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Dynamics at the Active Site of *N*-(4-Fluorophenyl)-*N*-(2,6-difluorophenyl) carbamoyl-α-chymotrypsin

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***N*-(4-Fluorophenyl)-*N*-(2,6-difluorophenyl)carbamoyl chloride was synthesized and used to inactivate the serine protease α-chymotrypsin. The derivatized enzyme reactivates sufficiently slowly at pH 5.2 that extended fluorine NMR studies of the system are possible. Such studies show that the 4-fluorophenyl ring can be found in two enzyme environments which are characterized by chemical shifts and relaxation behaviors that are very similar to those obtained in work with related compounds reported previously. The 2,6-difluorophenyl ring is also found in two environments, with rotation of the difluoroaromatic ring being slow in both. Saturation transfer methods were used to estimate the rate of interconversion between environments and the rate of aromatic ring rotation. Experiments with the protein dissolved in 8 M urea, conditions that normally lead to protein denaturation, show that sufficient structure remains about the difluorophenyl ring that rotation is slow under these conditions also, even though the structural features that produce the large fluorine chemical shift effects seen in the native enzyme are absent.**

KEY WORDS NMR, ^{19}F NMR, enzyme inactivation; protein dynamics; chymotrypsin; conformational changes; saturation transfer; diphenylcarbamoyl group.

INTRODUCTION

A number of experimental methods have enriched understanding of protein structure and dynamics beyond the conclusions provided by crystallographic observations. These include fluorescence techniques and the analysis of NMR relaxation parameters (for an example of the coordinated application of fluorescence and NMR relaxation methods to study of motions in a protein, see Ref. 1). Such experiments and others confirm that proteins are highly mobile systems, with conformational processes that take place on a time scale that stretches from nanoseconds to hours.[2] However, the relevance of a particular motion to the biological function of a specific protein generally still remains hidden from understanding.[3,4]

Processes occurring at the active site of an enzyme are of particular interest, as there are probably considerable changes in dynamics that occur during the course of the enzymatic catalysis. Initially, the enzyme must be flexible enough to allow rapid rotational and translational diffusion of molecules as they are screened in the substrate recognition process. Upon recognition, the active site must take on a sufficiently stable configuration that substrate and enzyme functional groups necessary for catalytic action are in the required relative alignment for the requisite time. Once catalysis has occurred, there must be weakening of protein–substrate interactions so that products are released and the catalytic cycle can start anew.[5,6]

Erlanger and co-workers[7–11] have shown that diphenylcarbamoyl chloride (**1**) reacts stoichiometrically with the serine protease α-chymotrypsin, presumably at the active site residue serine-195, to give a catalytically inactive protein. Previous work from our laboratory has extended their work by demonstrating that various fluorine-substituted diphenylcarbamoyl chlorides also inactivate this enzyme.[12–16] Fluorine NMR studies of those fluorinated derivatives (**2**–**10**) have provided evidence (1) that two magnetically distinguishable sites for each of the aromatic rings of the diphenylcarbamoyl group are present in the enzyme, (2) that appreciable protein-induced fluorine chemical shift effects characterize these sites, (3) that the relative populations of the conformations of the fluorinated diphenylcarbamoyl group depends on the position(s) of fluorine substitution, with the effects of a 2-fluoro substituent dominating all other effects, (4) that the rates of interconversion of conformations available to the diphenylcarbamoyl group are similar in native and unfolded enzyme (8 M urea) and (5) that the rate of rotation about the C_{phenyl}—N bond in at least one of the protein sites for the phenyl rings is probably slow.

The previous conclusions with regard to rotation of phenyl rings about their C-1—C-4 axis were based on observations with 2-fluorophenyl groups.[14] For example, with enzyme derivative **4**, interpretation of the spectra indicated that in one protein site the 2-fluorophenyl ring gives rise to two signals and, thus, the conclusion that ring rotation is slow in this site. However, the 2-fluorophenyl ring in the second site was

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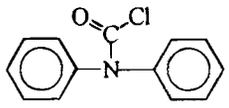

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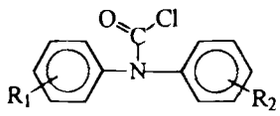
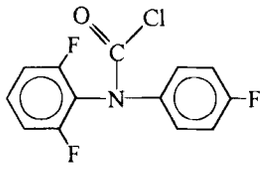


	R_1	R_2
2	H	4-F
3	4-F	4-F
4	H	2-F
5	2-F	2-F
6	H	3-F
7	3-F	3-F
8	2-F	3-F
9	2-F	4-F
10	3-F	4-F

characterized by a single resonance, an observation that is consistent with either rapid rotation of the ring, producing a single, averaged fluorine resonance, or with slow rotation of the ring, but a strong preference of the 2-fluorophenyl ring for one conformation. The present work was intended to distinguish between these possibilities.

