 133.7 (C-2,6), 145.0 (C-4′), 155.0 (C-1′), 168.1 (C-γ). Anal. Calcd for $C_{14}H_{11}NO_4Se$: C, 50.01; H, 3.30; Se, 23.49. Found: C, 49.92; H, 3.45; Se, 23.19.

[^{77}Se]-p-Nitrophenyl (Phenylselenyl)acetate. ^{77}Se (100 mg, 1.3 mmol) was placed in a glass-blown side arm on a one-neck 25-mL flask. Resublimed Mg (32 mg, 1.3 mmol) and a micro stir bar were placed in the bottom of the flask. The flask was attached to a reflux condenser that was attached to a high-vacuum line. The apparatus was flamed out under dynamic vacuum (taking care not to vaporize the selenium) and allowed to cool to room temperature. Bromobenzene (220 mg, 1.4 mmol), followed by tetrahydrofuran (1 mL), was introduced into the bottom of the flask, which had been cooled to liquid nitrogen temperature (−196 °C). The frozen mixture was isolated below the reflux condenser, allowed to warm, and heated at a gentle reflux with magnetic stirring. After all the Mg had reacted, the Grignard solution was frozen at −196 °C and the selenium was condensed onto the reaction mixture. On allowing the mixture to warm, the finely coated reddish gray selenium reacted as the isolated flask was swirled. The flask was capped with a septum, and a stream of nitrogen was introduced. Methanol (2 mL) was added, followed by a few drops of a $NaBH_4$ solution (enough to produce a solution containing a white precipitate), and argonated water (2 mL) for total dissolution of the precipitate. Borate buffer (0.5 mL, .05 M, pH 10) containing iodoacetate (705 mg, 3.8 mmol, 3× excess) was gradually syringed into the magnetically stirred solution and allowed to react overnight at room temperature. The cooled and stirred solution was acidified with concentrated HCl, extracted with CH_2Cl_2 in the same flask (syringing off the aqueous layer), and dried with $MgSO_4$. After the solution was concentrated to 3 mL, the synthesis of the *p*-nitrophenyl

ester was performed as in the previous procedure (vide supra) by using dicyclohexylcarbodiimide (300 mg, 1.4 mmol) in CH_2Cl_2 (2 mL), *p*-nitrophenol (210 mg, 1.5 mmol) in CH_2Cl_2 (2 mL), and washing with 0.2 M HCl, 1% $NaHCO_3$, and water. In the purification of the crude product, addition of ethanol produced a dark oil, which was dissolved in ether (100 mL) and filtered to remove intractable material. The ethereal and ethanolic supernatants were combined, evaporated to an orange oil, and crystallized by trituration with ethanol at −10 °C, yielding 170 mg of an off-white powder. The mother liquor gave a second crop of 55 mg upon crystallization from ethanol/H_2O. The combined yield was 53% from elemental selenium-77.

Kinetics. The kinetics of all reactions were monitored spectrophotometrically on a Hewlett-Packard HP-8450 spectrophotometer equipped with a thermostated cell compartment that maintained the temperature at ±0.1 °C. All data were analyzed graphically after being plotted on a Hewlett-Packard 7470A plotter by extrapolating the initial rate of the steady-state reaction, usually from the first 100 s of the reaction period. Due to the low solubility of the *p*-nitrophenyl (phenylselenyl)acetate in water, a 33% acetonitrile solution was employed. The extinction coefficient (ϵ) for *p*-nitrophenolate was determined at pH 8.62 in 33% acetonitrile by hydrolyzing a stock solution of *p*-nitrophenyl acetate (recrystallized from hexane/chloroform, mp 79.0–79.5 °C [lit. mp 79.5–80.0 °C (Kézdy & Bender, 1962)]) in 1.0 N NaOH and diluting appropriately. At λ_{max} 400 nm, an $\epsilon_{400} = 1.93 \times 10^4$ M^{-1} cm^{-1} was determined from a Beer's law plot of the data. α-Chymotrypsin active-site concentrations were determined with cinnamoylimidazole (Schonbaum et al., 1961). The extinction coefficient at 335 nm of cinnamoylimidazole at pH 5.09 (0.1 M total acetate buffer, 3.3% CH_3CN) as determined by the titration method was found to be 9.02×10^3 M^{-1} cm^{-1}. The type II α-chymotrypsin normally titrated between 78 and 85% active sites on a weight basis (M_r 24 800).

The following experimental technique was used in monitoring the enzyme kinetics. Acetonitrile, the appropriate aliquot of *p*-nitrophenyl (phenylselenyl)acetate dissolved in acetonitrile, and 2 mL of the buffer solution (Tris, 0.08 M; $CaCl_2$, 0.1 M; pH 7.77) were added to a cuvette containing a magnetic stirrer. The solution was thermostated and stirred in the cell compartment for 5–8 min, recording at 400 nm was begun, and an 80 or 50-μL aliquot of the enzyme solution was added from the tip of a stirring rod and stirred vigorously for a few seconds. Delivery volumes were calibrated, and ideal solutions were assumed.

