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Structure and dynamics of α-chymotrypsin-*N*-trifluoroacetyl-4-fluorophenylalanine complexes

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SUMMARY

Fluorine NMR lineshape, relaxation and Overhauser effect data collected at 282 and 470 MHz have been used to obtain information about the nature of complexes formed between *N*-trifluoroacetyl-4-fluorophenylalanine and the enzyme chymotrypsin. Systems involving both enantiomers have been examined as well as derivatives of these in which the aromatic ring hydrogens have been replaced by deuterium. The enzyme-induced fluorine chemical shift effects and the dynamics of molecular motions of the fluorophenyl ring at the respective binding sites appear to be similar in both complexes and, where comparable, the results are in agreement with data obtained at lower frequencies that have been reported by other workers. The dynamics of the fluoroaromatic ring in these complexes are significantly different from those observed in a closely related acylated enzyme.

INTRODUCTION

α-Chymotrypsin, a digestive enzyme found in the bovine gut, catalyzes the hydrolysis of peptide bonds. It is representative of a number of enzymes known as serine proteases which have many structural and mechanistic features in common (Creighton, 1983; Steitz and Shulman, 1982). The action of chymotrypsin involves, minimally, recognition of an appropriate substrate by formation of a Michaelis complex, covalent attachment of the acyl part of the substrate to the protein, and hydrolysis of the resulting acylenzyme to form an enzyme-product complex which dissociates to regenerate the native, active enzyme. Intuitively, each phase of this mechanistic sequence likely involves a distinct set of protein-substrate interactions and the conformational dynamics of the various identifiable intermediates should be substantially different. Thus, for example, the ways in which the protein moves, especially near the active site, during the recognition part of the mechanism are presumably different from the molecular motions that achieve the correct orientation of

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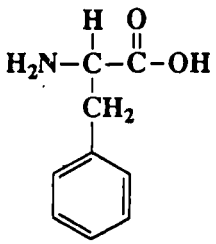
functional groups needed for the catalytic step(s) or that encourage the release of reaction products from the enzyme environment. Crystallographic results have provided evidence that as many as 170 of the amino acids present in chymotrypsin participate in a conformational change that follows binding of a specific substrate to the protein and have supported the suggestion that extensive vibrational coupling somehow related to this motion positions the substrate for the subsequent events in the catalytic cycle (Havsteen, 1989). However, the requirements for diffraction limit the range of molecular motions that can be examined crystallographically and it falls to other experimental techniques or computational methods to provide information about protein conformational behavior in solution.

Phenylalanine (**I**) derivatives are among the specific substrates for chymotrypsin (Blow, 1976; Fersht, 1977). 4-Fluorophenylalanine (**II**), an analog of phenylalanine accepted by the enzyme (Bennett and Nieman, 1950), provides a structural unit whose dynamics can be examined by fluorine NMR spectroscopy. We have previously reported a study of an acylated chymotrypsin (**III**) in which a close analog of fluorophenylalanine formed the acylating group (Gerig and Hammond, 1984). This work showed that the dynamics of enzyme-acyl group interactions in this acylenzyme were such that rotation of the fluoroaromatic ring takes place slowly, if at all. We have now studied an enzyme-product complex formed by interaction of *N*-trifluoroacetyl-4-fluorophenylalanine (**IV**) with α-chymotrypsin and find that the fluoroaromatic ring dynamics in this system are strikingly different from those detected in acylenzyme **III**.



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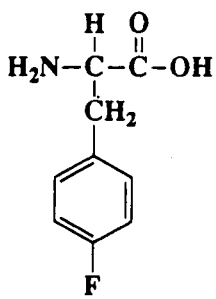

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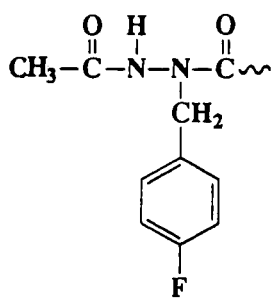

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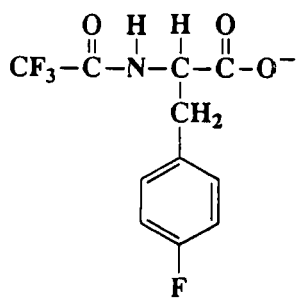

IV

Aspects of the interaction of N-trifluoroacetyl-4-fluorophenylalanine with chymotrypsin have been examined previously (Gammon et al., 1972; Tsavalos et al., 1978; Nicholson and Spotswood, 1978). Those efforts, often focused on observations of the trifluoroacetyl resonance, produced data at magnetic fields corresponding to fluorine Larmor frequencies in the range 56.4 to 94.1 MHz and provided important underpinnings for the present work, which uses observations of the aromatic fluorine signal at higher operating frequencies.

EXPERIMENTAL SECTION

α-*Chymotrypsin* (Type II, Sigma) was purified by exclusion chromatography on Sephadex G-25 according to the procedure described by Wilcox (1970). The appropriate fraction was lyophilized to dryness, dissolved in deuterium oxide (Aldrich, 99.9%), and again lyophilized to exchange solvent-exchangeable protons for deuterons. The exchange procedure was repeated a second time.