The inactivator *N*-4-fluorophenyl-*N*-2,6-difluorophenyl carbamoyl chloride (**11**) has been prepared and shown to undergo reaction with α-chymotrypsin. In this diphenylcarbamoyl system, the 2,6-difluoro-substituted ring will report whether or not aromatic ring rotation is rapid; slow rotation of this ring would be expected to give rise to two fluorine signals of equal intensity,[14,15] whereas rapid ring rotation would give only a single, averaged resonance. Because of the symmetrical substitution there cannot be a preference for one orientation of the ring within the enzyme site. The fluorine NMR characteristics of the 4-fluorophenyl ring in the binding loci of chymotrypsin are well characterized and it was expected that signals from this fluorinated ring would provide indications of the relative amounts of conformations present and their rate of interconversion. Because of the position of the fluorine atom, fluorine spectra of the 4-fluorophenyl ring will not be affected by the rate of aromatic ring rotation.



11

EXPERIMENTAL

Materials

Common salts, buffers, and organic solvents were the highest grade commercially available. All water was deionized and then distilled in glass. Deuterium oxide (99.8% D) was obtained from Aldrich. α-Chymotrypsin was a three-times recrystallized salt-free product from Sigma (lot 71H7110) and was either used without purification or purified as described below.

***N*-(2,6-Difluorophenyl)-*N*-(4-fluorophenyl)acetamide.** A 3.05 g amount of *N*,*N*-(2,6-difluorophenyl)acetamide (17.9 mmol), prepared from 2,6-difluoroaniline (Aldrich) and acetic anhydride, 0.70 g of freshly recrystallized CuI (3.7 mmol) (Alfa), 0.41 g of Cu metal powder (6.5 mmol) (Aldrich), 2.60 g of K_2CO_3 (18.9 mmol) (Mallinckrodt), 0.43 g of KI (2.6 mmol) (Baker & Adamson) and four grains of I_2 were dried over P_2O_5 under high vacuum overnight. These ingredients were combined with 8.5 g of 4-bromo-1-fluorobenzene (Aldrich) and 10 ml of nitrobenzene and the mixture was heated under reflux for 48 h. The nitrobenzene and remaining 4-bromo-1-fluorobenzene were removed by vacuum distillation, then steam distillation. Water was added to the residue, the black solids were removed by vacuum filtration and the filtrate was extracted with 5 × 30 ml of diethyl ether. The combined ether extracts were rinsed with water and saturated NaCl solution, then dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue remaining was 5–10 ml of dark-brown oil which gave a ^1H NMR spectrum consistent with the expected structure; this crude material was used directly in the next step.

***N*-(2,6-Difluorophenyl)-*N*-(4-fluorophenyl)amine.** To the dark oil described above were added 5.3 g of KOH in 30 ml of absolute ethanol and the mixture was heated under reflux for 5 h. Water (50 ml) was added to the cooled reaction mixture and the system then extracted with diethyl ether (5 × 30 ml). The combined ether layers were extracted with 1 M HCl (3 × 30 ml) and, saturated NaCl solution and, after drying over anhydrous Na_2SO_4, concentrated *in vacuo* to yield a dark-brown oil. The ^1H NMR spectrum of the crude product at 200 MHz showed two multiplets in the aromatic proton region centered at 6.75 and 6.93 ppm. The $^{13}C\{^1H\}$ spectrum exhibited multiplets due to coupling to fluorine at δ 117.2 (t, $J_{AF} = 14.5$ Hz), 153.7 (d,d, $J_{BF} = 245$ Hz, $J_{BF'} = 5.8$ Hz), 109.3 (d, $J_{CF} = 22.6$), 120.3 (t, $J_{DF} = 9.9$ Hz), 137.0 (d, $J_{A'F} = 2.8$ Hz), 115.0 (d, $J_{B'F} = 7.6$), 112.9 (d, $J_{C'F} = 22.2$ Hz) and 155.1 (d, $J_{D'F} = 259$ Hz).