NMR Sample Preparation. Two procedures were used in the preparation of the acyl-α-chymotrypsin. (1) α-Chymotrypsin (type II) was dissolved to a concentration of 4.5 mM (6.3 mL, 2.8×10^{-5} mol) in D_2O containing 0.1 M acetate at an apparent pH of 4.9 and readjusted to pH 5.0. [^{77}Se]-*p*-nitrophenyl (phenylselenyl)acetate (14.4 mg, 4.2×10^{-5} mol) was dissolved in CH_3CN (0.7 mL) and added to the chilled enzyme solution (0–3 °C) with vigorous vortexing. The solution was frozen, lyophilized to remove CH_3CN, and redissolved, while cold, to an acyl-enzyme concentration of 2.6 mM. (2) In the second procedure, α-chymotrypsin (1.6×10^{-5} mol) was reacted as in procedure 1 with [^{77}Se]-*p*-nitrophenyl (phenylselenyl)acetate (14.6 mg, 4.4×10^{-5} mol) at room temperature.

The preparation of the denatured acyl-α-chymotrypsin followed procedure 1 with α-chymotrypsin (1.6×10^{-5} mol) and *p*-nitrophenyl (phenylselenyl)acetate (6.8 mg, 2.0×10^{-5} mol) being used. The acyl-α-chymotrypsin solution was adjusted to pH 2 and denatured with an acidic solution of urea, which resulted in an 8 M urea solution. This solution was subsequently adjusted in pH.

In order to prepare samples for a ^{77}Se NMR titration of (phenylselenyl)acetate in the presence of denatured enzyme, α-chymotrypsin (5.4 mM in D_2O containing 0.1 M acetate buffer, pH 4.4) was reacted with [^{77}Se]-*p*-nitrophenyl (phenylselenyl)acetate (12.1 mg, 3.5×10^{-5} mol) at room temperature, and the solution was adjusted to pH 4.8 with 6 M NaOH. Selenium-77 NMR spectroscopy at 19 ± 1 °C showed a single resonance at 303.4 ppm. Denaturation of the protein was performed by the addition of urea (2.67 g, sufficient to make the solution 7.4 M in urea), yielding a solution of pH 6.45. Subsequent pH adjustments were made with concentrated HCl.

NMR Measurements. ^{77}Se NMR spectra were obtained at 38.168 MHz in the Fourier transform mode on a Bruker WP-200 with 15-mm sample tubes. For a few of the low molecular weight model compounds, ^{77}Se NMR spectra were obtained at 15.27 MHz in the Fourier transform mode on an IBM NR-80B spectrometer with 10-mm sample tubes. The following acquisition parameters were employed for the acyl-α-chymotrypsin samples: flip angle 90°, spectral window 15 000 Hz, acquisition time 0.54 s, and gated proton decoupling (delays of 2.0, 0.5, and 1.0 s for the acyl-α-chymotrypsin at pH 5, the acyl-α-chymotrypsin at pH 3.83 and pH 1.99, and denatured acyl-α-chymotrypsin, respectively). The number of scans varied between 10 000 and 58 000 for the acyl-α-chymotrypsin and between 7500 and 25 000 for the denatured acyl-enzyme. Data points in the FID due to pulse breakthrough were eliminated, and exponential multiplication producing a line broadening equal to 30 and 10 Hz was used for the native and denatured acyl-enzyme species, respectively. The average sample temperature in these runs including decoupler heating was 4.5 ± 1.0 °C. In the experiment in which the temperature dependence of the acyl-enzyme was monitored at pH 3.19, the following acquisition parameters were employed: flip angle 90°, spectral window 29 400 Hz, acquisition time 0.278 s, and proton gated decoupling (0.25 s delay). An exponential multiplication producing a line broadening of 100 Hz was applied to each FID. Chemical shifts were externally referenced to a 60% $(CH_3)_2Se$ solution in $CDCl_3$ (Luthra et al., 1983) and were uncorrected for the change in the deuterium-lock solvent.

Results

p-Nitrophenyl (Phenylselenyl)acetate as a Substrate for α-Chymotrypsin. The steady-state hydrolysis of *p*-nitrophenyl (phenylselenyl)acetate by α-chymotrypsin was monitored by measuring the increase in the absorbance of *p*-nitrophenolate ion at 400 nm and correcting for the background rates of hydrolysis of this substrate. The second product of the reaction, (phenylselenyl)acetate and the substrate, did not absorb at 400 nm. A Lineweaver–Burk plot of the data obtained at pH 7.77 for two enzyme concentrations with $E_0 \ll S_0$ is shown in Figure 1. The rate constants, an average of those obtained from these two enzyme concentrations, are $K_M^{app} = 5.16$ (±0.22) $\times 10^{-6}$ M, $k_{cat} = 7.86$ (±0.14) $\times 10^{-2}$ s^{-1}, and $k_{cat}/K_M^{app} = 15.2$ (±0.9) $\times 10^3$ M^{-1} s^{-1}. The experimental finding that the α-chymotrypsin-catalyzed hydrolysis of this substrate fits Michaelis–Menten kinetics indicates that the substrate is being processed by the active site of the enzyme. In analogy to the known α-chymotrypsin mechanism, the reaction of *p*-nitrophenyl (phenylselenyl)acetate with the enzyme should apparently proceed in three steps: (1) formation of an en-

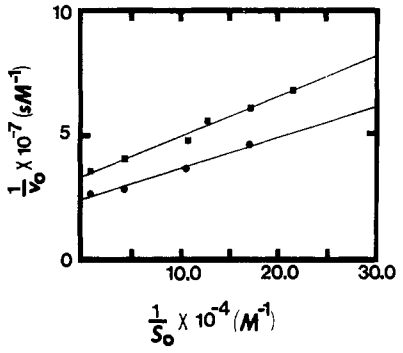

FIGURE 1: Lineweaver–Burk plot of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl (phenylselenyl)acetate at 25.0 °C, pH 7.77, 0.08 M Tris, and 0.10 M $CaCl_2$: ●, 32.8% CH_3CN, $E_0 = 3.82 \times 10^{-7}$ M; ■, 32.5% CH_3CN, $E_0 = 5.54 \times 10^{-7}$ M.