Preparation of N-*trifluoroacetyl-4-fluorophenylalanine enantiomers.* Racemic 4-fluorophenylalanine (Calbiochem) was esterified by heating 6 g (33 mmol) to reflux in 60 mL of absolute ethanol containing several drops of sulfuric acid. After careful neutralization with NH_4OH the free base (5.5 g, 81%) was obtained as a white crystalline material and used without further purification.

Resolution of the racemate was accomplished by enzymatic hydrolysis with chymotrypsin according to the procedure of Sheardy et al. (1986). After neutralization, L-4-fluorophenylalanine precipitated from the reaction mixture. The mother liquor was made basic and the ester of the D-isomer was extracted with chloroform and then saponified with 1N NaOH.

Trifluoroacetylation of each enantiomer was accomplished by heating a mixture of the amino acid and 1.1 equivalents of diisopropylethylamine in an excess of ethyl trifluoroacetate (Aldrich). Each trifluoroacetate was crystallized from an ethyl ether/hexanes mixture to give off-white materials. *N*-trifluoroacetyl-D-4-fluorophenylalanine was obtained in 82% yield, mp 137-138°, $[\alpha_D^{25}] = -51.2$ (c = 0.25, ethyl ether), $[\alpha_D^{25}] = -9.6$ (c = 2, methanol) (Lit: mp 134-135° $[\alpha_D^{25}] = -10$ (methanol); Vine et al., 1973) while the corresponding L-isomer was obtained in 73% yield, mp 138-139°, $[\alpha_D^{25}] = +50.4$ (c = 0.25, ethyl ether).

Ring-deuterated 4-fluorophenylalanine was prepared by exchange with deuterium sulfate as described previously (Hiyama et al., 1986). The material was resolved and converted to the corresponding trifluoroacetates following the procedures described above. Mass spectral analysis indicated that the ratios d_0, d_1, d_2, d_3, and d_4 species in the D-isomer were 0.5:1:4.5:3:1, while in the L-isomer these ratios were 1:1:4:3:1. ^1H NMR spectra indicated that deuteration was most extensive at the 3- and 5- ring positions.

NMR samples. All samples were contained in 10 mm od tubes and were made up in D_2O buffered with sodium citrate (0.1 M) at an apparent pH ('pD') of 6.10. The sodium citrate was exchanged twice before use by dissolving in D_2O with subsequent lyophilizing. Sample 'pD' was adjusted by addition of small amounts of 1 N DCl. The 'pD' of a sample was checked before and after an experimental run. Concentration of enzyme in stock solutions was determined by the absorbance at 282 nm using an extinction coefficient of 5×10^4 (Wilcox, 1970). Samples were stored at 4° between experiments and, as judged by fluorine NMR spectroscopy, were stable for 2-3 months.

NMR spectroscopy. Fluorine spectra at 282 MHz were recorded with a Nicolet NT300 or a GE GN300 spectrometer. Experiments at 470 MHz were carried out on a GE GN500 instrument. In all cases, the temperature controllers supplied with the instruments were used to maintain the sample temperature at 35°C. Spin-lattice relaxation times (T_1) were determined using the inversion-recovery method with a composite 180° pulse (Freeman, Kempsell and Levitt, 1980) and phase shifting (Cutnell, Bleich and Glasel, 1976). Experimental data were fit to the appropriate three-parameter function by routines provided with the vendor's software (Levy and Peat, 1975). T_1 data obtained were generally reproducible to better than 5%. $^{19}F\{^1H\}$ steady state nuclear Overhauser effects (NOEs) were determined by comparison of spectra accumulated with and without ^1H irradiation during the pre-acquisition period. An alternating data accumulation (multi-block mode) was used so that the collection of an FID with NOE was obtained immediately after the collection of an FID without NOE, thus minimizing the effects of thermal and instrumental drifts. A delay of at least $10 \times T_1$ was employed between each accumulation. The NOEs reported are believed to be accurate to within 5%. NOE build-up data were obtained by making

the length of the proton irradiation period a variable (Opella et al., 1979); a delay of 10-12 × T_1 was used between scans. The intensities of the fluorine resonance at each irradiation time were compared by integration, using the integral at the shortest irradiation time as the standard. Under the conditions of the build-up experiments peak heights were reproducible to about ±5%. Although the details varied slightly from experiment to experiment, collection of the various fluorine NMR data typically involved 16K data points to represent the FID, exponential apodization that gave a linebroadening of 5-10 Hz, and ±5 kHz spectral windows. Responses of the singlet representing the trifluoromethyl group were not carefully monitored in this work and experiments were generally set up to optimize the detection of the 4-fluorophenyl resonance.

Data analysis. Theoretical line shapes for a system undergoing two-site exchange were generated using a program based on the derivation of Johnson (1965) and run on an IBM-PC. The program used to predict spin-lattice relaxation behavior (T_1, $^{19}F\{^1H\}$ NOEs) was a modernized version of the one described previously (Gerig, Luk and Roe, 1979). The programs for data fitting described below were developed in this laboratory.