***N*-(2,6-Difluorophenyl)-*N*-(4-fluorophenyl)carbamoyl chloride.** In a three-necked 50 ml flask connected to a fritted glas tube and two serial aqueous ammonia traps were combined 2.0 g of crude *N*-(2,6-difluorophenyl)-*N*-(4-fluorophenyl)amine, prepared as described above, 10 ml of $CHCl_3$ and 0.79 ml of pyridine. The reaction mixture was flushed with nitrogen and cooled in an ice–NaCl bath. Phosgene gas (Matheson) was passed into the flask until approximately 10 ml had condensed. The ice bath was removed and the mixture stirred magnetically for 5 h while the mixture warmed and the excess phosgene evaporated. The apparatus was then flushed thoroughly with nitrogen. The chloroform was removed by rotary evaporation *in vacuo*. The oily residue was suspended in benzene and insoluble material (presumably pyridinium hydrochloride) removed by vacuum filtration. Benzene was removed from the clear solution by evaporation *in vacuo*, to afford 2.1 g of a dark red oil. TLC on silica gel (20% ethyl acetate–hexane mobile phase) was used to monitor the progress of the reaction, with the product appearing at $R_f \approx 0.3$. The product was isolated as a white, crystalline solid by flash chromatography on silica gel of the red oil using 20% ethyl acetate–hexane as the eluent. The solid was recrystallized twice from absolute ethanol to yield 0.46 g of crystalline chloride (18% based on crude starting amine), m.p. 63–66 °C. The ^1H NMR spectrum (500 MHz) showed three aromatic multiplets centered at 7.70, 7.95 and 8.80 ppm while the ^{19}F NMR (470 MHz) showed signals at −21.7, −20.0, −15.0 and −14.5 ppm downfield from the signal for *o*-fluoroaniline, with intensities in the ratio 1:2:2:4. The first two signals are assigned to the 4-fluorophenyl group while the remainder arise from the 2,6-difluorophenyl group, signals are doubled for each ring because of the two possible conformations for the carbamoyl chloride group, which are present in the ratio of 1:2. The infrared spectrum of the product [1750 (C=O), 1600, 1500, 1220, 1000, 850, 820 and 790 cm^{-1}] was consistent with the expected structure and the IR spectra from similar fluorine-substituted diphenylcarbamoyl chlorides prepared previously in this laboratory.

Instrumentation

Melting points were determined on a Mel-Temp apparatus and are uncorrected. Routine proton NMR spectra

were recorded on GN-500 and Gemini 200 spectrometers. ^{19}F NMR experiments at 282 MHz and 470 MHz were performed on General Electric GN-300 and GN-500 spectrometers, respectively. UV–visible spectra were determined on a HP Model 8452 diode-array spectrometer equipped with an HP 89500 UV–visible ChemStation. pH measurements were made with a Markson Model 88 pH meter connected to a Fisher gel-filled electrode. IR spectra were recorded on a Perkin-Elmer Model 283 spectrometer. Ion-exchange liquid chromatography was performed using a Waters HPLC system. Concentration of enzyme solutions employed Amicon Centriprep 10 ultracentrifuge filters.

Methods

Enzyme modification. Enzyme modification (inactivation) with *N*-(2,6-difluorophenyl)-*N*-(4-fluorophenyl)carbamoyl chloride was carried out by stirring 1 g of α-chymotrypsin dissolved in 30 ml of water with 41 mg of the inhibitor dissolved in 1.5 ml of methanol (fourfold molar excess) for 3 h. The pH was maintained at 7.7 by the addition of small amounts of 1 M NaOH. At the end of 3 h the enzyme exhibited less than 1% of its initial activity toward the substrate *N*-glutarylphenylalanine-*p*-nitroanilide (Sigma).[17] Excess inhibitor was removed by centrifugation. The supernatant solution was brought to pH 5.2 with 1 M HCl and then stored at 4 °C. The protein concentration of the resultant solution was determined to be 0.80 mM using the absorption at 280 nm and a molar absorptivity of 50 000 l mol^{-1} cm^{-1}.[18]

Stability of inactivated enzyme. At pH 5.2 the inactivated enzyme regained less than 0.3% of its initial activity in 5 days when stored at 4 °C, whereas a sample stored under identical conditions at pH 7.4 regained 12% activity in the same time period.

Purification of inactivated enzyme. Cation-exchange chromatographic purifications of the inactivated chymotrypsin were performed using Bio-Rad Econopac CM weak cation-exchange columns. The inhibited protein (3 mg in 15 mM Tris buffer, pH 4.98) was applied to the column. A 15 min 0.0–0.5 M non-linear NaCl gradient (gradient 2 on the Waters gradient maker) at a flow rate of 1 ml min^{-1} was started. Many contaminants were eluted prior to two major protein fractions which appeared as overlapping bands at 4.1 and 4.9 min. Fluorine NMR spectra of material collected during the early stages of elution of the 4.1 min peak were identical with those obtained from material that eluted at the tail of the 4.9 min peak. No chromatographic conditions were found which were able to effect baseline separation of these materials, so they were collected together and used to prepare the NMR samples. A number of minor fluorine signals in the range −10 to −22 ppm from *o*-fluoroaniline appeared in the spectrum of the crude modified enzyme.

Fluorine NMR experiments with modified enzyme. Enzyme samples contained typically *ca* 0.3 mM of chromatographically purified protein, 0.05 M NaCl and 10% D_2O to provide a lock signal. All enzyme samples were contained in 10 mm NMR tubes and were adjusted to pH 5.2 by the addition of small amounts of 1 M HCl or NaOH as necessary. Fluorine spectra of the enzyme systems were not proton decoupled as this made no detectable difference in the observed linewidths. Procedures for collection of the T_1 data and for DANTE selective excitations were the same as those described by Gerig and Hammond.[19]