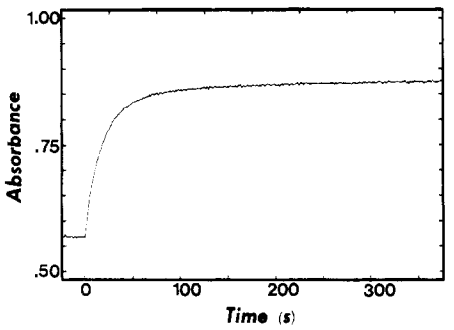

FIGURE 2: Burst titration of *p*-nitrophenyl (phenylselenyl)acetate with α-chymotrypsin measured at 318 nm under conditions similar to those for the selenium-77 NMR sample preparation.

zyme–substrate complex, (2) acylation of the enzyme to form an acyl-enzyme intermediate, and (3) deacylation of the acyl-enzyme to form the carboxylic acid (or carboxylate) and the active enzyme.

Formation of the stabilized acyl-α-chymotrypsin will occur if the *p*-nitrophenyl (phenylselenyl)acetate substrate is an active-site titrant and if the turnover of the substrate after the burst is slow. Titration of α-chymotrypsin depends on deacylation of the acyl-enzyme being a slower step than acylation of the enzyme. Titration of α-chymotrypsin with *p*-nitrophenyl (phenylselenyl)acetate was performed under two sets of conditions. The results are compared to the titration of the enzyme with cinnamoylimidazole. Liberation of *p*-nitrophenol was monitored at 318 nm ($\epsilon_{318} = 9.63 \times 10^3$ M^{-1} cm^{-1}; Kezdy & Bender, 1962). The titration procedure followed that previously reported for cinnamoylimidazole (Schonbaum et al., 1961). The deacylation product, (phenylselenyl)acetate, does not absorb at 318 nm; however, *p*-nitrophenyl (phenylselenyl)acetate, with an absorption maximum at 270 nm ($\epsilon_{270} = 1.15 \times 10^4$ M^{-1} cm^{-1}), has a tail absorption at 318 nm ($\epsilon_{318} = 1.88 \times 10^3$ M^{-1} cm^{-1}). The absorption of substrate should cause a 20% underestimate in the active-site concentration as determined from a burst of *p*-nitrophenol at 318 nm. Titration at 25.0 °C for a pH 4.13 solution (0.1 M acetate containing 32% acetonitrile) gave an active-site concentration that was 5% less than of that determined with cinnamoylimidazole when a 3-fold excess of the substrate to the enzyme was used. This result is consistent with an absorption of the acyl-enzyme at this wavelength. A titration at 3.0 °C for an apparent pH 5.0 solution (0.1 M acetate, D_2O containing 33% acetonitrile) gave similar results when a 4-fold excess of the substrate to enzyme was used. The plot of this burst titration is shown in Figure 2. The slow turnover rate (3.9×10^{-9} M s^{-1}) after the burst for an initial α-chymotrypsin concentration of 6.6×10^{-5} M is evidence that this substrate is forming a stabilized acyl-

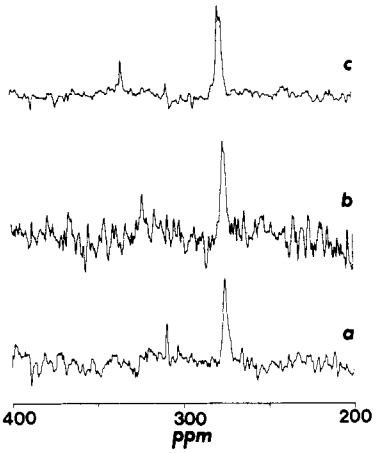

FIGURE 3: ^{77}Se spectra of the (phenylselenyl)acetyl-α-chymotrypsin intermediate (2.6 mM): (a) after preparation at pH^{app} 5.0, 4.5 °C, 11 460 scans; (b) at pH^{app} 3.83, 4.5 °C, 10 352 scans; (c) at pH^{app} 1.99, 4.5 °C, 58 138 scans, for D_2O solutions (0.1 M acetate), line broadening (30 Hz) in each spectrum.

enzyme species under these conditions. When the use of the ^{77}Se-labeled substrate was further optimized, it was found that a burst of the same magnitude could be obtained for an *equivalent amount* of the substrate to the enzyme over a 300-s period under the second set of conditions. The use of less than a 3-fold excess of the substrate to the enzyme required a 10% acetonitrile solution for substrate solubility. For the NMR experiments, the acetonitrile was removed by lyophilization after acyl-enzyme formation.

