The reaction was brought to room temperature and stirred for 18 h. Excess diborane was destroyed by cautious addition of water until there was no further evolution of hydrogen. The reaction was concentrated in vacuo and the residue added to 100 mL of 6 N HCl. The solution was heated at reflux (1 h), cooled, and neutralized with NaOH pellets. The aqueous phase was extracted with CH_2Cl_2 (3 × 100 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. A bulb-to-bulb distillation (Kugelrohr apparatus, 144–146 °C (0.05 mm)) of the residue afforded **8** (3.7 g, 97%) as a transparent oil with physical properties identical with those reported.[12c]

***N,N'*-Bis(carbethoxymethyl)-4,13-diaza-18-crown-6 (9).** A solution of 4,13-diaza-18-crown-6 (6.0 g, 23 mmol), ethyl bromoacetate (3.4 g, 50 mmol), and Na_2CO_3 (5.4 g, 51 mmol) in MeCN (100 mL) was heated at reflux for 24 h. The reaction was then cooled, filtered and concentrated in vacuo. The residue was taken up in $CHCl_3$ (100 mL) and washed with H_2O (100 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. A bulb-to-bulb distillation (Kugelrohr apparatus, 195–197 °C (0.18 mm)) of the residue afforded **9** (9.2 g, 92%) as a transparent oil with physical properties identical with those already reported.[12c]

***N,N'*-Bis(carboxymethyl)-4,13-diaza-18-crown-6 (10).** A solution of **9** (3.0 g, 6.9 mmol) in water (22 mL) was heated at reflux temperature for 48 h. The reaction was cooled and concentrated in vacuo. Ethanol (40 mL) was added to the residue, and the mixture was left to stand overnight. The resulting crystals were filtered and dried in vacuo (100 °C (0.1 mm)) for 2 h to afford **10** (2.1 g, 81%) as a white solid (mp 173–175 °C) with physical properties identical with those reported.[12c]

***N,N'*-Bis(2-hydroxybenzyl)-4,13-diaza-18-crown-6 (11).** A solution of 4,13-diaza-18-crown-6 (3.00 g, 11.4 mmol), *o*-chloromethylphenyl acetate (4.69 g, 25.4 mmol), and Na_2CO_3 (2.69 g, 25.4 mmol) in MeCN (50 mL) was heated at reflux temperature for 20 h. The reaction was cooled, filtered, and concentrated in vacuo. The residue was taken up in $CHCl_3$ (100 mL) and washed with H_2O (100 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. The resulting yellow oil was identified as N,N'-bis(2-acetoxybenzyl)-4,13-diaza-18-crown-6: ^1H NMR ($CDCl_3$) δ 2.28 (s, 6 H, CH_3), 2.77 (t, 8 H, CH_2N), 3.59 (m, 20 H, CH_2O and benzyl), 6.80–7.50 (m, 8 H, aromatic); IR (neat) 3040, 2880, 1770 (s), 1495, 1460, 1375, 1215 (s), 1180, 1120, 1045, 920, 755 (s) cm^{-1}. Chromatography of the crude bis-acetate (alumina, 75% Et_2O:hexanes) resulted in acetate hydrolysis and afforded **11** (4.60 g, 85%) as a white solid (mp 120–122 °C): ^1H NMR ($CDCl_3$) δ 2.83 (t, 8 H, CH_2N), 3.62–3.79 (m, 20 H, CH_2O and benzyl), 6.56–7.36 (m, 8 H, aromatic), 9.90 (br s, 2 H, hydroxyl); IR (KBr) 3100 (br), 2980, 2960, 2920, 2840, 1620, 1590, 1490, 1260, 1250, 1150, 1130, 1120 cm^{-1}. Anal. Calcd for $C_{26}H_{38}N_2O_6$: C, 65.79; H, 8.09; N, 5.90. Found: C, 66.08; H, 8.35; N, 5.68.

***N*-(4-Methoxyphenyl)monoaza-9-crown-3 (12).** A solution of *p*-anisidine (1.2 g, 10 mmol), 1,2-bis(iodoethoxy)ethane (3.7 g, 10 mmol), and Na_2CO_3 (5.3 g, 50 mmol) in MeCN (45 mL) was heated at reflux temperature for 12 days. The reaction was cooled, filtered, and concentrated in vacuo. The residue was taken up in $CHCl_3$ (50 mL) and washed with H_2O (50 mL). The organic phase was dried with $MgSO_4$ and concentrated in vacuo to yield, after column chromatography (alumina, 4% EtOAc:hexanes), 0.3 g (13%) of the title compound as a yellow oil: ^1H NMR ($CDCl_3$) δ 3.42–3.83 (m, 15 H, CH_2O and CH_2N and CH_3O), 6.50–6.90 (m, 4 H, aromatic): IR (neat) 2900, 2860, 1615, 1510 (s), 1460, 1350, 1260, 1240, 1190, 1130, 1110, 1040, 1000, 930, 860, 810 cm^{-1}; mass spectrum M^+, 237. Anal. Calcd for $C_{13}H_{19}NO_3$: C, 65.79; H, 8.09; N, 5.90. Found: C, 65.70; H, 8.30; N, 5.60.

Acknowledgment. We thank the NIH for grants (GM 29150 and GM 31846) which supported this work. We thank Dr. D. M. Goli for determining the cation binding constants, Ms. Anne Ling Li for experimental assistance, and D. Mazzocchi and Dr. A. R. Browne for independently preparing samples of **3** by this reaction.

Registry No. 1, 69703-25-9; **2**, 93000-66-9; **3**, 93000-67-0; **4**, 90633-85-5; **5**, 69930-74-1; **5**·NaI, 87249-10-3; **6**·NaI, 93000-65-8; **7**, 23978-55-4; **8**, 72911-99-0; **9**, 62871-83-4; **10**, 72912-01-7; **11**, 88104-28-3; **12**, 93000-70-5; **13**, 36839-55-1; $PhCH_2NH_2$, 100-46-9; MeO-*o*-$C_6H_4CH_2NH_2$, 6850-57-3; CH_2=$CHCH_2NH_2$, 107-11-9; $NH_2(CH_2)_2$-OH, 141-43-5; $MeOCH_2C(O)Cl$, 38870-89-2; $BrCH_2C(O)OEt$, 105-36-2; $CH_3C(O)O$-*o*-$C_6H_4CH_2Cl$, 15068-08-3; MeO-*p*-$C_6H_4NH_2$, 104-94-9; Na^+, 17341-25-2; K^+, 24203-36-9; Ca^{2+}, 14127-61-8; 2-furanmethanamine, 617-89-0; *N,N'*-bis[(methoxymthyl)carbonyl]-4,13-diaza-18-crown-6, 93000-68-1; *N,N'*-bis(2-acetoxybenzyl)-4,13-diaza-18-crown-6, 93000-69-2; 2-pyridinemethanamine, 3731-51-9.

Dynamics at the Active Site of N^2-Acetyl-N^1-(4-fluorobenzyl)carbazoyl-α-chymotrypsin

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Abstract: N^2-Acetyl-N^1-(4-fluorobenzyl)carbazoyl-α-chymotrypsin is a catalytically inactive protein which should closely resemble the acylated enzyme intermediate formed during reaction of chymotrypsin with substrates derived from phenylalanine. Fluorine and deuterium nuclear relaxation in this carbazoylated enzyme and a specifically deuterated analogue have been examined. Analysis of the spin–lattice relaxation data (observed at several radio frequencies) and $^{19}F[^1H]$ nuclear Overhauser effects indicate that rotation of the aromatic ring of the carbazoyl group in the protein is slow relative to overall protein tumbling. However, observed fluorine line widths are larger than those predicted from this analysis. Experiments are described which suggest that the excess line widths are not due to heterogeneity of the protein, and exchange of the fluoroaromatic ring between environments characterized by different fluorine chemical shifts is proposed as a possible explanation. Fluorine NMR studies indicate that the acylated enzyme is resistant to loss of tertiary structure (denaturation) near the active site when the protein is dissolved in 8 M urea.