RESULTS

Fluorine NMR of modified α-chymotrypsin

The commercial native enzyme can be highly impure.[20] In contrast to our experience with other inactivated enzymes in the series **2–10**, fluorine spectra of the present derivative indicated contamination by impurities, or other forms of the enzyme, that are reactive to **11** by means of weak signals in the range of shifts shown in Fig. 1. It was also noted that fluorine NMR spectra of chymotrypsin inactivated by reaction with **11** were sensitive to protein concentration, with the observed signals being appreciably wider (×2) at protein concentrations near 1.5 mM. Modified protein purified by means of anion-exchange chromatography and examined in relatively dilute samples (0.3 mM) gave spectra in accord with expectations (Fig. 1). Although

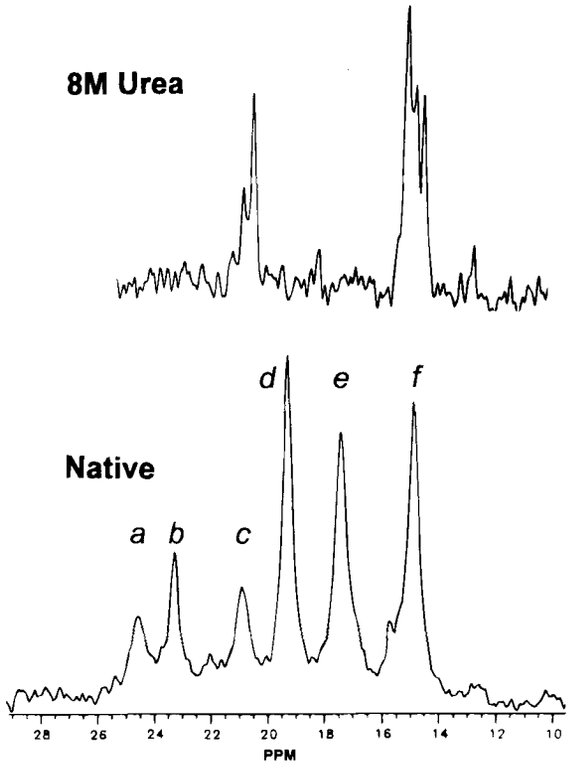


Figure 1. Fluorine-19 NMR spectra of *N*-(4-fluorophenyl)-*N*-(2,6-difluorophenyl)carbamoyl-α-chymotrypsin obtained at 470 MHz and 25 °C. The native modified enzyme spectrum was obtained at pH 5.2. Protein concentration in both samples was *ca* 0.3 mM.

within the expected range of chemical shifts, interpretation of this spectrum could not be made by inspection. For clarity in the discussion, the signals observed are labeled *a–f*, as indicated in Fig. 1.

Peaks *a–f* narrowed 20–50% as the sample temperature was raised from 0 to 25 °C at 470 MHz, presumably owing to the decrease in viscosity with increasing temperatures, then broadened as the temperature was increased to 45 °C. At this temperature, the low-field portion of the spectrum was so broadened as to be lost in the noise level. These changes in the spectra with temperature, while indicating exchange, were not sufficiently distinctive to lead to an estimate for the rates of conformational changes in this system by means of line shape analysis. The saturation transfer method at a sample temperature of −1 °C was adapted instead, since these conditions gave a convenient rate.

Figure 2 records the results of a typical series of saturation experiments with this system. Careful control of the intensity of the saturating field γB_2 was necessary because of the proximity and widths of the lines and the 'spillover' of the effects of irradiation on adjacent peaks. Without further analysis, however, it is clear that saturation of peak *f* leads to reduction in intensity of peak *a*. The species represented by peaks *b* and *d*, and also *c* and *e*, are similarly connected. Thus, two conformations or states for the fluorinated difluorophenylcarbamoyl system are present in this system, with the minor three signals at low field (*a–c*) corresponding to one conformation and the group of major signals (*d–f*) corresponding to the other. The ratio of the amount of the first conformation to the second was determined as part of the computer analysis described below, and found to be 0.30:0.70 at −1 °C. That three fluorine signals are observed for each conformation indicates that rotation of the 2,6-difluorophenyl group about its C-1–C-4 axis is slow in both.