Assignment of Selenium-77 Resonances after the α-Chymotrypsin Reaction with p-Nitrophenyl (Phenylselenyl)-acetate. ^{77}Se NMR spectra at 38.168 MHz after the reaction of α-chymotrypsin with [^{77}Se]-*p*-nitrophenyl (phenylselenyl)acetate under three conditions are shown in Figure 3. After the reaction at pH 5.0 a resonance at 275.1 ppm was observed after 8 h of acquisition at 4.5 ± 1 °C. This resonance has a half-height line width of 60.4 Hz. In addition, a lower intensity resonance was observed at 309.1 ppm (spectrum a). When the sample was prepared at pH 5.0 and the pH was lowered to 3.83, a resonance was observed at 276.0 ppm (half-height line width 83 Hz) with a second resonance of lesser intensity appearing at 323.2 ppm after a 3-h acquisition at 4.5 ± 1.0 °C (spectrum b). When the pH was changed to 1.99, the resonance at 276.0 ppm was observed to split into two resonances at 276.3 and 277.7 ppm. The half-height line width of this split resonance is estimated to be 100 ± 5 Hz. This was accompanied by a second resonance at 334.6 ppm (spectrum c). The ^{77}Se resonance for *p*-nitrophenyl (phenylselenyl)acetate has a chemical shift of 350.4 ppm in $CDCl_3$ and does not correlate to any of the chemical shifts observed upon reaction of the substrate with the enzyme (Table I). The chemical shift of the (phenylselenyl)acetate resonance, which would be observed as the second product in the α-chymotrypsin catalyzed reaction, was found to be pH dependent. The chemical shift vs. pH curve for the (phenylselenyl)acetate resonance is shown in Figure 4a. On the basis of this pH profile, we have assigned the pH-dependent resonances in Figure 3 to (phenylselenyl)acetate. It should be noted that an exact correlation of these chemical shifts (Figure 3) to those for (phenylselenyl)acetate in the titration curve (Figure 4a) is not expected. This is a result of obtaining the data in Figure 4 for (phenylselenyl)acetate (0.1M) in a 50% ethanol solution. A better correlation of the chemical shifts assigned to (phenylselenyl)acetate to those of the same species after denaturation of the enzyme is shown in Figure 4b.

Table I: Comparison of ^{77}Se Chemical Shifts for (Phenylselenyl)acetyl-α-chymotrypsin to Selected Models

species	^{77}Se chemical shift (ppm)	solvent
p-nitrophenyl (phenylselenyl)acetate	350.4	$CDCl_3$
(phenylselenyl)acetyl-α-chymotrypsin[a]	275.1	D_2O, pH 5.0
	276.0	D_2O, pH 3.83
	276.3, 277.7	D_2O, pH 1.99
(phenylselenyl)acetyl hydrazide[a,b]	332.3	D_2O, pH 7.85
denatured (phenylselenyl)acetyl-α-chymotrypsin[a] (urea, 8 M)	334.5	D_2O, pH 2.74
	334.6	D_2O, pH 4.90
	334.9, 337.0	D_2O, pH 6.73
	335.0, 336.9	D_2O, pH 9.16
(phenylselenyl)acetic acid	340.9	$CDCl_3$
sodium (phenylselenyl)acetate[a]	303.7	10% D_2O, pH 6.82
methyl (phenylselenyl)acetate	333.3	$CDCl_3$
phenylselenoacetaldehyde	257.3	$CDCl_3$

[a] 2.6 ppm must be subtracted from the chemical shifts of these species to make them directly comparable to the chemical shifts obtained for compounds in $CDCl_3$ due to the change in the deuterium lock. [b] This product was obtained by treating (phenylselenyl)acetyl-α-chymotrypsin with hydrazine sulfate (1 M).

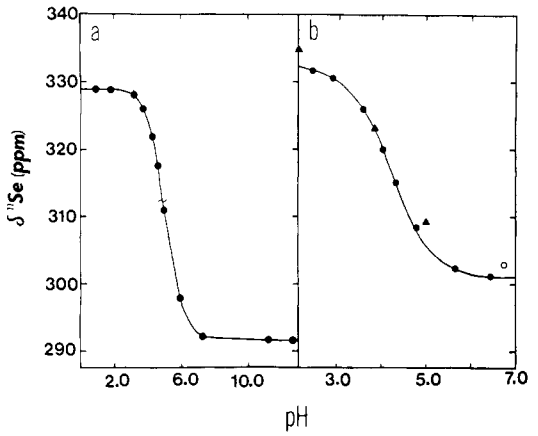

FIGURE 4: ^{77}Se chemical shift vs. pH^{app} curve for the titration of (a) sodium (phenylselenyl)acetate (0.1 M) in 50:50 D_2O/ethanol, pK_a = 4.9 ± 0.1, and (b) (phenylselenyl)acetate following chymotrypsin-catalyzed hydrolysis of the parent *p*-nitrophenyl ester and subsequent denaturation of the enzyme in 7.4 M urea. Chemical shifts of (phenylselenyl)acetate resulting from the chymotrypsin-catalyzed hydrolysis of the parent *p*-nitrophenyl ester at 4.5 ± 1 °C (▲). The chemical shift of (phenylselenyl)acetate (○) measured in the presence of denatured (phenylselenyl)acetyl-α-chymotrypsin.