The enzyme α-chymotrypsin cleaves amide and ester bonds by a mechanism which involves formation of an acylenzyme intermediate at the active site serine-195 residue. *N*-Acetyl-L-phenylalanine ethyl ester (**I**) is an excellent substrate for chymotrypsin and the acylenzyme that it forms is very rapidly hydrolyzed.[1] Kurtz and Niemann showed that when the methine group of **I** is substituted by a nitrogen atom, giving a carbazoic acid derivative (**II**), the resultant molecule binds at the enzyme active site but does not acylate the serine; that is, **II** is a competitive inhibitor of the enzyme.[2] Elmore and Smyth found that an aryl ester of the same carbazoic acid (**IIIa**) is sufficiently reactive to acylate chymotrypsin and that the acylenzyme which is formed does not readily undergo hydrolysis.[3] They used this reaction

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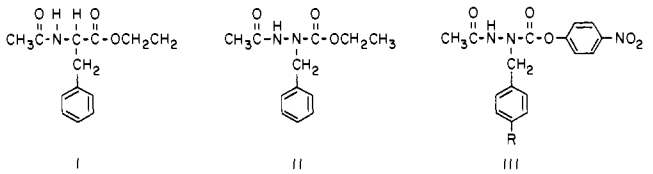
to develop a method for determining the absolute molarity of



I II III

a, R = H
b, R = F

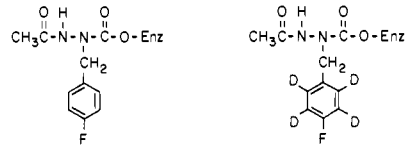
solutions of chymotrypsin, and similar chemistry has been used to construct a polymer which is useful in covalent affinity purification of this enzyme.[4] Most recently, Orr and Elmore have prepared a series of chymotrypsin derivatives using **III** with different para substituents on the aromatic ring of the acyl group.[5]

Chymotrypsin is specific for the cleavage of peptide bonds at aromatic amino acid residues. The structures of crystalline chymotrypsin, enzyme-inhibitor complexes, and acylated or otherwise modified chymotrypsins indicate that the aromatic side chain of specific substrates binds in a depression at the active site of the enzyme.[6-9] This pocket, sometimes referred to as the "tosyl hole", has an irregular, flattened shape that is long enough to accomodate indole and deep enough for tyrosine but is so narrow as to permit only one orientation of the plane of a bound aromatic group.[7] These crystallographic results imply that if the polypeptide structure around the tosyl hole is rigidly retained in solution, aromatic rings of substrates or inhibitors interacting with the tosyl hole will not be free to rotate around their local C_2 symmetry axis.

X-ray crystallographic studies provide an essentially static view of the average positions of atoms in a protein, but there is now ample evidence that in solution these atoms are in a state of constant motion and that they exhibit fluctuations of sizable amplitude about their average positions.[10-13] Some flexibility is, of course, required in both the structure of an enzyme and its substrate(s) so that catalysis can take place.[14-15]

NMR is a particularly powerful tool for the investigation of protein dynamics;[16-17] valuable sources of information are spin–lattice and spin–spin relaxation parameters, even though quantitative analysis of such data requires accurate knowledge of the dominant relaxation mechanism and the assumption of a specific model for molecular motion.[18-19] The application of proton NMR and natural abundance ^{13}C NMR spectroscopy to dynamical studies of proteins is often severely complicated by the problems of resolution and assignment in spectra which may contain hundreds of individual signals. Fluorine substitution provides a means to avoid some of these difficulties.[20-22]

In order to learn more about conformational dynamics at the active site of an acylated chymotrypsin that should strongly resemble the structure formed during hydrolysis of a specific substrate, we have prepared N^2-acetyl-N^1-(4-fluorobenzyl)carbazoyl-α-chymotrypsin (**IVa**) and an analogue in which the fluorophenyl group is deuterated (**IVb**). These enzymes are inactive, and we assume that the carbazoyl group is attached to serine-195. Fluorine and deuterium NMR were used to investigate the environment and motion of the fluorophenyl ring in these structures. The results indicate that this ring is considerably immobilized, probably by occupation of the tosyl hole, and are consistent with the notion that the conformational rigidity of the enzyme is greatly increased upon acylation.



IV a IV b

Experimental Section

Instrumentation. Melting points were measured on a Thomas-Hoover capillary melting point apparatus. Visible and UV spectra were recorded on Gilford 2000 or Cary 118 spectrometers. Mass spectra were obtained with a Micromass ZAB-2F instrument with use of electron impact or chemical ionization. Methane was the reactant gas in the latter instance. Routine ^1H NMR spectra were recorded on Varian EM360A, CFT20, or Nicolet NT300 instruments. Routine ^{13}C spectra were obtained with the CFT20. Proton and carbon-13 chemical shifts are reported relative to Me_4Si.

Fluorine-19 spectra at 94 MHz were accumulated with a Varian XL-100 as described previously with use of 12 mm o.d. sample tubes.[23] Fluorine spectra at 282 MHz were recorded with the Nicolet NT300 with use of 10 mm o.d. samples. Experiments at 470 MHz used 5 mm o.d. tubes and were carried out on the Bruker WM-500 at the Southern California Regional NMR Facility, California Institute of Technology. In all cases, the temperature controllers supplied with the instruments were used to maintain the sample temperature at 25 °C, unless otherwise stated.

Deuterium spectroscopy at 46 MHz employed a broad-band probe on the NT-300 which was run unlocked.

A Radiometer PHM52 meter was used to record pH.

Materials. All water was deionized and then distilled in glass. Deuterium oxide (99.8 atom % D) and sulfuric acid-d_2 (99.5± atom % D) were purchased from Aldrich. Organic solvents, inorganic salts, and buffers were obtained from a variety of commercial suppliers and were generally of the highest available grade. These materials were used as supplied except where indicated. Acetylhydrazine was synthesized by the procedure of Kurtz and Niemann.[2]

N^2-Acetyl-N^1-(4-fluorobenzylidene)hydrazine. Freshly distilled 4-fluorobenzaldehyde (31 g, 0.25 mol, Aldrich) was added in small portions to an ice-cold solution of acetylhydrazine (18.5 g, 0.25 mol) in 50 mL of water. The mixture was shaken vigorously and became viscous with the formation of a white precipitate. The solid was isolated by filtration, washed with diethyl ether to remove a yellow impurity, and recrystallized twice from aqueous ethanol. White needles were obtained in 74% yield, mp 168–169 °C. ^1H and ^{13}C NMR spectra in $(CD_3)_2SO$ showed that this material was a mixture of isomers in a ratio of 2:1. The major isomer is assigned the *trans* configuration on the basis of a chemical shift calculation for the methine proton.[24] ^1H NMR (60 MHz, Me_2SO-d_6, major isomer) 2.2 (s, CH_3CO), 7.1–7.9 (m, 4 H, aryl), 8.0 (s, methine), 11.3 ppm (br s, NH). In the spectrum of the minor isomer, a singlet at 2.0 ppm was observed for the acetyl protons with the signals for the other protons of this form intermingled with those of the major isomer. ^{13}C NMR (20 MHz, Me_2SO-d_6) 20.0 and 21.5 (CH_3, cis and trans isomers), 114.9 and 116.0 (d, ^2J(C–F) = 22 Hz, C-3), 128.4 and 128.8 (d, ^3J(C–F)

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= 8 Hz, C-2), 130.8 and 131.0 (d, ^4J(C–F) = 3 Hz, C-1), 141.3 (s, methine), 144.6 and 156.6 (d, ^1J(C–F) = 241 Hz, C-4), 169.0 and 171.8 ppm (carbonyl, cis and trans isomers). The ^{1}H NMR spectrum in $CDCl_3$ showed resonances for only a single isomer, possibly due to HCl-catalyzed equilibration to the more stable trans isomer in this solvent.