RESULTS

The interaction of *N*-trifluoroacetyl-4-fluorophenylalanine with chymotrypsin can be described by the equilibrium

$$EA \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} E + A \qquad (1)$$

where EA, E, and A represent the complex, the free enzyme, and the unbound fluoroamino acid derivative, respectively (At the pH of these experiments A is present as the carboxylate anion; this will not be shown explicitly). Previous fluorine NMR studies of this system have shown that dimerization of the free enzyme is a complication in this scheme and have provided quantitative information about dimerization behavior and the dissociation constants descriptive of equilibrium (1) for the D- and L-forms of **IV** (Gammon, Smallcombe and Richards, 1972). In order to employ the data provided by these previous efforts we have used closely comparable experimental conditions in the present work, including the presence of citrate buffer. The isotopic composition (H_2O/D_2O) of the solvent used in the earlier work is not clear although at least some solvent deuterium must have been present. Since relaxation phenomena were of primary interest for the present study, the solvent was as highly deuterated as could be conveniently achieved. Lastly, the previous work used a sample temperature of 32°C while our work was carried out at 35°C. Thus, a reasonably close congruence of experimental conditions is present but exact equivalence has not been attempted.

In equilibrium (1) the small molecule (A) is exchanged between solvent and enzyme-bound environments. The resonances from a fluorine nucleus on A will depend upon the NMR parameters (chemical shift, relaxation times) that characterize the environment for this nucleus in the bound state (EA) *and* in the free state (A), as well as the rate of exchange of the nucleus between these states. If exchange is rapid enough, experimental determination of a parameter gives a value that is the average of the values characteristic of the free and bound states, the contribution of each being weighted by the fraction of A molecules in that state. How fast exchange must be to achieve

this averaging depends upon the parameter being measured; the requirements for observation of an averaged linewidth are more stringent than the requirements that must be met to have the observed line appear at the averaged chemical shift (Gerig and Stock, 1975). Whether or not an averaged linewidth and line position are observed depends critically on the difference in the chemical shifts (Δ) the spin experiences in each environment. The requirements for fast exchange averaging are progressively harder to meet as the magnetic field strength (B) used to observe the resonance(s) increases, since the shift difference increases with this change (Δ (Hz) = δ (ppm)*B, where δ is the shift difference expressed in ppm).

Bound chemical shifts and linewidths, exchange rates. Samples of *N*-trifluoroacetyl-D (or L)-4-fluorophenylalanine and α-chymotrypsin in D_2O were prepared at a variety of concentration ratios, including some where the concentration of enzyme was in excess of the concentration of small molecule. Fluorine NMR spectra of these samples were obtained at 282 and 470 MHz. To analyze our observations we developed a procedure based on simulation of complete lineshapes, where 'shape' is taken to include both the position of the line and descriptors of its shape. The line position(s) and lineshape(s) will depend on the dissociation constant (K_D = [E][A]/[EA]) for (1), the chemical shift difference between the two environments in the absence of any exchange (Δ), the line widths ($w_{\frac{1}{2}} = 1/\pi T_2^*$) for both the free and bound spins, and the rate constant for dissociation of the complex, k_{off}. A program was prepared that used values for K_D, k_{off}, $w_{\frac{1}{2}(EA)}$, and δ to compute the expected lineshape for a nucleus undergoing two-site exchange; the width at half-maximum intensity and the position of the peak maximum of the computed lineshape were then compared to experimental observations. The values of these parameters were adjusted until optimum agreement between observed and calculated lineshapes, as judged by a least-squares criterion, was achieved for the spectra collected at 282 and 470 MHz. The program included the effects of protein dimerization as elucidated by Gammon, Smallcombe and Richards (1972). Table 1 records the results of this fitting process applied to data obtained with both the D and L forms of **IV** in the presence of the enzyme, while Fig. 1 indicates the agreement achieved between the experimental lineshapes and those computed with the parameters given in Table 1. It was found that the dissociation rate constant (k_{off}) for both systems must be greater than 10^6 s^{-1}, a value large enough to place both systems in the fast exchange regime as regards both chemical shift and relaxation parameters. Given fast exchange conditions, consideration of line position or shape cannot provide more than a lower limit for the dissociation rate constant. Values for the equilibrium con-

TABLE 1
DISSOCIATION OF ENZYME COMPLEXES[a]

	D-Isomer	L-Isomer
K_D, mM	0.53 ± 0.04	3.7 ± 0.2
δ, ppm	−0.79 ± 0.05	−0.98 ± 0.05
$W_{\frac{1}{2}(EA)}$, Hz.	110 (282 MHz) 190 (470 MHz)	17 (282 MHz) 28 (470 MHz)
K_{off}, s^{-1}	> 10^6	> 10^6

[a] From analysis of fluorine spectral data obtained at 282 and 470 MHz.