The resonance at ~275 ppm has been assigned to the (phenylselenyl)acetyl-α-chymotrypsin, i.e., the acyl-enzyme, on the basis of the following results. (1) The species which gives rise to this resonance is transitory. A series of spectra at increasing temperatures is shown in Figure 5. It can be seen that a diminution of the resonance due to the acyl-enzyme occurs with a concomitant increase in the resonance due to (phenylselenyl)acetate. (2) On saturating the acyl-enzyme solution with hydrazine sulfate (~1 M) and raising the pH to 7.85, resonances at 332.3 and 302.2 ppm were observed (Table I). The resonance at 332.2 ppm has been assigned to the protonated (phenylselenyl)acetyl hydrazide and the resonance at 302.2 ppm corresponds to (phenylselenyl)acetate at this pH. (3) Denaturation of the acyl-enzyme species produces a sharpening of the resonance (12 Hz) and a shift of the resonance to 334.6 ppm at pH 4.90 (Figure 6). (4) The denatured (phenylselenyl)acetyl-α-chymotrypsin was stable upon raising the pH to 9.16 within the time frame of the NMR experiment.

In the spectrum of the denatured acyl-enzyme at pH 2.74 the peak at 352.6 ppm has been assigned to the excess *p*-nitrophenyl (phenylselenyl)acetate in solution. Observation of this resonance is a result of immediately denaturing the acyl-enzyme at low pH after having formed this intermediate, thereby halting turnover of excess substrate. The signal-to-noise ratio in the spectrum at a pH of 4.90 does not allow observation of the *p*-nitrophenyl (phenylselenyl)acetate, while at pH 6.73 a resonance appears at 302.9 ppm. The assignment of this resonance to the hydrolysis product of the *p*-nitrophenyl ester, i.e., the (phenylselenyl)acetate, has been made on the

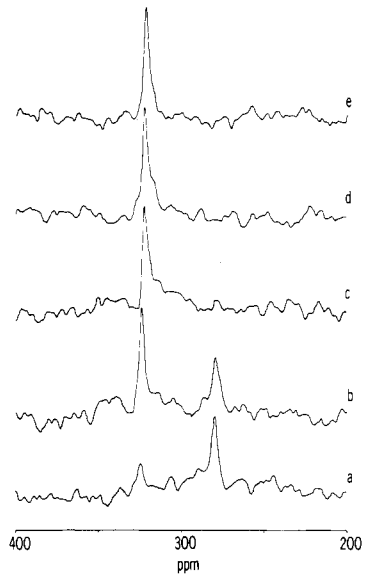

FIGURE 5: ^{77}Se spectra of (phenylselenyl)acetyl-α-chymotrypsin and the (phenylselenyl)acetic acid-α-chymotrypsin complex at pH 3.19 for the following (phenylselenyl)acetic acid-α-chymotrypsin complex at pH 3.19 for the following temperatures and number of scans: (a) 277 ± 2 K, 72 690 scans; (b) 280 ± 2 K, 132 006 scans; (c) 282 ± 2 K, 172 356 scans; (d) 287 ± 2 K, 159 799 scans; (e) 292 ± 2 K, 144 829 scans. The spectra were obtained sequentially in the order of increasing temperature.

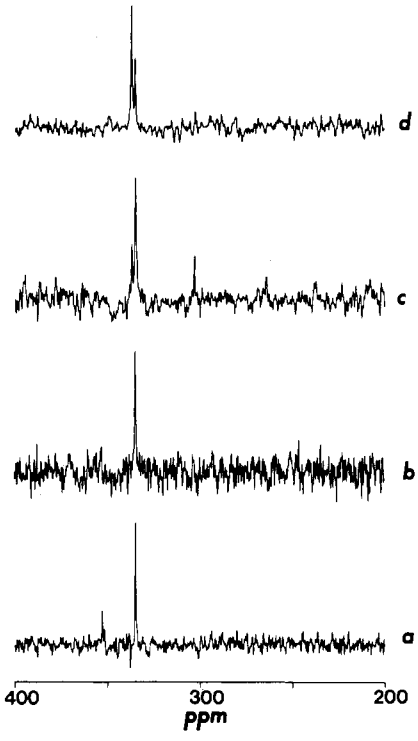

FIGURE 6: ^{77}Se spectra of the denatured (phenylselenyl)acetyl-α-chymotrypsin intermediate (2.6 mM) at (a) pH^{app} 2.74, 24 737 scans; (b) pH^{app} 4.90, 12 066 scans; (c) pH^{app} 6.73, 14 291 scans; (d) pH^{app} 9.16, 7581 scans, line broadening (10 Hz) in each spectrum.

Table II: Comparison of Kinetic Results for the Hydrolysis of *p*-Nitrophenyl Ester Substrates of α-Chymotrypsin

substrate	$k_2/K_M \times 10^3$ (M^{-1} s^{-1})	$K_M^{app} \times 10^6$ (M)	pH	cosolvent (% CH_3CN)
p-nitrophenyl acetate[a]	3.39 ± 2.8	1.59	7.8	1.6
p-nitrophenyl cinnamate[b]	11.3 ± 0.4		8.34	10
p-nitrophenyl (phenylselenyl)-acetate	15.2 ± 0.9	5.16 ± 0.22	7.77	33

[a] Kézdy & Bender, 1962. [b] Bender et al., 1962.

basis of the results of the ^{77}Se NMR pH titration of (phenylselenyl)acetate in the presence of denatured enzyme (Figure 4b. At a higher pH of 9.16 this resonance anomalously disappears, and can possibly be explained by a longer T_1 at this pH resulting in a saturation of the resonance. This is an area that requires further investigation. Although the resonance of the denatured acyl-enzyme shows only a slight chemical shift dependence on pH (Table I), it is interesting that a splitting of the resonance is observed at the two highest pH values. This could be the result of two conformationally different forms of the denatured enzyme at these pH values, i.e., incomplete motional averaging on the NMR time scale.