N^2-Acetyl-N^1-(4-fluorobenzyl)hydrazine. N^2-Acetyl-N^1-(4-fluorobenzylidene)hydrazine (10.7 g, 59 mmol) was partially dissolved in 250 mL of absolute ethanol and shaken with 1 g of 10% palladium on charcoal (Matheson, Coleman and Bell) for 24 h under an atmosphere of hydrogen at 3.4×10^5 Pa (Parr hydrogenator). The catalyst was removed by filtration and the solvent evaporated under reduced pressure to leave a solid residue which was crystallized from CH_2Cl_2/light petroleum in 77% yield: mp 107–108 °C; ^{1}H NMR (60 MHz, $CDCl_3$) 1.9 (s, CH_3CO, 3 H), 3.9 (br s, 2 H, CH_2), 6.8–7.4 ppm (4 H, m, aryl). ^{13}C NMR (20 MHz, Me_2SO-d_6 20.3 (CH_3), 53.8 (CH_2), 114.0 and 115.1 (d, ^2J(C–F) = 21 Hz, C-3), 129.9 and 130.3 (d, ^3J(C–F) = 8 Hz, C-2), 134.6 and 134.7 (d, ^4J(C–F) = 3 Hz, C-1), 155.2 and 167.3 (d, ^1J(C–F) = 242 Hz, C-4), 168.3 ppm (carbonyl).

4-Nitrophenyl N^2-Acetyl-N^1-(4-fluorobenzyl)carbazate. N^2-Acetyl-N^1-(4-fluorobenzyl)hydrazine (1.82 g, 10 mmol) and freshly distilled triethylamine (1.0 g, 10 mmol, Mallinckrodt) were dissolved in 60 mL of $CHCl_3$ (dried by distillation from P_2O_5) and the solution cooled in an ice bath. A cooled solution of 4-nitrophenyl chloroformate (2.02 g, 10 mmol, Aldrich) in 15 mL of dry $CHCl_3$ was added dropwise with stirring over 30 min. The reaction mixture was stirred for another 60 min while it reached ambient temperature. The mixture was washed successively with dilute HCl, water, saturated $NaHCO_3$, and water. The organic phase was dried ($MgSO_4$) and the solvent removed under vacuum at low temperature to leave a pale yellow solid which was crystallized from CH_2Cl_2/light petroleum, mp 154–157 °C. A second crop of material was collected from the crystallization mother liquors and had mp 168-169 °C. No difference between these two crops of crystals could be detected by TLC or ^{1}H NMR. A sample of the product that was recrystallized several times from CH_2Cl_2/light petroleum (mp 169–170 °C) had the following elemental analysis (figures in parentheses calculated for $C_{16}H_{14}N_3O_5F$): C, 55.3 (55.3); H, 4.3 (4.1); N 11.9 (12.1). ^{1}H NMR (60 MHz, $CDCl_3$) 2.0 (s, CH_3CO, 3 H), 4.7 (br s, CH_2, 2 H), 7.0–7.6 (aryl and NH, 7 H), 8.3–8.5 ppm (aryl, 2 H). ^{13}C NMR (20 MHz, $CDCl_3$) 20.6 (CH_3), 53.3 (CH_2), 115.3 and 116.4 (d, ^2J(C–F) = 22 Hz, C-3), 121.7 (C-2′), 122.4 (C-3′), 125.2 and 125.6 (d, ^3J(C-F) = 7 Hz, C-2), 130.5 ppm (C-1); other carbon signals were not detectable above noise. An EI mass spectrum at a source temperature of 200 °C did not exhibit a molecular ion but had a peak at m/z 208 with 7% intensity of the base peak at m/z 109. With use of chemical ionization, a mass spectrum of the carbazate gave a peak at m/z 348, corresponding to (M + H). The carbazate readily undergoes cyclization and elimination of 4-nitrophenol to form 2-methyl-4-(*p*-fluorobenzyl)-1,3,4-oxadiazol-5-one[25] which apparently is responsible for the m/z 208 peak in the EI mass spectrum. We also observed this decomposition in several organic solvents at ambient temperature using ^{1}H and ^{19}F NMR. The carbazate is stable for at least several hours in methanol, tetrahydrofuran, and acetonitrile but has a half-life of less than 1 h in dimethyl sulfoxide.

2,3,5,6-Tetradeuterio-4-fluorobenzoic Acid. 4-Fluorobenzoic acid (5 g, Aldrich) was dissolved in 18 g of D_2SO_4 and the solution heated under N_2 on an oil bath at 140 °C for 48 h and then at 180 °C for another 12 h. During this time the reaction mixture becomes dark brown. The mixture was poured onto ice (50 g). The solid which formed was taken up in ether, the solution dried ($MgSO_4$), and the solvent evaporated to afford 3.5 g of product. (Attempts to directly extract the product into ether without filtering led to the formation of an emulsion.) The extent to which the aromatic protons were exchanged was judged by comparing their integrated intensites in the ^{1}H NMR spectrum to that of the carboxyl proton.

It was found in repeating this preparation a number of times that the degree of deuterium incorporation at each position on the ring and the percentage recovery of 4-fluorobenzoic acid are dependent on the temperature at which the equilibration is effected; the degree of incorporation can be increased by raising the temperature in the range 120–180 °C but the recovery of acid decreases. The exchange was repeated two or three times for a particular sample of acid in order to increase the total amount of deuterium incorporated.

Several batches of deuterated 4-fluorobenzoic acid were combined and crystallized from aqueous ethanol. Proton-decoupled ^{19}F NMR spectra of this material contained three peaks separated by 0.27 ppm, corresponding to species in which the number of deuterons substituted for protons ortho to fluorine is zero, one, or two, with the resonance at lowest field corresponding to the nondeuterated species. Integration of these three peaks indicated that the proportions of molecules that are non-, mono-, and dideuterated ortho to fluorine are 2%, 23%, and 75% (each ±2%), respectively.

2,3,5,6-Tetradeuterio-4-fluorobenzyl Alcohol. Deuterated 4-fluorobenzoic acid (2.5 g, 17.5 mmol) in 30 mL of THF (dried by distillation from $LiAlH_4$) was added under N_2 and with stirring at 0 °C to a suspension of 1.4 g (37 mmol) of $LiAlH_4$ in THF. When the addition was complete the mixture was allowed to reach ambient temperature and stirred overnight. Water (1.4 mL), 1.4 mL of 15% NaOH, and 4.2 mL of water were added sequentially, dropwise, to give a granular precipitate of inorganic material. This precipitate was separated on a sintered glass filter, digested in refluxing THF, and the mixture extracted again in the same way. The organic filtrates were combined and evaporated under vacuum to leave a liquid residue of crude alcohol which was purified by distillation at 150 °C (2 mmHg) to afford 1.2 g of alcohol (53%). ^{1}H NMR (100 MHz, $CDCl_3$), 3.0 (br s, 2 H, CH_2), 7.0 (m, 0.4 H, H3,5), 7.2 ppm (d, 1.2 H, H 2,6).