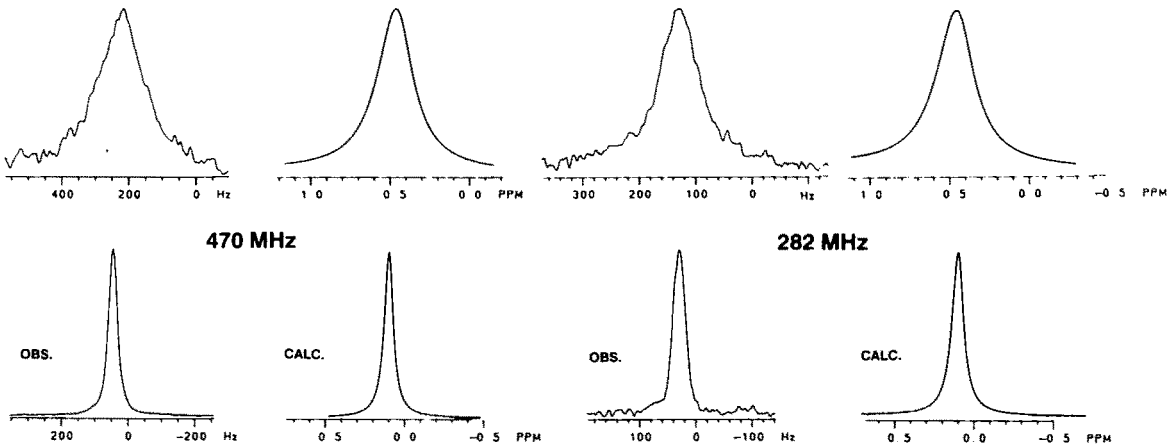

Fig. 1. Comparison of experimental and theoretical lineshapes for complexes of *N*-trifluoroacetyl-4-fluorophenylalanine with α-chymotrypsin at 35°C. The top traces are for complexes of the D-isomer, with the enzyme concentration at 3.8 mM and the fluoroamino acid derivative at 0.2 mM. Under these conditions the fraction of small molecule bound to the enzyme is 0.53. The lower curves are for the L-isomer at the same concentrations of protein and small molecule; because of the larger dissociation constant the fraction of the L-isomer bound is 0.14. Spectra were not proton-decoupled. Calculated curves were prepared as described in the text.

stants (K_D) obtained by the procedure used are in good agreement with those reported by Gammon, Smallcombe and Richards (1972).

These authors found that the change in the chemical shift of the aromatic fluorine is approximately 1.2 ppm downfield within enzyme complexes of either enantiomer. Tsavalos et al. (1978) and Nicholson and Spotswood (1978) reported that this shift for the complex of the D-isomer is -0.88 ± 0.02 ppm under conditions of slightly lower temperature and pH. Our results (-0.79 ± 0.05 ppm for the complex of the D-isomer, -0.98 ± 0.05 ppm for the L-isomer) are in good qualitative agreement with these previous results; that better quantitative agreement has not been achieved is presumably related to experimental errors and the non-identity of experimental conditions. Tsavalos et al. (1978) indicate that the exchange rate for the D-complex is rapid enough that there is no exchange contribution to the observed linewidth at an operating frequency of 84.67 MHz. Our results show that these systems are also in the fast exchange limit at 282 and 470 MHz. However, a substantial broadening of the (averaged) fluorine resonance is observed, especially in the case of the complexes of the D-isomer, and this must be a reflection of the dynamics of the fluoroaromatic ring within the complex EA.

Spin-lattice relaxation, NOEs for bound molecules. Chemical exchange also merges the spin-lattice relaxation behaviors of the free and protein-bound molecules, although the conditions for achieving fast-exchange averaging in this case are different and less restrictive than those required for averaging of peak positions or line widths (Gerig and Stock, 1975; Leigh Jr., 1971; McLaughlin and Leigh Jr., 1973). While there is no dependence of the (averaged) T_1 on magnetic field due to variation in Δ, T_1 of the bound nuclei are expected to be field-dependent because of the long correlation time experienced in the enzyme-bound state. Fluorine-proton nuclear Overhauser effects arise from dipolar interactions with nearby protons. Such protons include those at the 3- and 5- positions of the aromatic ring and, in the bound state, protons attached to

amino acids at the enzyme-small molecule binding site. In the exchanging system defined by (1), the NOEs generated in the bound state (EA) would be expected to dominantly contribute to the observed effect (Borzo and Maciel, 1981; Clore and Gronenborn, 1983; Neuhaus and Williamson, 1989). Because of the strong distance dependence of those interactions that give rise to T_1 and T_2 relaxation effects and the NOE, analysis of these effects can give information about structure and dynamics of the binding site for the fluoroaromatic ring in the enzyme complex.

We determined fluorine T_1 relaxation times, steady state $^{19}F\{^1H\}$ NOEs, and the time dependence of the formation of $^{19}F\{^1H\}$ NOEs at 282 and 470 MHz for the samples used for the lineshape analyses discussed above. In order to interpret these data it is necessary to assume a theoretical model for the system. We adopted the model depicted in Fig. 2 for the protein-bound 4-fluorophenyl group and for this group in the free state as well. As in previous work with a protein-bound 4-fluorophenyl ring (Gerig and Hammond, 1984; Cairi and Gerig, 1983), proton-fluorine dipolar interactions and chemical shift anisotropy (csa) were the only relaxation mechanisms considered for the fluorine nucleus. In each case the correlation time τ_i describes the 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 using F-H distances and angles found in the structure of fluorobenzene (Nygaard et al., 1968). The relaxation due to dipolar interactions with protons of the protein and residual protons in the solvent is 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. The parameters needed to compute the contributions of the csa mechanism were taken from a study of 4-fluorophenylalanine (Hiyama et al., 1986). For systems where the ring protons have been replaced by deuterium the residual protons on the ring were approximated by the inclusion of a single *meta* proton in the calculations. Two regimes for motion of the fluoroaromatic ring were considered. In one, rotation was assumed to be diffusive in nature while a second model involved 180° flips of the aromatic ring. The treatments differ only in the correlation functions used to describe this motion (London, 1980).