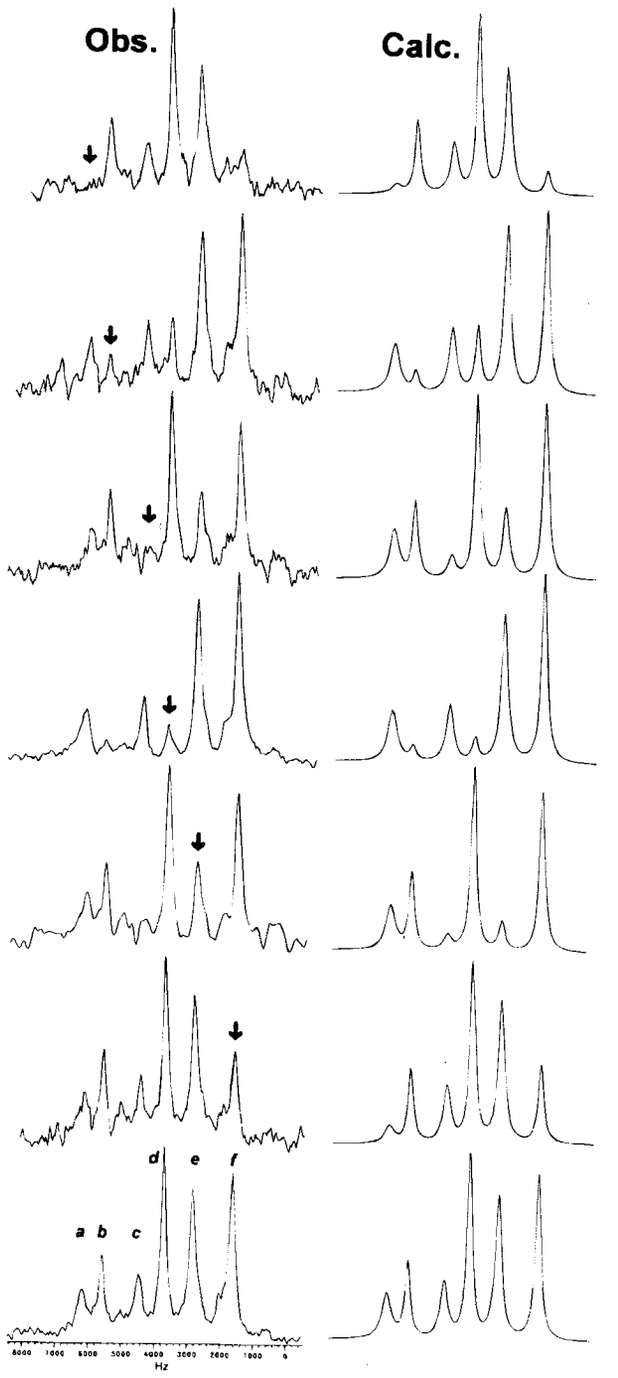


Figure 2. Saturation transfer studies of *N*-(4-fluorophenyl)-*N*-(2,6-difluorophenyl)carbamoyl-α-chymotrypsin at −1 °C and 470 MHz. An average r.f. field of 13 Hz was applied at the positions indicated for 2 s by means of the DANTE sequence followed by a 90° hard pulse. Approximately 1600 scans were averaged to obtain the experimental spectra, shown on the right. Calculated spectra (left) were prepared using the program described in the text.

Analysis of saturation transfer results

It was necessary to go beyond a qualitative analysis of the saturation transfer data to find those signals which could be assigned to each fluorine of the *N*-(2,6-difluorophenyl)-*N*-(4-fluorophenyl)carbamoyl group attached to the enzyme. To this end, a computer program based on solution of the Bloch equations in the presence of an arbitrary r.f. field for a set of interconverting species was devised.

Consider a set of n species that are each represented in the NMR spectrum by a single resonance. If interconversion between species is present, the behavior of the components of the sample magnetization vector u_i, v_i and $M_{z,i}$ in a frame of reference rotating at ω_0 for the ith species is given by

$$\frac{du_i}{dt} = \frac{-u_i}{T_{2,i}} + (\omega_0 - \omega_i)v_i - u_i \sum_{i(i\neq j)}^{n} k_{ij} + \sum_{j(j\neq i)}^{n} k_{ji}u_j \quad (1)$$

$$\frac{dv_i}{dt} = -(\omega_0 - \omega_i)u_i - \frac{v_i}{T_{2,i}} - \gamma B_2 M_{z,i} - k_{ij}\sum_{i(i\neq j)}^{n} v_i + \sum_{j(j\neq i)}^{n} k_{ji}v_j \quad (2)$$

$$\frac{dM_{z,i}}{dt} = \gamma B_2 v_i - \frac{(M_{z,i} - M_{0,i})}{T_{1,i}} - k_{ij}\sum_{i(i\neq j)}^{n} M_{z,i} + \sum_{j(j\neq i)}^{n} k_{ji}M_{z,j} \quad (3)$$

where ω_i is the Larmor frequency for site i, $T_{1,i}$ and $T_{2,i}$ are the spin–lattice and transverse relaxation times

characteristic of site i, respectively, γB_2 is the magnitude of the saturating field and $M_{0,i}$ is the equilibrium amount of species i present, here set equal to the mole fraction of species i present at equilibrium. The rate constants k_{ij} quantitate the rate of transfer of magnetization from site i to site j. For the present work, simulation of the saturation phase of an experiment was accomplished by solving the system of equations based on Eqns (1)–(3) for exchange between six sites using experimental inversion–recovery and linewidth data to estimate $T_{1,i}$ and $T_{2,i}$. As with the experimental spectra, ω_0 was the frequency of γB_2. It was then assumed that at the end of the saturation period a perfect 90° pulse rotated the z-components to the y' axis in the rotating frame, while rotating the y' components to the z-axis. Another solution of a system of equations based on Eqns (1) and (2) was then done to compute the lineshape for the spectrum that would be observed by Fourier transformation of the free induction decay. The system of 18 equations needed to describe the effect of the r.f. field and the system of 12 equations to describe the spectrum obtained after saturation were solved by a PC program which employed the EISPACK routines for diagonalizing non-Hermitian matrices.[21]