2,3,5,6-Tetradeuterio-4-fluorobenzaldehyde. Pyridinium dichromate was prepared as described by Corey and Schmidt[26] and used as recommended by these authors. 2,3,5,6-Tetradeuterio-4-fluorobenzyl alcohol (1.2 g, 9.2 mmol) in 10 mL of CH_2Cl_2 (distilled from P_2O_5) was added under N_2 to a suspension of pyridinium dichromate (5.2 g, 13.8 mmol) in 40 mL of CH_2Cl_2. TLC and GC indicated that oxidation of the alcohol was complete within 18 h. A solid was filtered off and the volume of the remaining solution reduced by evaporation. Ether was added to precipitate more inorganic material and $MgSO_4$ added as a drying agent. The solids were removed by filtration and the solvent evaporated to leave the fluorobenzaldehyde (0.93 g, 79%). The ^{1}H NMR spectrum (100 MHz, $CDCl_3$ of this product was consistent with the expected structure, with the integrated intensities showing 60% deuteration at protons 3 and 5 (7.2 ppm) and 40% deuteration at protons 2 and 6 (7.9 ppm) by comparison to the intensity of the aldehyde resonance (10 ppm).

The procedures used to convert the deuterated fluorobenzaldehyde into the deuterated inhibitor were the same as those used for the nondeuterated material. Proton and fluorine spectra of the intermediates involved showed no significant loss of deuterium.

Enzyme Activity. Chymotryptic activity was assayed by using *N*-glutarylphenylalanine *p*-nitroanilide (GPNA) as a substrate. All assays were carried out at 25 °C with use of a 0.2 mM solution of GPNA in 0.05 M Tris, pH 7.6, with 10% Me_2SO added. A solution of the enzyme was added (100 μL or less) to 3 mL of GPNA in each of 3 cuvettes and formation of *p*-nitroaniline monitored at 410 nm. An equivalent volume of buffer was added to a fourth cuvette to provide a control for spontaneous hydrolysis. The activity was calculated as an average of the three rates for solutions containing enzyme. Hydrolysis in the absence of enzyme was negligible under these conditions.

Enzyme Modification. A stock solution of carbazate in THF was added to 0.05 M phosphate buffer to give a substrate concentration of around 0.5 mM and an organic co-solvent content of 10%. Hydrolysis or cyclization/elimination reactions of the carbazate were followed by monitoring the appearance of *p*-nitrophenol at 400 nm. Loss of the carbazate was pseudo first order with a half-life of 15 min at pH 7.25 and 53 min at pH 6.15. Addition of chymotrypsin to the solution during one of these experiments caused a burst of *p*-nitrophenol formation, indicating a rapid reaction between the carbazate and enzyme, as shown by previous workers.[3-5]

The enzyme was carbazoylated on a preparative scale at room temperature by dissolving α-chymotrypsin (Sigma, type II) in water (pH 6) at a concentration of 0.5 g/25 mL. With magnetic stirring approximately 2 molar equiv of inhibitor dissolved in a minimum amount of methanol was added dropwise to the enzyme solution. After being stirred for 30 min the solution was acidified to pH 3–4, centrifuged if necessary, and the inactivated enzyme purified by gel filtration chromatography on a 36 × 2.5 cm column of Bio-Gel P-10 with 1 mM HCl as the eluent. Column effluent was monitored at 280 nm. The protein eluted before the low molecular weight materials present in the reaction mixture. Alternatively, the reaction mixture was dialyzed against 1 mM HCl at 4 °C with use of tubing of 6000–8000 D molecular weight cut-off. Both of these methods were found to yield a product with one major ^{19}F NMR resonance.

Stoichiometry of Carbazoylation. To measure the stoichiometry of the inactivation of chymotrypsin with the carbazate a known amount of 4-fluorophenylalanine was added to a solution of IVa to provide an intensity reference. Integration of the ^{19}F NMR spectrum of the mixture indicated 1 mol of fluorophenyl becomes bound to 1 mol of chymotrypsin, within an error of ±5%.

Modified Enzyme Stability. Solutions of purified carbazoylated enzyme were prepared at a concentration of about 20 mg/5 mL in phos-

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phate buffer at pH 4.0 or 7.0. Each solution was divided in half and the two portions maintained at 25 and 6 °C, respectively. The activity of each solution immediately after preparation was 4% (±2%) of that of a fresh solution of native α-chymotrypsin. At pH 7.0, 25% activity was recovered in 1 day at 25 °C and 10% activity after 3 days at 6 °C. At pH 4.0, activity increased to 7% in 3 days at 25 °C and did not increase detectably when stored at 6 °C for the same period.

Solutions of carbazoyl enzyme used for NMR experiments were adjusted to pH 4.0 and stored at 6 °C when not in use. No change in the ^{19}F NMR spectrum of IVa was detectable either in the probe (25 °C) over several days or after storage under refrigeration (6 °C) for 2–3 weeks. Samples kept for longer periods generally developed one or more sharp peaks in the region 0.5–2.0 ppm upfield of the characteristic IVa resonance. Most samples for NMR were either freshly made or used within 2–3 weeks of preparation.

Samples for NMR Spectroscopy. Samples of carbazoylated α-chymotrypsin for NMR were prepared from purified, lyophilized protein by dissolution in either 95% H_2O/5% D_2O or 100% D_2O. Protein concentration was measured from the absorbance at 280 nm with use of an extinction coefficient of 50000 M^{-1} cm^{-1} for those experiments requiring an accurate knowledge of concentration;[27] otherwise samples were prepared gravimetrically.

Sample pH was adjusted by using microliter amounts of NaOH or HCl. In 100% D_2O pD was taken to be 0.41 unit higher than the pH meter reading.[28] No correction to pH was made for the deuterium content of solutions in 5% D_2O. A capillary of 4-fluoroacetophenone (Aldrich) provided a ^{19}F chemical shift reference signal.

Samples of the enzyme for ^2H NMR were prepared in ^2H-depleted water (Aldrich). The concentration of these samples was 1 mM and the pH was not adjusted from the value observed (3.6) upon dissolving the lyophilized protein.

NMR Experiments. Spin-lattice relaxation times ($T_1 = 1/R_1$) were determined by using the inversion–recovery method with a composite 180° pulse[29] and phase shifting.[30] Experimental data were fit to the appropriate three-parameter function by routines provided with the Nicolet software. T_1 values obtained were generally reproducible to better than 5% and are estimated to be accurate within at least ±10%.[31] Spin–spin relaxation times ($T_2 = 1/R_2$) at 282 MHZ were determined by Hahn two-pulse spin-echo experiments with a compensated 180° pulse[32] or the Carr-Purcell sequence with phase alternation of the 180° refocussing pulses. Both experiments were tried with and without sample spinning, with equivalent results within experimental uncertainty. Both the spin-echo and Carr-Purcell experiments are sensitive to the accuracy and homogeneity of the 90° and 180° pulses used;[33] pulse widths were determined for each sample before the start of the experiments. Transverse relaxation data were fit to the function $M(t) = A \exp(-R_2 t)) + B$ to compensate for imperfections in the experiment. R_2 values so obtained were reproducible within ±15%, and we take this to be an indication of their accuracy. A delay of 5–10 × T_1 was allowed between accumulations in all experiments to measure relaxation parameters.