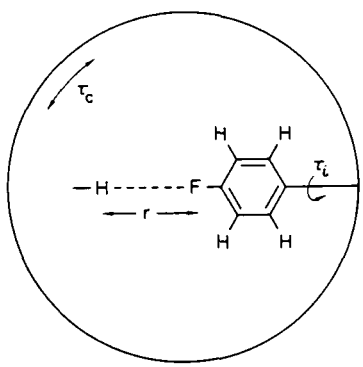

Fig. 2. Model used to analyze the spin-lattice relaxation and Overhauser effect data. The fluorophenyl ring is assumed to rotate either diffusively or in 180° jumps about an axis that is attached to a sphere undergoing diffusive reorientation characterized by a single correlation time τ_c. A single proton at a distance r from the fluorine is used to represent the protons of the enzyme or solvent that might interact with the fluorine.

Analysis of relaxation data for the free molecules (D and L isomers) was done by adjusting the correlation times and r until good agreement with data collected at 282 and 470 MHz was obtained. The values of these parameters giving the best fit are given in Table 2. The diffusive rotation and 180°-flip models for ring rotation required different values for the correlation times and r to fit the observed data, but there was no clear superiority of one model over the other. It would be injudicious to interpret any of these data as being accurately reflective of the dynamics of free **IV** since our goal was simply to find parameters that characterize the relaxation observed for the free small molecule under our experimental conditions. More detailed studies involving specific deuterium labeling or observation of carbon-13 relaxation behavior would be needed before reliable information on the dynamics or anisotropy of motion in this molecule could be obtainable (Gerig, Halley and Reimer, 1977).

It became apparent early on that it would not be possible to find a set of the parameters τ_c, τ_i, and r which would be consistent with the spin-lattice relaxation and NOE data as well as the observed linewidths. It has been frequently observed that fluorine linewidths in protein systems are substantially broader than would be anticipated from spin-lattice relaxation behavior (for examples see Kimber et al. (1977), Post et al. (1984), and Ando, Gerig and Luk (1986)) and we ignored the linewidth data in attempting to analyze the dynamics of these systems. Relaxation of aromatic fluorine of **IV** in the presence of the enzyme was examined by setting up a program to compute T_1 relaxation behavior and time dependence of $^{19}F\{^1H\}$ NOE formation using the model system described above. The program assumed that spins of the free molecule (A), tumbling with the correlation times given in Table 2, are in exchange with spins attached to a protein-bound molecule (EA), with a rate of exchange sufficient to give fast exchange conditions. The correlation times τ_c, τ_i, and the distance r for the bound spins were adjusted until, by the least-squares criterion, the agreement between experimental T_1 fluorine magnetization recovery curves, $^{19}F\{^1H\}$

TABLE 2
PARAMETERS DESCRIBING RELAXATION OF **IV**[a]

	Diffusive rotation			180° Ring flips		
	τ_c,ns	τ_i,ns	r,nm	τ_c,ns	τ_i,ns	r,nm
Free						
D- or L-Isomer	0.021	0.16	0.207	0.024	0.16	0.218
Ring deuterated	0.038**	0.16	0.252	0.038**	0.16	0.252
Enzyme-bound						
D-Isomer	16.	0.12	0.244	12.*	0.15*	0.320
L-Isomer	5.5	0.38*	0.247	5.7	0.67	0.294
D-Isomer, Ring deuterated	12.**	0.29	0.285	10.**	0.29	0.288
L-isomer, Ring deuterated	7.6**	0.35	0.259	6.7**	0.15	0.260

[a] Parameters marked ** could be varied ±20% without producing significant degradation of the least-squares fits. Parameters marked * could be varied ±10% before significant reductions in quality of fits were observed. Parameters that are not marked could not be varied more than ±5% from the values given without noticeable effects on the quality of the agreement between experimental and calculated data.

NOE build-up curves, and steady state NOEs, and the theoretical values for these quantities, calculated from the model was maximized. This computationally-intensive process considered experimental results obtained at 282 and 470 MHz for samples providing several different fractions of bound **IV** and was guided by a SIMPLEX algorithm (Becsey, Berke and Callan, 1986). The fitting procedure was applied to complexes of D- and L-forms of **IV** and to complexes of both enantiomers of **IV** in which the majority of the aromatic ring hydrogen atoms had been replaced by deuterium. The intent of the deuteration in the latter systems was to reduce the contributions of dipolar interactions between the aromatic fluorine and adjacent aromatic ring protons in order to enhance the influence of interactions with protons of the protein on fluorine relaxation.

The correlation times and r values that fit the experimental data are given in Table 2. Data sets for a given sample consisted of 30-70 experimental points and the points were generally fit with an rms agreement of 2-4%. Figure 3 indicates graphically the agreement with experiment that was achieved by this process. It should be borne in mind that the experimental data have some unavoidable inaccuracies. For the enzyme-containing systems, especially those involving the D-isomer of **IV**, the line widths of the fluorine signal observed are broad so that, at the signal-to-noise ratios achievable within practical experimental times, determination of peak intensities or integrals has limited accuracy; in those parts of an experiment where the signal strengths are high (at the beginning or end of an inversion-recovery sequence, or at the beginning of a NOE build-up curve) it is estimated that the experimental uncertainty is roughly $\pm$ 5%, but it is probably larger than this in the phases of these experiments where signal strength is less than the maximum. In this light, the agreement between observed and calculated fluorine signal intensities is good and tends to substantiate the model used.