For comparison purposes, we have determined the ^{77}Se chemical shifts of two compounds with structures similar to that of (phenylselenyl)acetate. In Table I are shown the chemical shifts for methyl (phenylselenyl)acetate and (phenylseleno)acetaldehyde.

Discussion

For deacylation being the rate-determining step in an α-chymotrypsin-catalyzed hydrolysis of esters, it has been shown that $k_{cat}/K_M^{app} = k_2/K_M$ (Kézdy & Bender, 1962). Rate-limiting deacylation has been observed at pH 4.1, 25 °C and at pH 5.0, 3.0 °C for the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl (phenylselenyl)acetate. By analogy to other *p*-nitrophenyl ester substrates, we assume that deacylation is rate-determining at pH 7.77. On this basis, we have made a comparison of the kinetic results for the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl (phenylselenyl)acetate with *p*-nitrophenyl cinnamate and *p*-nitrophenyl acetate (Table II). The values of k_2/K_M in the catalyzed reaction for both *p*-nitrophenyl (phenylselenyl)acetate and *p*-nitrophenyl acetate (Kézdy & Bender, 1962) were obtained under turnover or steady-state conditions, while the k_2/K_M of *p*-nitrophenyl cinnamate (Bender et al., 1962) was obtained under second-order conditions. Kézdy and Bender (1962) have previously shown that for *p*-nitrophenyl acetate, consistent values of k_2/K_M can be obtained under a number of conditions in the α-chymotrypsin-catalyzed hydrolysis reaction. The specificity of this selenium-containing substrate can be assessed if we assume no appreciable changes in K_M^{app} due to the differing percentages of acetonitrile used (both pH and temperature being constant). The k_2/K_M for *p*-nitrophenyl (phenylselenyl)acetate is of the same order of magnitude as that of *p*-nitrophenyl cinnamate and is larger than that of *p*-nitrophenyl acetate as would be expected for a tighter binding (more specific) substrate (Table II). Substitution of the selenium–methylene bridge for the olefinic group in *p*-nitrophenyl cinnamate produced little overall change in the affinity of these two *p*-nitrophenyl esters for the enzyme. To our knowledge, no kinetic data for the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl 3-phenylpropanoate have been reported. We have previously determined the K_I for (phenylselenyl)acetate (20 mM) (Mullen et al., 1985), and this is in good agreement with the K_I for the methylene congener, 3-phenylpropanoate (25 mM). The subtle changes observed in the kinetic parameters upon substitution of selenium for carbon, i.e., in the form of a $-CH_2-$ or $=CH-$ group, are likely to be the result of the softness of selenium and the similar carbon and selenium electronegativities.

We have demonstrated that the ~275 ppm resonance is a result of the acyl-enzyme intermediate. This acyl-enzyme adduct is by analogy to other fully characterized acyl-enzyme species defined as the serine-195 ester of (phenylselenyl)-acetate. Such an intermediate was first characterized for the reaction of *p*-nitrophenyl acetate with α-chymotrypsin (Balls & Aldrich, 1955; Hartley & Kilby, 1954), and the hydrolysis of this substrate has been regarded as a simple prototype for all α-chymotrypsin-catalyzed reactions (Kézdy & Bender, 1962). The chemical shifts of the denatured acyl-enzyme at several pH's are well represented by the chemical shift of the model ester, methyl (phenylselenyl)acetate (Table I). The stability of this species with increasing pH is consistent with the denaturation of the enzyme and loss of its catalytic activity.

This is the first report of the observation of selenium-77 resonances for selenium covalently attached to a native protein system. These results are encouraging and show that relaxation mechanisms are sufficient to permit a ready observation (rapid data acquisition without saturation) of selenium resonances on a molecule with a large rotational correlation time. There are several prominent features that are apparent in comparing the native acyl-enzyme spectra (Figure 3) to the denatured acyl-enzyme spectra (Figure 6). The most startling of these is the very large (58 ppm) chemical shift difference that has been observed between the native and denatured states of the acyl-enzyme. Next, the resonance line in the native acyl-enzyme spectrum at pH 5.0 is much broader than that in the denatured acyl-enzyme spectra. The additional broadening and splitting of the native acyl-enzyme resonance on lowering the pH leads us to believe that the width of the resonance at pH 5.0 (60 Hz) may be the result of two overlapping resonances or two resonances not yet at coalescence. Undoubtedly, fast transverse relaxation rates may also contribute to this broadening. It is expected that at 4.5 °C the solution viscosity and the correlation time of the protein will be substantially greater than the 11 ns/rad value at 20 °C (Kuznetsov et al., 1975) and thereby cause broader resonance absorptions. On the basis of resonance Raman results, MacClement et al. (1981) have suggested that furyl- and thienylacryloyl-α-chymotrypsins adopt two conformations in solution, one characterized by strong hydrogen bonds to the carbonyl oxygen and the other with a non-hydrogen-bonding hydrophobic environment. In ^{13}C NMR titrations of 5-*n*-propyl-2-furoyl-α-chymotrypsin (McWhirter et al., 1985), splitting of the acyl-enzyme resonance was observed above pH 6.7 and below pH 5.25. Splitting of resonances for *p*-(trifluoromethyl)-benzenesulfonyl-α-chymotrypsin (pH 3.8) and *p*-(trifluoromethyl)cinnamoyl- α-chymotrypsin (pH 3.6) has also been observed by ^{19}F NMR spectroscopy (Maddox et al., 1975). Our results do not establish the source of the pH-dependent splitting of the (phenylselenyl)acetyl-α-chymotrypsin resonance.