"Hole burning" experiments were carried out by using the DANTE sequence.[34] A train of 100, 250, or 500 pulses of length 2 μs (9° flip) and spaced at 0.5 ms was used with the resonance offset from the transmitter frequency by 2000 Hz.

Nicolet programs were used to estimate line widths by fitting an observed resonance to a Lorentzian curve, using the least-squares criterion. In the case of the line shapes at 282 MHz (10-mm sample in a narrow-bore magnet) spinning side bands were unavoidably 5–10% of the main signal. For an estimate of the line widths in this case, the proton spectrum of the HOD line and its side bands were recorded by using the observe coil, and immediately thereafter the fluorine spectrum of the enzyme was recorded without altering any experimental conditions including the sample spinning rate. The HOD line, typically 1–2 Hz wide, was broadened by multiplying the proton fid by a single exponential.[35] The resulting line shape was compared by superimposition to the fluorine line shape; the amount of line broadening was adjusted until a good fit was obtained. It is estimated that the fluorine line widths could be determined to within ±5Hz by this procedure.

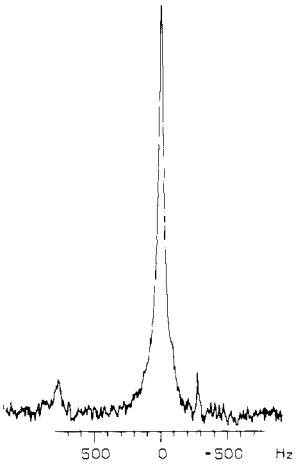

Figure 1. Fluorine-19 spectrum at 282 MHz of N^2-acetyl-N^1-(4-fluorobenzyl)carbazoyl-α-chymotrypsin (1 mM) at pH 4 in 95% H_2O. The fid was multiplied by an exponential function which broadened the line 5 Hz.

$^{19}F[^1H]$ NOEs were determined by comparison of spectra accumulated with and without ^1H irradiation during the pre-acquisition period. An alternating data accumulation was used so that the collection of an fid with NOE was obtained not more than a few seconds after the collection of an fid without NOE, minimizing the effects of thermal and instrumental drifts. A delay of at least 10 × T_1 was observed between each accumulation. The NOEs reported are believed to be accurate to within 2%.

Deuterium spectra were obtained by application of single 90° pulses or by using a 180°–t–90° sequence with the value of t chosen to null the signal from HOD. A spectral width of 10–15 kHz was used and data accumulated into 4K or 8K points. Collection of at least 50K transients was necessary for an adequate signal/noise ratio. Deuterium spectra observed were simulated by using the Nicolet program NMCCAP.

Results

The carbazate IIIb was prepared by conventional means and can be crystallized to analytical purity. It is unstable toward decomposition by cyclization and elimination of 4-nitrophenol to an oxadiazolone, a reaction which occurs upon heating, in the EI source of a mass spectrometer, and at room temperature in a variety of solvents. The reaction of IIIb with chymotrypsin was stoichiometric, and the inactive acylated enzyme so formed is sufficiently stable to permit NMR experiments of several days duration.

Initial Fluorine NMR Observations. Freshly prepared samples of modified enzyme showed a single major fluorine resonance. Depending on the method of isolation and sample pH, minor resonances (<5%) appeared in the spectrum, most often at 2.8 ppm to low field of this signal and 0.9 ppm to high field (Figure 1). Fluorine spectroscopy of another fluorine-containing serine-modified crymotrypsin indicates the presence of several reactive proteins in commercial chrymotrypsin,[36] and presumably these are also reactive with the carbazate. No attempt was made in the present case to identify the species responsible for the minor peaks or to remove them from the samples.

At 1 mM concentration in 5% D_2O at 282 MHz, the major resonance of IVa had a width at half-height of about 35 Hz in the pH range 2.5–4.0, but between pH 4.0 and 7.0 there is a reversible increase in the width to nearly double this at the highest pH. The acylenzyme likely aggregates more in neutral solution than at lower pH, a conclusion consistent with the findings of previous workers on the association of active site derivatives of α-chymotrypsin.[37-39]

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Table I. Enzyme Relaxation Data[a]

system	radio frequency, MHz	$W_{1/2}$, Hz	R_2, s^{-1}	R_1, s^{-1}	NOE
IVa, H_2O[b]	94.1	12	38 (39)	10.0 (12.4)	−0.87 (−0.89)
	282.3	35	110 (60)	3.5 (4.4)	−0.98 (−0.95)
	470.6	75	236 (110)	2.5 (2.0)	(−0.94)
IVa, D_2O[c]	94.1	18	56 (26)	7.1 (7.8)	−0.90 (−0.89)
	282.3	27	85 (48)	3.0 (2.8)	−0.98 (−0.94)
IVb, H_2O[d]	94.1	16	50 (24)	6.7 (6.9)	−0.88 (−0.89)
	282.3	e	(53)	2.5 (2.5)	−0.93 (−0.94)
	46.1[f]	900	2830 (2180)		

[a] At 25 °C. Values in parentheses are those calculated by using the model for relaxation described in the text. The solvent "H_2O" consisted of 95% H_2O/5% D_2O. Line widths were measured with proton decoupling at 94 and 282 MHz. For calculated values τ_c = 14 ns and τ_i = 30 μs. [b] Theoretical values obtained with r = 0.20 nm. [c] Calculated values obtained with r = 0.24 nm. [d] Data fit with r = 0.21 nm. [e] Overlapping lines due to isotope effects on the fluorine shift; line width could not be reliably determined by the procedure described in the Experimental Section. [f] Deuterium signals observed from a sample made up with deuterium-depleted H_2O.

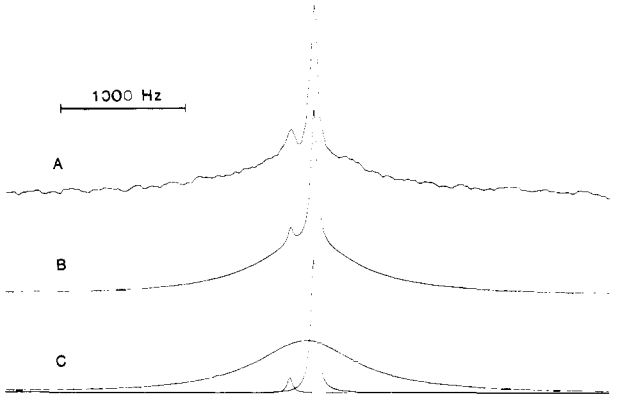

Figure 2. (A) Deuterium spectrum of enzyme derivative IVb obtained at 46 MHz. The center curve, formed by summing the Lorentzian functions indicated in trace C, shows a simulation of the observed line shape. Lines were broadened by 50 Hz in the experimental spectrum by exponential multiplication.