It was found that on the spectrometer used the short pulses employed in the DANTE method for generation of the field γB_2 during the saturation experiment were not square, so that the magnitude of γB_2 could not be computed reliably from the experimental pulse length and inter-pulse delays.[22] In modeling the experimental spectra, the magnitude of γB_2 and the values for the rate constants k_{ij} were adjusted until computed spectra based on a single set of values for all of these produced lineshapes that agreed with all spectra in a series of experimental saturation results. Figure 2 illustrates the agreement that could be obtained between such simulations and experimental results.

In this way it was determined that peaks b and d of the spectrum shown in Fig. 1 have only one interconversion pathway and therefore must arise from the 4-fluorophenyl group in the enzyme derivative. In confirmation of this conclusion, we note that the chemical shifts and relaxation behavior observed for these signals are very similar to corresponding data obtained for 4-fluorophenyl rings in other chymotrypsin derivatives containing the 4-fluorophenyl ring, as shown in Table 1. The simulations showed that the rate constant for conversion from the major to the minor conformation of the diphenylcarbamoyl group in this inactivated chymotrypsin is 8.7 s^{-1} with an estimated error of about 10%. This rate corresponds to an activation free energy $\Delta G^{\ddagger} = 59.7$ kJ mol^{-1}. Assuming that the entropy term is negligible, this activation free energy would correspond to a rate constant for interconversion at 25 °C of about 60 s^{-1}.

Peaks a and c originate from the 2,6-difluorophenyl ring in the minor conformation, while the same ring in the major conformation gives rise to peaks e and f. There are no previous shift or relaxation data for comparison to these results. However, the 2-fluorophenyl ring in other systems typically exhibits a *ca* 8 ppm difference between the shift observed for the major and minor forms (Table 1), which is in accord with the 6–7 ppm differences observed with the 2,6-difluoro system.

Rotation of the difluoroaromatic rings in either conformation was found to be significantly slower than the rate of interconversion between conformations. The data analysis described led to a value of $k_{ac} = k_{ef}$ of about 0.12 s^{-1}. There error in this estimate is probably ±25%, based on the sensitivity of the simulations to this parameter. We could not detect a difference in the rate of ring rotation in the two interaction sites in these experiments.

The rate of ring rotation observed corresponds to $\Delta G^{\ddagger} = 71$ kJ mol^{-1} and a rate constant for rotation of 2 s^{-1} at 25 °C if the entropy term for rotation is neglected.

Fluorine NMR of modified α-chymotrypsin in 8 M urea

The fluorine NMR spectrum of *N*-(2,6-difluorophenyl)-*N*-(4-fluorophenyl)carbamoyl-α-chymotrypsin dissolved in 8 M urea is shown in Fig. 1. The chemical shifts are significantly changed under these presumably strongly denaturing conditions, with signals being much sharper and, thus, consistent with appreciably unfolding of the protein. By referring to previous studies of fluorinated

Table 1. Native fluorodiphenylcarbamoylchymotrypsins[a]

	2-Fluoro-substituted ring				4-Fluoro-substituted ring			
Enzyme	Fraction	Shift (ppm)	$w_{1/2}$ (Hz) (470)	T_1 (s^{-1}) (470)	Fraction	Shift (ppm)	$w_{1/2}$ (Hz) (470)	T_1 (s^{-1}) (470)
R_1 = 2-F, R_2 = H	0.22	19.15	660	0.9	—	—	—	—
	0.02	15.25	230	0.9	—	—	—	—
	0.76	11.10	230	0.9	—	—	—	—
R_1 = 4-F, R_2 = H	—	—	—	—	0.70	23.8	200	—
	—	—	—	—	0.30	20.5	280	—
R_1 = 2-F, R_2 = 4-F	0.29	19.40	300	0.9	0.31	22.50	310	1.0
	0.02	15.10	—	—	0.69	19.45	220	1.0
	0.69	11.16	520	0.9	—	—	—	—
R_1 = 4-F, R_2 = 2,6-diF	0.30	24.57, 20.87	325, 260	0.98, 0.93	0.30	23.23	200	0.84
	0.70	17.32, 14.79	235, 200	0.79, 0.90	0.70	19.25	190	1.2

[a] Chemical shifts are downfield relative to the signal from *o*-fluoroaniline. Linewidths are those obtained without proton decoupling but are corrected for any line broadening introduced by apodization during data process. Data for the first three systems come from Refs 14–16; data for the last system are from the present work.