Both the spin rotation (SR) and chemical shift anisotropy (CSA) mechanisms of spin–lattice relaxation (T_1) have been shown to be important for the selenium-77 nucleus (Dawson & Odom, 1977; Odom et al., 1979; Wong & Ang, 1984). Values of T_1 on the order of 0.1–25 s have been determined for small molecules (Dawson & Odom, 1977; Pan & Fackler, 1978; Odom et al., 1979; Wong & Ang, 1984). The relative

contributions of the SR or CSA mechanisms to the rate of spin–lattice relaxation of the selenium nucleus depend on the correlation time of the molecule, the chemical shift anisotropy at the selenium nucleus, and the temperature and field strength at which the NMR experiment is performed. The line widths (and presumably spin–spin relaxation times) for the native acyl-enzyme at various pH values are consistent with a CSA relaxation mechanism. We believe that the CSA relaxation mechanism is the major spin–spin and spin–lattice relaxation mechanism. Presumably then, the T_1 is short for the acyl-enzyme as predicted by our previous considerations (Odom et al., 1979). However, we cannot totally rule out the dipole–dipole relaxation mechanism for the native acyl-enzyme, since this mechanism has a strong dependence on the correlation time of the molecule. We believe that the CSA mechanism of relaxation is also significant for the denatured acyl-enzyme at 4.5 °C and that the spin–lattice relaxation time is short for this randomly oriented peptide chain. Further studies at variable field and/or temperature are required to evaluate these hypotheses.

An evaluation of the chemical shifts for (phenylselenyl)-acetic acid, (phenylselenyl)acetate, and phenylselenoacetaldehyde readily shows that the chemical shift of the selenium resonance in these structurally similar compounds is very sensitive to the distribution of π electron density at the carbonyl group. For the same solvent conditions, the selenium resonance of (phenylselenyl)acetate is 37.3 ppm *shielded* from (phenylselenyl)acetic acid. Carbonyl ^{13}C resonances for aliphatic carboxylate anions are ~5 ppm more *deshielded* than the corresponding carboxylic acid resonances (Hagen & Roberts, 1969). Thus, for the selenium-77 probe β to the carbonyl carbon a 7-fold increase in chemical shift sensitivity is observed. The selenium-77 resonance for phenylselenoacetaldehyde is 76.0 ppm shielded from the selenium-77 resonance of methyl (phenylselenyl)acetate. This shift is >2-fold larger than the corresponding shifts for carbonyl ^{13}C resonances of aliphatic methyl esters and the corresponding aldehydes (Levy & Nelson, 1972) and again occurs in the opposite direction. It is also important to note that this 76.0 ppm shift occurs in the absence of one of the γ-substituents that was present in the methyl (phenylselenyl)acetate. We have previously reported that a γ-effect for a methyl group can produce 28–37 ppm of shielding depending on the alkyl chain (Luthra & Odom, 1986).

We now discuss the reasons for ruling out an assignment of the 275 ppm resonance to a tetrahedral intermediate.[2] It is clear from the titration results at pH 5.0 and 3.0 °C that the possible observation of a tetrahedral intermediate would be the result of the hydrolysis of the acyl-enzyme, since *p*-nitrophenol is liberated in the acylation step. This hydrolysis can be represented by the equilibrium

$$ES' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES'^{\ddagger} \xrightarrow{k_2} EP$$

where $ES'^{\ddagger}$ is the tetrahedral species. If there is a lower activation barrier for the formation of the tetrahedral intermediate than for its breakdown to products,[3] three resonances would be observed for a system out of equilibrium for ES'

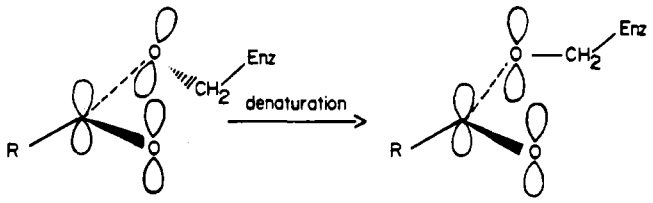

FIGURE 7: Representation of the orbitals participating in resonance overlap at the acyl moiety before and after denaturation as derived from the proposal by Bernhard et al. (1971, 1974).

reacting to form EP or for species in slow exchange. At 4.5 °C (Figure 3) and for increasing temperatures (Figure 5), three sufficiently different resonances are not observed.