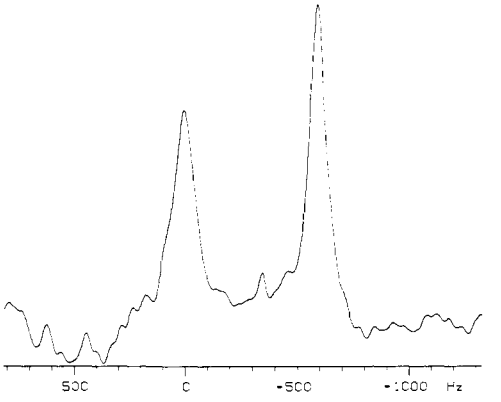

Figure 3. Fluorine spectrum at 282 MHz of the carbazoylated enzyme IVa dissolved in 8 M urea. Exponential multiplication broadened the lines by 50 Hz.

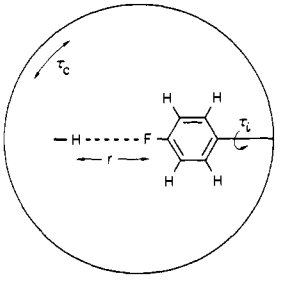

Figure 4. Dynamical model used to analyze the relaxation data. The fluorophenyl ring is assumed to rotate about an axis which is attached to a sphere undergoing reorientation characterized by the correlation time τ_c. A single proton at a distance r from the fluorine is used to represent the various protons of the enzyme that might alter relaxation by dipole–dipole interactions.

At pH 4 the observed line width did not change within experimental error over a sample concentration range of 0.2–2.0 mM, suggesting that there is little change in the degree of association of the acylenzyme over this range of protein concentrations. The conditions adopted as standard for the NMR experiments were pH 4.0 and a concentration of 1.0 mM.

The chemical shift of 1 mM **IV**a under the standard conditions was 7.19 ± 0.03 ppm upfield of the fluorine signal of 4-fluoroacetophenone (external reference). The shift was independent of concentration over the range noted and did not change detectably when the solvent was changed from 95% H_2O/D_2O to 100% D_2O. The carbazate inhibitor in acetone appeared 1.3 ppm upfield of the main signal of **IV**a while a sample of N^2-acetyl-N^1-(4-fluorobenzyl)hydrazide, the expected product of hydrolysis of **IV**a, had a chemical shift 0.9 ppm upfield of **IV**a.

The fluorine spectrum of the deuterated form of the enzyme (**IV**b) at 282 MHz had two overlapping resonances due to species with one or two deuterium atoms ortho to the fluorine nucleus. With use of line shape simulations it was determined that the chemical shift of the major peak is 7.76 ± 0.05 ppm upfield of 4-fluoroacetophenone, an isotope consistent with the shift of 0.28 ppm per ortho deuteron observed in the inhibitor and its precursors. The proportion of enzyme molecules without any deuterons ortho to fluorine is less than 5%, and this species could not be detected above the noise level in spectra of **IV**b.

The deuterium spectrum **IV**b (Figure 2) shows two sharp peaks superimposed on a broad line. The most intense sharp peak is due to residual deuterium in the solvent and, if this is assigned a chemical shift of 4.7 ppm, the other sharp peak occurs at 7.2 ppm. The experimental spectrum was simulated by a sum of three Lorentzian lines (Figure 2) to obtain estimates of peak widths and areas. The area of the sharp aromatic peak was less than 5% of the area of the broad protein resonance and is believed to arise from minor, non-active enzyme forms or a product of hydrolysis of the acylenzyme.

Attempts were made to denature the acylenzyme by dissolving **IV**a in a solution of 8 M urea. Even after 2 weeks at room temperature the resonance of the native enzyme persisted while a second resonance of a shape and width similar to the native enzyme signal appeared at 2.1 ppm to higher field (Figure 3). A much sharper resonance near the chemical shift of the free inhibitor would be expected if complete unfolding of the protein had taken place.[40,41]

Fluorine and Deuterium Relaxation. Fluorine spin–lattice relaxation rates (R_1), spin–spin relaxation rates (R_2), and fluorine–protons were determined at several field strengths for both **IV**a and the deuterated form **IV**b. The data obtained are presented in Table I. It is seen that replacement of either solvent protons or the protons on the carbazate ortho to the fluorine nucleus has a detectable effect on fluorine spin–lattice relaxation, showing

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that these protons are close enough to the fluorine atom in the modified enzyme to significantly influence its relaxation behavior.

In order to analyze the relaxation data in terms of the motion of the 4-fluorophenyl ring, it is necessary to assume a theoretical model for the system. As in previous work with a protein-bound 4-fluorophenyl ring,[40] we have adopted the model depicted in Figure 4. The correlation time τ_i describes the free rotation of the fluorophenyl ring about its symmetry axis, which is attached to a sphere which tumbles isotropically with a correlation time τ_c. The protons of the fluorophenyl ring were explicitly included in the calculation by using F–H distances and angles based on the structure of fluorobenzene,[42] although the protons meta to fluorine are at such a distance (0.45 nm) that neglect of them has little effect on calculated relaxation parameters. The 37% deuterium substitution at the meta position in **IVb** was approximated by the inclusion of a single proton in the calculations for this system. The relaxation due to protons of the protein and solvent was accounted for by assuming the presence of one other proton at a distance r from the fluorine along an extension of the symmetry axis of the fluorophenyl ring. This simplifies the theoretical treatment because distances between this proton and the nuclei on the ring are not modulated by ring rotation.

Proton–fluorine dipolar interactions and chemical shift anisotropy (csa) were the only relaxation mechanisms considered for the fluorine nucleus. The parameters used to compute the contributions of the csa mechanism were, in the notation of Hull and Sykes,[43] $\delta_z = 4.5 \times 10^1$, $C_0 = 3.06 \times 10^{-2}$, $C_2 = 1.58$. These constants were derived from preliminary results of a study of 4-fluorophenylalanine in the solid state.[44] When the formulae of Hull and Sykes are used there is only a small difference in calculated relaxation rates with use of these values and the csa parameters for fluorobenzene.[43] For spin–lattice relaxation, the calculated csa contribution did not exceed 5% of the total relaxation at the highest frequency used in this work.

Spin–spin relaxation of the aromatic deuterium nuclei was also computed in our analysis. This process is dominated by the quadrupolar mechanism with the width at half-height of the resonance given by[45]

$$w_{1/2} = \frac{1}{160} Q_D^2 (1 + \eta^2/3)[9J(0) + 15J(\omega) + 6J(2\omega)] \quad (1)$$

where Q_D is the quadrupolar coupling constant for a given deuteron and η is an asymmetry parameter. The value of Q_D for **IVb** is not known, but initial studies of 2,3,5,6-tetradeuterio-4-fluorophenylalanine indicate a value of 173 ± 5 kHz,[44] in good agreement with 180 kHz observed for ring-deuterated phenylalanine.[46] Small motions of the fluorophenyl ring may still be present at the temperature of this determination, and we used a value of 178 kHz in the analysis described below. The asymmetry parameter for an aromatic deuteron is sufficiently small (<-0.06[46,14]) that it can reasonably be neglected.[48] The spectral densities are given by[49]

$$J(\omega) = \frac{(3\cos^2\theta - 1)^2}{4} \frac{\tau_c}{1 + (\omega\tau_c)^2} + (3\sin^2\theta\cos^2\theta) \times \frac{\frac{6\tau_c\tau_i}{\tau_c + 6\tau_i}}{1 + \left(\frac{\omega\tau_c\tau_i}{\tau_c + 6\tau_i}\right)^2} + \frac{3\sin^4\theta}{4} \frac{\frac{3\tau_c\tau_i}{2\tau_c + 3\tau_i}}{1 + \left(\frac{3\omega\tau_c\tau_i}{2\tau_c + 3\tau_i}\right)^2} \quad (2)$$

where θ is the angle between a carbon–deuterium bond and the axis of rotation; for the model used in this work θ is 60° for a deuteron at any position on the ring. The analysis of deuterium relaxation is relatively simple because dipolar interactions do not contribute significantly and there are no internuclear distances involved in the calculation; a consideration of the observed deuterium line width indicates that neither of the two correlation times in the model can be less than ~10 ns.