The samples containing the L-isomer of **IV** showed small steady state NOEs ($+0.04$ to -0.10) depending on the fraction bound and the magnetic field strength. These NOEs were so small that reliable NOE build-up data could not be collected and the fitting process for the complexes of the L-isomer involved only inversion-recovery (T_1) and steady state fluorine-proton NOE data.

It should also be noted that the fitting procedure would occasionally explore regions of the parameter space that contained absurd values for a given parameter. For some combinations of parameters the calculations would have to be halted because of the limitations imposed by the number of significant digits the computer could represent. There appeared to be many local minima in the least-squares agreement function used and we made arbitrary choices to ignore some of these and 'push' the fitting process in a particular direction, keeping in mind that the values for the correlation times and r found would have to be 'reasonable', particularly as regards the value for the overall correlation time of the protein τ_c. Values of τ_c that were considered credible were those near the known correlation time of the enzyme (about 15 ns) or the expected correlation time for the enzyme dimer (about 30 ns) (Vaz and Schoellmann, 1976). Values for τ_i and r were allowed to vary over a much wider range. Thus, the parameters given in Table 2 are not the result of an exhaustive global search over a wide range for each parameter but rather, for each system, are values that characterize a local minimum, are of a reasonable magnitude, and give a good representation of the (imperfect) experimental data.

As can be seen from Fig. 3 the modeling process used is less successful with data from the ring-deuterated materials. It should be recalled that the experimental data used in these cases arises from a mixture of partially deuterated molecules. Each member of this set will have different relaxation behaviors but what is measured is only an aggregate response. For reasons of economy