Table 2. Fluorodiphenylcarbamoylchymotrypsins in 8 M urea[a]

	2-Fluoro-substituted ring			4-Fluoro-substituted ring		
Enzyme	Fraction	Shift (ppm)	$w_{1/2}$ (Hz) (470)	Fraction	Shift (ppm)	$w_{1/2}$ (Hz) (470)
R_1 = 2-F, R_2 = H	0.53	12.51	20	—	—	—
	0.47	11.62	23	—	—	—
R_1 = 4-F, R_2 = H	—	—	—	0.50	21.1	—
	—	—	—	0.50	20.9	—
R_1 = 2-F, R_2 = 4-F	0.45	12.86	20	0.45	20.46	20
	0.55	12.04	20	0.55	20.35	20
R_1 = 4-F, R_2 = 2,6-diF	0.36	15.15, 15.23	25, 25	0.36	21.0	25
	0.64	14.61, 14.90	25, 25	0.64	20.7	25

[a] Chemical shifts are downfield relative to the signal from *o*-fluoroaniline. Linewidths are those obtained without proton decoupling but are corrected for any line broadening introduced by apodization during data processing. Data for the first three systems come from Refs 14–16; data for the last system are from the present work.

diphenylcarbamoylchymotrypsins in 8 M urea (Table 2), the pair of signals at 20.7 and 21.0 ppm are readily assigned to the 4-fluorophenyl ring. From these, it is apparent that two conformations are present in the ratio of 0.36:0.64. The collection of signals from 14.5 to 15.5 ppm must be assigned to the fluorines of the 2,6-difluorophenyl ring. It is clear from this part of the spectrum that rotation of these aromatic rings remains slow even under the denaturing conditions.

The fluorine spectrum of the modified enzyme in 8 M urea is strongly temperature dependent, with the set of lines for the 4-fluorophenyl ring undergoing the broadening and coalescence behavior typical of an exchanging system. A rough analysis of the changes of lineshape in this part of the spectrum indicated that the rate of interconversion between the two conformations detected is 20 s^{-1} at 25 °C. This rate was consistent with the lineshape changes observed in the 2,6-difluorophenyl region of the spectrum, indicating that, again, the rate of rotation of the 2,6-difluorophenyl ring about its C-1–C-4 axis is considerably slower than the rate of interconversion between conformations under these denaturing conditions.

DISCUSSION

A crevice on the protein surface at the active site of α-chymotrypsin provides a pocket for the binding of the side-chains of aromatic amino acid residues; it is presumably interactions with this site that are responsible for the substrate specificity this enzyme exhibits. Covalent linkage of the diphenylamino group to the serine residue at the active site through a carbamoyl function could position one of the aromatic rings of the diphenylamino moiety within this pocket. Because of the inherent magnetic asymmetry of the protein an aromatic ring of the diphenylamino group will be found in two distinguishable environments, as indicated in Fig. 3. Interconversion of the possible environments can only take place by rotation about the amino nitrogen–carbonyl carbon bond (Fig. 3). If the structure of the protein observed in the crystal is retained, this rotation would also be expected to be slow because an appreciable amount of structured polypeptide must be moved or circumvented to move one aromatic ring into the location occupied by the other.

Our previous NMR studies with the various fluorine-substituted diphenylcarbamoyl-α-chymotrypsins (**2–10**) have shown that the rate constant at 25 °C for interconversion of fluorinated aromatic rings by this rotation ranges from 4 to 126 s^{-1}, with the average being 42 s^{-1}. Beyond the contribution of experimental uncertainties to the spread of rates observed for these various systems, some effect of fluorine substitution on electron delocalization in the diphenylcarbamoyl group would be expected and this could have an impact on the rotation rate. The presence of fluorine could alter interactions between an aromatic ring and the protein either by participating in new interactions or altering the strength of existing ones. Thus, in this context, there is nothing remarkable about the rate of rotation about the amino nitrogen–carbonyl carbon bond of *N*-(4-fluorophenyl)-*N*-(2,6-difluorophenyl)carbamoyl-α-chymotrypsin (60 s^{-1} at 25 °C) that we estimate by the saturation transfer experiments.

Dissolution of the carbamoylated enzyme in 8 M urea is expected to produce protein molecules largely in random conformations. The removal of the large protein-induced fluorine shifts in this solvent observed with enzyme derivatized with **11** (Fig. 1) is consistent with this expectation. Even when unfolded, the enzyme provides two magnetically distinguishable environments for an aromatic ring of the diphenylcarbamoyl group and it is possible to estimate the rate of rotation about the amino nitrogen–carbonyl carbon bond under denaturing conditions. In six systems the rate constants

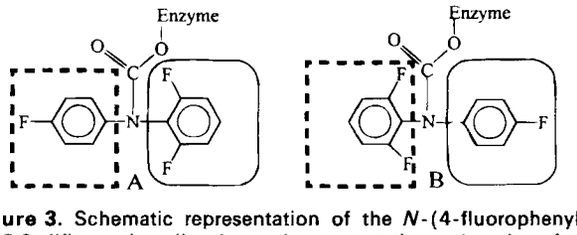


Figure 3. Schematic representation of the *N*-(4-fluorophenyl)-*N*-(2,6-difluorophenyl)carbamoyl group at the active site of the enzyme. Since both sites available to the 2,6-difluorophenyl ring are inherently magnetically anisotropic, separate NMR signals will be observed for the fluorines of this ring if ring rotation is slow.