The sets of data for the fully protonated enzyme in H_2O, the deuterated protein in H_2O, and the fully protonated system in D_2O (Table I) were initially analyzed separately. The parameters τ_c, τ_i, and r were varied until the agreement between the experimental fluorine R_1, NOE, and deuterium R_2 data for **IVb** and those computed with the model was optimum by the least-squares criterion. In order to estimate the reliability of the parameters so determined, the fitting process was repeated several times using experimental values increased or decreased by the amount of the estimated experimental error. The correlation times obtained in each analysis were so similar that these were averaged and the analysis repeated with only r being varied. Optimum agreement for the three systems was found with $\tau_c = 14 \pm 2$ ns, $\tau_i > 10$ μs, and $r = 0.20 \pm 0.01$ nm, but when the solvent is D_2O, r increases to 0.24 nm. The quality of the fits was insensitive to the value of τ_i as long as this parameter was substantially larger than τ_c. A comparison of the observed relaxation data and those calculated with the best-fit parameters is given in Table I; most fluorine R_1 values, the Overhauser effects, and the deuterium line width are reproduced by the calculation to within experimental error.

A range of correlation times (τ_c) corresponding to oligomeric forms of the enzyme and a model involving 180° flips of the aromatic ring were examined. Only values of τ_c near that noted above gave a reasonable reproduction of the Overhauser effects as well as the other classes of data mentioned. The ring-flip model did not give results appreciably different from those computed by using the diffusive rotation model described, indicating that ring rotation is so slow that the details of its motion(s) are irrelevant to relaxation processes.

Transverse Relaxation. The analysis described above is consistent with the notion that the *p*-fluorophenyl ring rotates within the protein structure at a rate less than the overall tumbling of the protein. However, the model developed in the analysis is not consistent with the line widths of the fluorine signals observed at three radio frequencies (Table I), predicting line widths that are much narrower than those observed. At 470 MHz the csa contribution to transverse relaxation is computed to make up 74% of total relaxation. However, reasonable adjustment of the parameters used in the csa calculation does not appreciably alter the situation. If we presume that the analysis is basically correct, then there must be additional factors which lead to broadening of the fluorine resonances in excess of that predicted by proton–fluorine dipolar interactions and the csa effect on transverse relaxation. Two possible factors are chemical exchange processes and the presence of unresolved resonances which fortuitously combine to give the appearance of a single Lorentzian line.

To investigate the possibility that the observed signal arises from a set of overlapping resonances, we attempted to "burn a hole" in it by selective irradiation. For comparison the same experiment was also carried out on a sample of poly(4-fluorostyrene) that had R_1 and R_2 values at 282 MHz roughly comparable to those of the enzyme derivative and a ^{19}F spectrum consisting of a broad, major signal which other experimentation shows is clearly heterogeneous.[50] A hole can be burnt in the polymer resonance (Figure 5A), but an attempt to burn a hole in the protein resonance only causes partial saturation of the entire signal (Figure 5B) with the resonance being reduced to 15–20% of the intensity observed in the absence of irradiation. A calculation of the saturation factor, $[(1 + {}^2H_1^2)/R_1R_2]^{-1}$, from the measured R_1 and R_2 values predicts a reduction to 10–23%. These results would appear to eliminate the possibility that the observed protein resonance is composed

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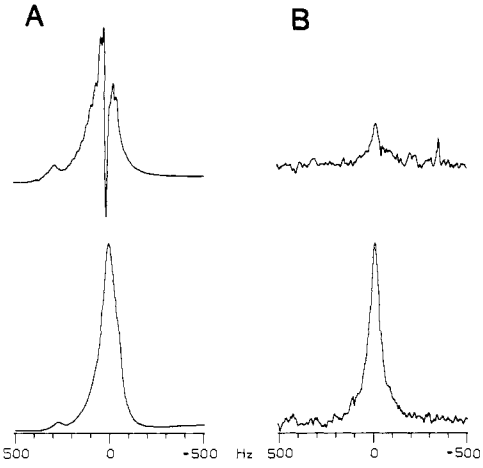

Figure 5. "Hole-burning" experiments with (A) poly(*p*-fluorostyrene) dissolved in $CDCl_3$ and (B) carbazoylated chymotrypsin IVa. The bottom curves show unperturbed spectra while the top traces are the result of the DANTE sequence described in the Experimental Section; the "hole" in the polymer line shape is consistent with the extensive heterogeneity of the polymer.

Table II. Fluorine Spin–Spin Relaxation[a]

radio freq., MHz	line width[b] R_2- (obsd)	line width[b] R_2- (calcd)	Hahn echo R_2- (obsd)	Hahn echo R_2- (calcd)	Carr–Purcell[c] R_2- (obsd)	Carr–Purcell[c] R_2- (calcd)
94.1	38	5				
282.3	110	106	112	106	77	104
470.6	236	236			220	153

[a] Calculated values were obtained for the two-site exchange situation described on the text. [b] Data from Table I for IVa in 95% H_2O. [c] Carr–Purcell experiments used times between the refocusing 180° pulses of 0.3–0.6 ms. There was no detectable dependence of R_2 observed in these experiments on the time between pulses.

of a number of non-exchanging components of fortuitously similar chemical shifts. We, therefore, reached the conclusion that the extra broadening of the fluorine lines is due in some way to an exchange process.

Observed transverse relaxation rates R_2 at 282 and 470 MHz were determined by the Hahn two-pulse spin-echo and Carr–Purchell methods (Table II). At 282 MHz R_2 from the Hahn echo experiment agreed within experimental error with that expected from the observed line width for **IV**a in 95% H_2O and 100% D_2O, reinforcing the conclusion that this signal is not composed of several sharper components. The Carr–Purcell experiments gave R_2 values that were somewhat smaller than those found with the Hahn experiments. However, in systems where $R_2 > R_1$ imperfections in the pulse train can produce apparent R_2 values that are smaller than the correct one.[33] Moreover, the Carr–Purcell R_2 can be reduced by chemical-exchange phenomena.[51]

In an attempt to rationalize the R_2 results we have made a brief theoretical analysis of spin–spin relaxation in a system involving exchange between two sites, one of which is dominant. A system like this could arise, for example, from exchange between a major and a minor conformation form of the protein when the fluorine environment is different in each conformation. Transverse relaxation in this situation is defined by the intrinsic R_2 for each site, the mole fraction (x_i) of molecules in each state, their lifetimes (τ_A or τ_B) before exchange to the other state, and the difference in the chemical shifts of the fluorine nuclei in the two states.[52] The fractional population of the minor site (x_B), its lifetime (τ_B), and its chemical shift (δ) from that of the major site were adjusted with use of a computer program until optimum agreement with our experimental R_2 results was obtained. In making this analysis we took R_2 in the absence of exchange at each frequency to be that predicted by the analysis of the other relaxation data given in Table I. As indicated in Table II reasonably good agreement with the experimental data could be obtained in this fitting exercise with $x_B = 0.15$–0.45, $\tau_B = 0.05$–0.15 ms, and $\delta = 1.0$–1.6 ppm. Simulated line shapes for this exchange situation at each of the frequencies used in our work are single peaks for which the non-Lorentzian character due to exchange broadening is barely discernible.