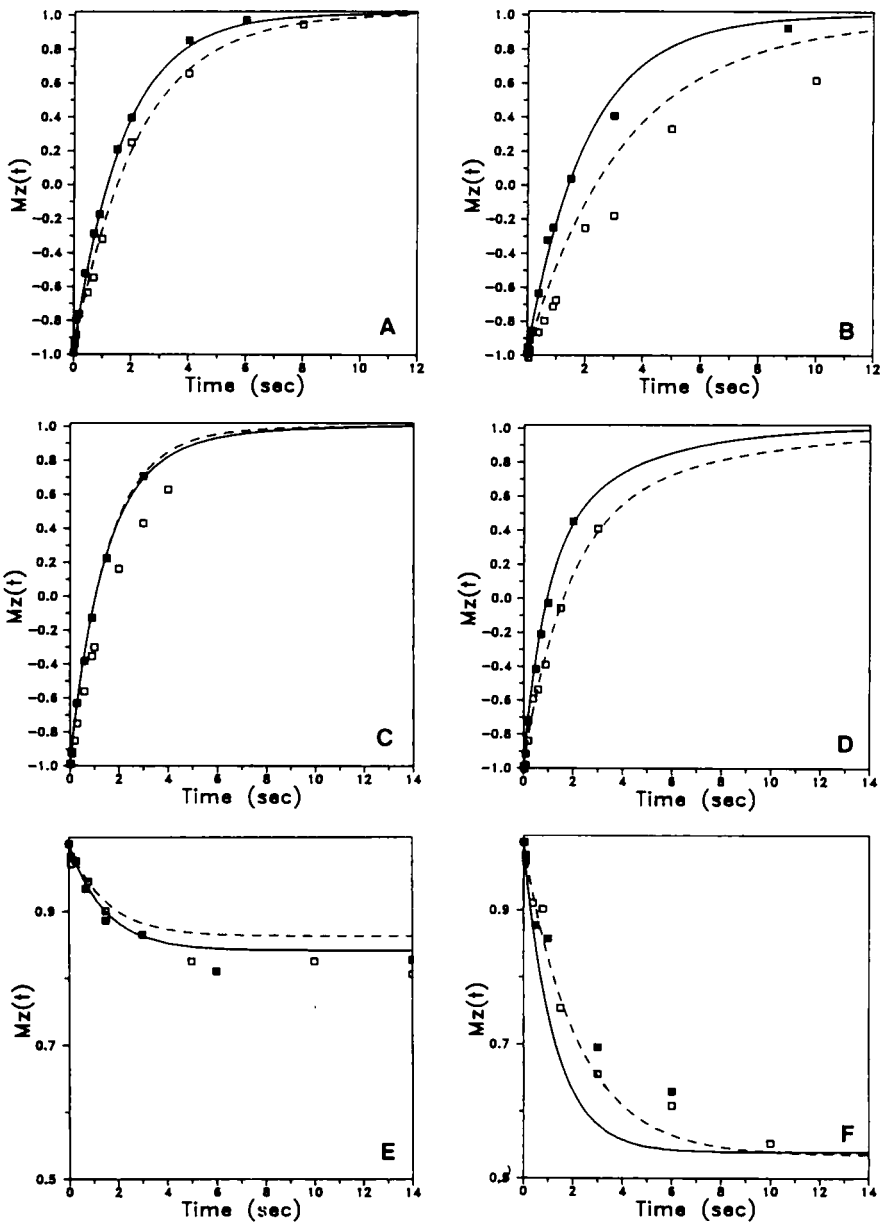

Fig. 3. Comparison of experimental and theoretical relaxation data for complexes of *N*-trifluoroacetyl-4-fluorophenylalanine with α-chymotrypsin. (A) Inversion-recovery T_1 experiment at 470 MHz with a sample containing 3.8 mM enzyme and 0.2 mM of the L-isomer; (B) same experiment with the same sample at 282 MHz. (C) Inversion-recovery T_1 experiment at 470 MHz with a sample containing 3.8 mM enzyme and 0.2 mM of the D-form of the small molecule; (D) same experiment at 282 MHz; (E) formation of the $^{19}F\{^1H\}$ NOE after start of proton irradiation at $t=0$ for the same sample at 470 MHz; (F) NOE formation at 282 MHz. The $^{19}F\{^1H\}$ NOE with samples involving the L-isomer was so small that the time dependence of its formation could not be reliably determined. In each panel the experimental data are indicated by the square symbols and the lines are those predicted using the parameters given in Table 2. The solid lines and symbols are for systems with all aromatic protons of **IV** present while the open symbols and dashed lines correspond to the form of **IV** in which most of the aromatic protons have been replaced by deuterium.

only a single, partially deuterated model system was used to represent this collection. We also assumed that the binding constants for the deuterated forms of **IV** are the same as those for the normal isotopomers, although there are examples known where placement of deuterium in an aromatic ring produces significant isotope effects on the binding of small molecules to proteins (Cherrah et al., 1988). In this light the results obtained with the deuterated systems are in reasonable agreement with the conclusions from analysis of the all-proton systems.

Table 2 contains the results of fitting the experimental data using both a model for the bound fluoroaromatic ring in which rotation of the ring was diffusive and a model in which this ring flips in 180° increments. Although the parameters that characterized each fit are different for the two models, the agreement between experimental and calculated data was virtually the same for each data set and there appeared to be no basis on which to decide which of these models is most appropriate for description of the fluoroaromatic ring motion at the enzyme binding site.

Relaxation parameters for bound species. The correlation times and r values found in the analysis of the T_1 and NOE data (Table 2) lead to the computed relaxation times and steady-state NOEs for the fluorine of enzyme-bound **IV** shown in Table 3. The predicted aromatic fluorine linewidths at each observation frequency computed for the bound fluorine are much smaller than those that were defined by the lineshape, indicating that additional mechanisms contribute to the linewidth of the bound aromatic fluorine.

DISCUSSION

The pioneering studies of the interaction of *N*-trifluoroacetyl-4-fluorophenylalanine with chymotrypsin by Gammon, Smallcombe and Richards (1972), focused primarily on enzyme-induced changes in the resonance frequency of the trifluoromethyl group. They noted a downfield shift of 0.69 ppm of this signal when the D-isomer was bound to the enzyme, while a shift of less than 0.05 ppm downfield was detected when the L-complex formed. With the assumption of rapid exchange between the free and protein-bound forms of the fluoroamino acid derivative the shift effects ob-

TABLE 3
COMPUTED RELAXATION PARAMETERS FOR BOUND **IV**

	282 MHz	470 MHz
D-Isomer[a]		
T_1,s	0.70	0.97
$W_{\frac{1}{2}(EA)}$,Hz	2.9	3.7
$^{19}F\{^1H\}$NOE	−0.61	−0.34
L-Isomer[b]		
T_1,s	0.52	0.50
$W_{\frac{1}{2}(EA)}$,Hz	1.6	2.0
$^{19}F\{^1H\}$NOE	−0.41	−0.28

[a] Calculated using $\tau_c = 14.0$ ns, $\tau_i = 0.12$ ns, r = 0.244 nm.
[b] Calculated using $\tau_c = 6.0$ ns, $\tau_i = 0.38$ ns, r = 0.247 nm.

served could be used to (1) show that only the monomeric form of the enzyme bound either enantiomer of **IV**, (2) provide a quantitative description of dimerization and (3) determine the pH dependence of dissociation and dimerization constants. Because our data for the same systems were collected at higher operating frequencies, the conditions for fast exchange averaging of NMR parameters such as chemical shifts and linewidths could be more difficult to meet. However, analysis of our results confirms the dissociation constants obtained previously by Gammon, Smallcombe and Richards (1972), and shows that rapid exchange conditions are extant even at 470 MHz.

Collection and analysis of spin-lattice relaxation data were carried out to provide further information about the structure and local dynamics of **IV** as it is bound to the enzyme. Regardless of system examined or model chosen for analysis of data the overall correlation time τ_c for the fluoroaromatic ring in the complex of the D-isomer of **IV** is about 14 ns, a value in agreement with the results from ESR and fluorescence studies (Vaz and Schoellman, 1976), as well as values for τ_c found for the 4-fluorophenyl group in the acylchymotrypsin **III** (Gerig and Hammond, 1984) or for the 4-fluorophenyl group in the 4-fluorocinnamic acid complex of chymotrypsin (Gerig, Halley and Ortiz, 1977). The correlation time (τ_i) used to characterize internal rotation of the aromatic ring in this complex is about 0.2 ns whether rotation is considered to be diffusive or to take place by 180° ring flips. In complexes of the L-form of **IV** with the enzyme the overall correlation time for the ring (5.5 ns) appears to be smaller than it is in the D-complex, but the rate of ring rotation ($\cong$ 0.4 ns) appears to be slower than observed for the complex of the D-isomer. It is recognized that the structural model used is crude, but it gives theoretical relaxation behavior that is in good agreement with experimental data for the full proton model (Fig. 3) and indicates that either enantiomer of **IV**, upon entering the enzyme binding site, enters a structure that places the fluoroaromatic ring into about the same environment, with similar local dynamics. Protein-induced chemical shift effects are usually dominated by van der Waals interactions with surrouding nuclei (Gregory and Gerig, 1990; Rummens, 1975) and these effects are strong functions of internuclear proton-fluorine distances in proteins. The same fluorine-proton interactions that produce the chemical shift effects also lead to fluorine dipolar relaxation and a correlation between shifts and T_1 relaxation times has been noted (Hull and Sykes, 1976). Thus, the high similarity of the r parameters for both the D- and L-complexes and the aromatic fluorine chemical shifts in the complexes of **IV** with the enzyme is consistent.