for this rotation at 25 °C vary from 42 to 590 s^{-1}, with an average of 243 s^{-1}. The tendency for a more rapid rate of rotation under denaturing conditions is consistent with removal of some or all of the tertiary structure of the protein present in the native state, but the close similarity of the rates and activation parameters for the rotation under denaturing conditions to those characteristic of the native protein have led us to postulate that local unfolding of the part of the protein to which the diphenylcarbomoyl group is attached is part of the process by which the phenyl or fluorophenyl rings of the diphenylcarbamoyl derivative exchange aromatic ring environments. *N*-(4-Fluorophenyl)-*N*-(2,6-difluorophenyl)carbamoylchymotrypsin in 8 M urea appears to undergo rotation at a rate (about 20 s^{-1}) that appears to be slower than for the other systems in this series even if a generous allowance is made for experimental uncertainties. Further, for the first time, the rate appears to be less than the rate of rotation found with the corresponding native structure.

Ab initio molecular orbital studies of diphenylamine derivatives suggest that the energy barrier to rotation of an aromatic ring about the bond between C-1 of the aromatic ring and the carbamoyl nitrogen should be of the order of 13 kJ mol^{-1}.[23] Translated to a free energy of activation, a barrier of this magnitude would correspond to a rate constant for aromatic ring rotation of about 4×10^{10} s^{-1} at 25 °C. In *N*,*N*′-bis(2-fluorophenyl)carbamoyl chloride a process detected by fluorine NMR that was assigned to rotation of the aromatic rings takes place at a rate of about 10^8 s^{-1}.[24] The order of magnitude for the rate of aromatic ring rotation in α-chymotrypsin derivatized with *N*,*N*-bis(2-fluorophenyl)carbamoyl chloride is around 10 s^{-1}, if it is assumed that part of the dynamic processes detected by saturation transfer and lineshape studies of that system are, in fact, due to this motion.[14] The rate of fluoroaromatic ring rotation is about 0.1 s^{-1} in the present system, a reduction in rate of about nine orders of magnitude that must be ascribed to interactions between the 2,6-difluorinated ring and the enzyme surface.

We have no quantitative information on the rate of aromatic ring rotation in derivatized protein dissolved in 8 M urea. The rate of rotation must be appreciably less than 150 s^{-1} or else slow exchange spectra would not be observed for the 2,6-difluorophenyl region of the spectrum (Fig. 1). Protein–diphenylcarbamoyl group interactions may remain strong enough in 8 M urea to prevent significant rotation. It is known that hydrophobic groups bound to the active site of the enzyme seem to stabilize this part of the protein to 8 M urea so that the structure is not completely unfolded.[19] Fluorine substitution increases the hydrophobicity of aromatic rings[25] and this could lead to enhancement of the interactions between enzyme and the diphenylamino aromatic rings to the extent that enough native structure around the active site is retained that aromatic ring rotation is slowed. However, retention of structure cannot be complete, for those protein–ring interactions that produce the large chemical shift effects seen in the native protein disappear in 8 M urea.

The very broad lines observed in the fluorine spectrum of native enzyme inactivated with the carbamoyl chloride **11** relative to those observed when the modified protein is dissolved in 8 M urea are striking (Fig. 1). At 470 MHz and the correlation time expected for a protein of the size of α-chymotrypsin (*ca* 15 ns), a linewidth of about 35 Hz is expected on the basis of the fluorine–proton dipolar contributions and the fluorine chemical shift anisotropy contributions to transverse relaxation. The expected linewidth is fairly close to what is observed with other kinds of fluorophenyl-containing derivatives of the enzyme.[19] However, all of the fluorinated diphenylcarbamoyl derivatives of chymotrypsin that have been examined exhibit linewidths many times larger than this estimate. The extraordinarily wide lines found experimentally have not been adequately explained but may originate from protein inhomogeneity or additional exchange processes.

CONCLUSIONS

Our results indicate that the rate of rotation about the carbonyl carbon–nitrogen bond of the *N*-(4-fluorophenyl)-*N*-(2,6-difluorophenyl)carbamoyl group when covalently bound to α-chymotrypsin is similar to rates for this process that have been observed for a variety of fluorine-substituted diphenylcarbamoyl derivatives of this protein. Our observations provide strong evidence that, nonetheless, the rate of rotation of the aromatic rings about the ring–carbamoyl nitrogen bond in a diphenylcarbamoyl group is reduced by about 10^9 when this group is covalently bound to the active site of α-chymotrypsin. The phenyl–enzyme interactions responsible for the restriction of aromatic ring rotation must persist, at least in part, in protein dissolved in 8 M urea, indicating that some local tertiary structure persists under normally strongly denaturing conditions.

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