Discussion

The ^{19}F NMR resonance of the acylenzyme appears about 1.3 ppm downfield of the shift of the free inhibitor. Fluoroaromatic amino acids non-covalently bound at the active site of α-chymotrypsin generally display a downfield shift of 1.2–1.4 ppm with respect to the free amino acid whereas covalent attachment to the active site serine of the group containing a 4-fluorophenyl ring causes an enzyme-induced downfield shift of 4–7 ppm.[22] (For recent examples see ref 53–55.) The enzyme-induced shift may be due to the hydrophobic environment of the tosyl hole or hydrogen bond formation, or, because the fluorine label could make contact with atoms of the protein, it may arise from the van der Waals term.[55] Although the direction of the shift is always downfield, no correlation between the size of the effect and the position of a fluorophenyl ring in the tosyl hole has been developed. The covalent inactivator studied in the present work is a close analogue of a true substrate, and we have evidence from a two-dimensional $^{19}F[^1H]$ Overhauser experiment that the fluorine atom closely contacts several protons of the protein.[56] It is, therefore, intriguing that the shift of the fluorophenyl resonance in this system is so small.

Fluorine chemical shifts are sensitive to the isotopic composition of solvents,[57,58] and a fluorine nucleus that is exposed to an aqueous solvent experiences a 0.1–0.2 ppm upfield shift on changing from H_2O to D_2O.[59] There was no measurable (<0.05 ppm) D_2O/H_2O solvent effect on the ^{19}F chemical shift of **IV**a, and this suggests that the fluorine nucleus in this acylenzyme is at least partially shielded by the protein structure from interactions with the solvent. However, the spin–lattice relaxation rate of this fluorine is sensitive to the isotopic content of the solvent (Table I), and either the structure of the protein is altered enough when the solvent is changed from H_2O to D_2O to modify protein–fluorine interactions that are important for relaxation or there is a proton or set of protons derived from solvent (solvent exchangeable) which are close to the fluorine in the structure. The available data do not permit a distinction between these possibilities.

Native α-chymotrypsin denatures rapidly in 8 M urea.[60] Evidence from ESR and CD studies of chymotrypsins spin labeled at the active site suggest that denaturation is not an "all-or-nothing" process but passes through a variety of structures in which the region near the active site retains its tertiary structure to some extent while other parts of the molecule are unfolded.[61] Acylation of the enzyme with various carbazates has been shown by Orr and Elmore to stabilize the enzyme toward denaturation and to reduce the accessibility of tryptophanyl residues to solvent.[5] We were unable to detect denaturation of **IV**a by the criterion of a sharpened resonance at the appropriate chemical shift for samples kept in 8 M urea over a period of several weeks. Chymotrypsins modified with other acylating groups are known to also be resistant to denaturation,[40,41,62] but the degree of stability near the active

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site implied by our observation seems to be exceptional. The two distinct broad resonances in the ^{19}F spectrum of **IV**a in 8 M must be the signatures of enzyme forms that have tertiary structures of overall shapes very similar to that of the native enzyme rather than a random coil structure.

The analysis of fluorine and deuterium NMR relaxation data leads to a value for the correlation time τ_c which is consistent with the modified enzyme being present as the monomeric protein.[63,64] There has been a considerable amount of work on self-association of native chymotrypsin and derivatives formed by modification at the active site. Horbett and Teller[37] classified such derivatives according to their tendency to dimerize at pH 4.4 and concluded that groups which might be expected to occupy the enzymatic binding site usually eliminate dimerization of the enzyme. Neet and Brydon[38] studied the pH variation of the sedimentation velocity of some chemically modified chymotrypsins and found that tosylchymotrypsin shows increasing association between pH 4 and 6; NMR results are consistent with tosylchymotrypsin being monomeric at pH 4 but associated at pH 7.[39] The correlation time obtained from the relaxation data and the general observations regarding the pH dependence of the fluorine line width of **IV**a as described above are thus consonant with the anticipated behavior of a monomeric chymotrypsin chemically modified at the tosyl pocket.

Our analysis of the relaxation data suggests that the time scale for internal rotation of the fluorophenyl ring in **IV**a must be much slower than the correlation time for overall protein tumbling. The most likely explanation for the restricted motion of the ring is that it interacts strongly with the protein structure and, for at least a large fraction of the time, occupies the tosyl pocket of the enzyme. Motions of the fluoroaromatic ring would be appreciably constrained if the conformation of the tosyl pocket is similar to that which is observed in the solid state and if the rate of conformational change near this feature of the protein is slow.

The results we have gathered on the spin–spin relaxation rates and line widths as a function of radio frequency seem to require an exchange process that broadens the ^{19}F resonance of **IV**a, and we have formulated a minimal exchange process involving a minor component of the protein which is consistent with the available data. It would, of course, be possible to devise more elaborate schemes involving exchange between several fluorinated species that would be equally consistent with our observations. There is little hope of coming to a unique description of the exchange process without much additional experimentation; our goal at present is simply to show that such a process can account for our (otherwise) perplexing results.

A possible exchange process could be slow rotation about the amide or urethane carbonyl carbon–nitrogen bonds of the carbazate group as it is linked to the active site. The free energy of activation corresponding to the lifetime τ_B coming from our analysis is about 53 kJ mol^{-1}. This is substantially lower than a typical amide rotational barrier but similar to the barrier observed for rotation in urethanes.[40] However, rotation about either of these groups in **IV** would generate conformations ill-suited to occupy the active site pocket, and it seems probable that the enzyme would so favorably interact with a single rotational isomer that others would be present in concentrations too low to detect.

The furtive component in our model of the exchange process is not likely to be a protein dimer or oligomer since little concentration dependence of the line width is observed; instead it may be an alternative conformational form of the protein. The existence and interconversion of different conformations of enzymes including dihydrofolate reductase[65–67] and α-chymotrypsin[68–70] are well documented. The lifetime that we calculate for the minor component in our system is consistent with the finding that protein isomerization rates are often in the range 10^2–10^4 s^{-1},[71] and we hold open the possibility that the exchange process apparently present in the carbazoylated enzyme involves (subtle) conformational reorganization of the protein near the active site.

Summary. Our results suggest that during the reaction of specific substrates with chymotrypsin the aromatic ring of the substrate becomes significantly immobilized upon formation of the corresponding acylated enzyme. Although enzyme–acyl group interactions are strong enough to enhance the stability of this structure toward denaturation, conformational motions of the protein take place near the acyl group with sufficient rapidity to generate exchange-averaging effects in the NMR responses of nuclei in the acyl group.

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Registry No. IIIb, 92264-73-8; N^2-acetyl-N^1-(4-fluorobenzyl)-hydrazine, 93111-22-9; N^2-acetyl-N^1-(4-fluorobenzylidene)hydrazine, 93111-23-0; 4-fluorobenzaldehyde, 459-57-4; acetylhydrazine, 1068-57-1; 2-methyl-4-(*p*-fluorobenzyl)-1,3,4-oxadiazol-5-one, 93111-24-1; 2,3,5,6-tetradeuterio-4-fluorobenzoic acid, 93111-25-2; 2,3,5,6-tetradeuterio-4-fluorobenzyl alcohol, 93111-26-3; 2,3,5,6-tetradeuterio-4-fluorobenzaldehyde, 93111-27-4; 4-fluorobenzoic acid, 456-22-4.

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