There is no indication from our results, which pertain to the behavior of the aromatic ring, of the reason(s) why the D-isomer forms stronger complexes. The difference in the dissociation constants likely arises because the rate of dissociation of the D-complex is slower. If the local environments of the fluoroaromatic ring are similar in complexes of both the D- and L-forms of **IV**, the reduced rate of dissociation must be the result of interaction between those parts of the structure of **IV** that lie beyond the aromatic ring. With the fluoroaromatic rings constrained to the same binding region, the nature of the stereochemistry of the remainder of **IV** demands that the remaining groups attached to the α-carbon have different interactions with the enzyme in each complex. The much larger enzyme-induced shift effect on the trifluoroacetyl group of the D-isomer, presumably the result of van der Waals effects on shielding, indicates that appreciable CF_3-enzyme interactions are present in this system and the structure of the D-complex may be such as to place the hydrophobic CF_3 group into a position where interactions with the enzyme lead to some stabilization of this complex relative to the complex formed with the L-isomer, slowing the rate of dissociation and simultaneously producing the observed downfield shift of the

CF_3 resonance. Consistent with this notion is the observation that, under the same experimental conditions as used for the present work, *N*-trifluoroacetyl-D-4-fluorophenylalanine binds to chymotrypsin slightly better than the corresponding *N*-acetyl compound presumably because of the greater hydrophobicity of the CF_3 group relative to CH_3 (Tsavalos et al., 1978; Nicholson and Spotswood, 1978). Molecular mechanics studies of the type that has been applied to *N*-acyltryptophan complexes of chymotrypsin might suggest the locus of CF_3 binding and illuminate the energetics of binding in these complexes, as well as the cause(s) of the fluorine shift effects (Wipff et al., 1983).

Rotation of the fluoroaromatic ring in the acylchymotrypsin **III** was found to be characterized by a correlation time $> 10^4$ ns, a rate so slow that it could not be quantitated reliably by the relaxation experiments performed (Gerig and Hammond, 1984). There is, thus, a striking difference in the dynamics of the fluoroaromatic ring in this acylenzyme compared to the ring dynamics in the enzyme-product complexes studied here. Aromatic rings inside proteins must sweep out a volume containing a number of atoms in order to undergo rotation. The time scale for movements of neighboring atoms to open collectively a large enough void for ring rotation to be possible is typically in the range 10^5-10^9 ns (McCammon and Harvey, 1987; Williams, 1989), much too slow to permit rotations at the apparent rate observed for the 4-fluorophenyl ring in the **IV**-chymotrypsin complexes studied. Since there is no covalent bond permanently attaching *N*-trifluoroacetyl-4-fluorophenylalanine to the protein in the complex, it seems likely that the observed aromatic ring rotation takes place by a mechanism involving departures of the small molecule far enough from the enzyme binding site for ring rotation to become feasible. This 'partial dissociation' must take place at least 10^{10} times per second, with about every 10^4 such events having a trajectory sufficiently unencumbered that the small molecule becomes truly dissociated from the protein binding site and joins the pool of unbound molecules present in the medium. In making these excursions the small molecule must remain closely enough associated with the protein that the overall correlation time (τ_c) of the small molecule remains that of the protein.

The correlation times and internuclear distances that are consistent with the spin-lattice relaxation and NOE data obtained at two operating frequencies predict linewidths for the signal from the bound aromatic fluorine that are much smaller than those observed experimentally and there must be processes at work that lead to very significant additional linebroadening beyond that expected from fluorine-proton dipolar interactions and fluorine chemical shift anisotropy effects. A working hypothesis is that the observed excess fluorine linewidths are the result of exchange contributions from processes that place the fluorine nucleus into a set of structurally and dynamically similar but not identical protein environments or substates that have different chemical shifts. Dissociation from these states could be rapid but interconversion between them may be slow enough that the family of bound chemical shifts is incompletely averaged. The partial dissociation process suggested earlier could be consistent with this hypothesis if intermediate stages along the dissociation path are characterized by different chemical shifts. That the observed linewidths are invariably strongly dependent on the operating frequency is consistent with the suggestion that exchange processes are responsible for the observed broadenings. To the extent that this hypothesis is correct it appears that either the range of chemical shifts the aromatic fluorine is exposed to in the complexes of the L-form of **IV** is smaller than is the case in the D-complexes or else the rate(s) of exchange between the possible environments is faster in the L-complexes so that shift averaging is more complete, or both.

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