An alternate case to consider entails a possible observation of a rapid exchange equilibrium between the acyl-enzyme and the tetrahedral intermediate together with the observation of the enzyme–product complex. Again this requires that k_2 be less than k_1. For a rapid exchange of this type, a substantial pH dependence should be observed in the first equilibration step as a result of the general-base, general-acid catalysis that occurs in the k_1 and k_{-1} steps. Additionally, the temperature dependence of these rate constants should produce a temperature-dependent chemical shift for the averaged resonance. The small changes observed in the chemical shift of the resonance at 275 ppm for the pH range and the temperature range studied demonstrate that a possible contribution of a rapidly exchanging tetrahedral intermediate to the chemical shift of this resonances is minimal. It should be added that there is one report of the observation of both a tetrahedral intermediate and an acyl-enzyme intermediate by ^{13}C NMR spectroscopy at −63 °C after reaction of α-chymotrypsin and carbonyl ^{13}C enriched *p*-nitrophenyl acetate (Tobias et al., 1984). Apparently, the conditions of our experiments do not produce an activation energy barrier that permits trapping of a tetrahedral intermediate.

The selenium-77 chemical shift of the native (phenylselenyl)acetyl-α-chymotrypsin is more characteristic of the model aldehyde chemical shift and very much different from the chemical shift observed for the denatured acyl-enzyme and the model ester. The similarity of the selenium-77 chemical shifts for the denatured acyl-enzyme and the model ester clearly demonstrates that the native three-dimensional structure of the enzyme and, in particular, the active site is producing the 58 ppm chemical shift perturbation. It is not possible to determine the source of this perturbation, unambiguously; however, there are at least two effects that should be considered. First, a hypothesis has been forwarded by Bernhard et al. (1971, 1974) stating that, for acyl-α-chymotrypsins in which the acyl moiety is an arylacryloyl group, a decreased resonance overlap may be occurring between the seryl oxygen and the carbonyl group of the ester. This would be a result of the seryl oxygen–$^{\beta}CH_2$ bond being out of the plane of the three bonds centered at the trigonal carbonyl carbon as shown in Figure 7. This hypothesis is consistent with the X-ray crystallographic determination of indoleacryloyl-α-chymotrypsin (Henderson, 1970) and ultraviolet–visible absorption spectroscopy (Bernhard & Lau, 1971; Bernhard & Malhotra, 1974; Charney & Bernhard, 1967) on native and denatured arylacryloyl-α-chymotrypsins and their related model esters and aldehydes. Resonance Raman results for the carbonyl vibration and absorption spectra for native *p*-(dimethylamino)benzoyl-α-chymotrypsin also correlate with the spectra of the model aldehyde and not the ester (Argade et al., 1984). However, carbonyl ^{13}C NMR investigations of acetyl-α-chymotrypsin (Niu et al., 1977; Tobias et al., 1984)

[2] A referee has asked us to include a discussion on the possibility that this resonance may be due to a tetrahedral-like species.

[3] Kinetic studies of the α-chymotrypsin-catalyzed hydrolysis of several esters shows that deacylation occurs through a *transient* transition state (Bender et al., 1964), presumably a tetrahedral intermediate. Enzyme stabilization of this transition-state species could, in theory, allow for its observation.

and 5-*n*-propyl-2-furoyl-α-chymotrypsin (McWhirter et al., 1985) have given no evidence of a more keto-like carbonyl carbon for these acyl-enzyme intermediates. Thus, although the hypothesis of decreased resonance overlap is not consistent with all physical data for acyl-enzyme intermediates, it does qualitatively explain the shielding of the selenium-77 resonance observed in this system. Secondly, the shielding observed could be explained by an enhanced γ-effect or steric compression along a selenium nonbonding orbital. An enhanced γ-effect would be the result of the active site of the enzyme directing a γ-substituent toward the selenium nucleus. Such a constraint would, however, substantially reduce the γ-effect of the second γ-substituent. Steric compression along a selenium nonbonding orbital could also be the result of an interaction of a heavy atom on the protein itself. Finally, it should be added that the shielding effect cannot be the result of a simple solvation shift since the selenium-77 chemical shifts for the methyl (phenylselenyl)acetate and denatured acyl-enzyme vary at most by 2 ppm for a nonpolar vs. a very polar environment, respectively. Moreover, at low pH the denatured acyl-enzyme is in an environment that will allow for strong hydrogen bonds from protonated urea to the carbonyl oxygen of the acyl group. The small changes in the selenium-77 chemical shift observed over the pH range of 2.74–9.16 for the denatured acyl-enzyme leads us to believe that hydrogen bonding to the carbonyl oxygen in the native acyl-enzyme system alone is not causing the observed shielding effect.

In summary, we have used selenium-77 NMR spectroscopy to observe a (phenylselenyl)acetyl-α-chymotrypsin intermediate derived from the reaction of α-chymotrypsin with the kinetically competent substrate, *p*-nitrophenyl (phenylselenyl)-acetate. The results are in agreement with a resonance-perturbed ester linkage and/or steric compression of nonbonding orbitals at the selenium nucleus. Both of these mechanisms are consistent with high-energy states of the acyl-enzyme intermediate. Selenium-77 NMR spectroscopy has been electronically and structurally informative in regard to the possible physical nature of this highly labile species.

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