used for locking except when this signal was minimized in order to observe the signals from the X-proton of the inhibitor. In this latter instance 1% *tert*-butyl alcohol was used for a lock signal. A Varian C-1024 time-averaging computer was utilized for signal-to-noise enhancement. Samples were allowed to equilibrate to probe temperature (~34°) for about 1 hr before spectra were recorded, otherwise substantial drifts of peak positions were noted. Spectra were generally taken by accumulating three or more scans on the C-1024 at a sweep rate of 0.1 Hz/sec, a sweep width of 100 Hz, and a frequency response setting of 0.5. Spectral peak positions are believed to be accurate to at least 0.2 Hz.

Computer simulations were carried out with an IBM 360/75 computer interfaced to a Houston plotter and utilized a local version of the Ferguson–Marquardt program. Line-width determinations made by matching computed to experimental line shapes are estimated to be accurate to ~1 Hz.

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Nuclear Magnetic Resonance Studies of the Interaction of *N*-Trifluoroacetyltryptophanate with α-Chymotrypsin

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Abstract: The interaction of the D and L enantiomers of *N*-trifluoroacetyltryptophan with α-chymotrypsin has been studied by high-resolution proton and fluorine nuclear magnetic resonance techniques at apparent pH 6.2 in deuterium oxide or at pH 6.6 in normal water. Protein-induced chemical-shift and line-width effects in the pmr spectra of these inhibitors are similar to those found with the corresponding *N*-formyl derivatives. There is virtually no effect of enzyme on the chemical shift or line width of the fluorine resonance of the L isomer while a large downfield shift and some line broadening is observed with the D inhibitor. After consideration of the possible effects of enzyme oligomerization, these results are discussed with reference to suggested structures for the enzyme–inhibitor complexes that are similar to those found for the *N*-formyltryptophans.

A covalently bound fluorine atom is similar in size to a comparably attached hydrogen atom. Biochemists have made use of this fact to produce fluorinated substrates and inhibitors of enzymatic systems that are presumably nearly isosteric with the corresponding hydrogen-substituted compounds but which have distinctly different electronic properties. The result has often been a dramatic change in the properties of the biological system under investigation.[2-4] The introduction of fluorine atoms into an enzyme or an inhibitor of an enzyme offers several advantages when the system is to be investigated by nmr spectroscopic techniques. When observing the fluorine-19 nmr spectrum these include (1) enhanced chemical shift effects, (2) a spectrum that is unobscured by the multitudinous proton resonances of the protein, and (3) relaxation (or line width) effects that are somewhat diminished because of the smaller gyromagnetic ratio of the fluorine nucleus. Thus, fluorine nmr spectroscopy has been used to examine the Michaelis complexes formed between enzymes and inhibitors[5-8] or enzymes that have been chemically modified so that fluorine nuclei are covalently attached to the protein structure itself.[9,10] An important consideration in drawing conclusions from experiments of this nature is the extent to which the fluorine substitution has perturbed the system relative to the corresponding system which contains no fluorine. In an effort to illuminate this point and as an extension of previous work,[11] we have examined the interaction of *N*-trifluoroacetyl-D- and -L-tryptophan with the proteolytic enzyme, α-chymotrypsin. The results are described below; a subsequent paper will deal with similar experiments using the corresponding acetyl derivatives.

Results

The pmr spectrum of *N*-trifluoroacetyltryptophan (I) is quite similar to that found for tryptophan[11a] and *N*-formyltryptophan[11b] at similar solution acidities. The various regions of the pmr spectrum of I, recorded at 100 MHz and a concentration of I of 40 m*M* in 0.4 *M* phosphate buffered deuterium oxide solution (apparent pH 6.2), were analyzed by employing the Ferguson–Marquardt program;[12] the resulting chemical-shift and coupling-constant data are collected in Table I. There is no evidence in the proton spectrum of I for a second conformational isomer as was observed in the

(1) U. S. Public Health Service Predoctoral Fellow, 1968–1971. This work will form a portion of the Ph.D. thesis of R. A. R.

(2) (a) P. Goldman, *Science*, **164**, 1123 (1969); (b) R. M. Hochster and J. H. Quastel, Ed., "Metabolic Inhibitors," Vol. I, Academic Press, New York, N. Y., 1963, Chapters 4 and 6.

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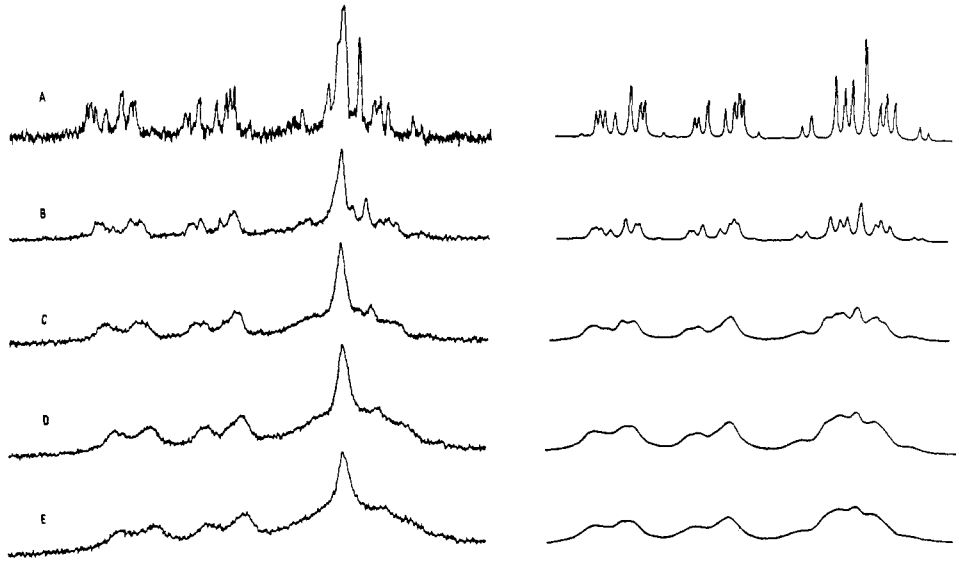


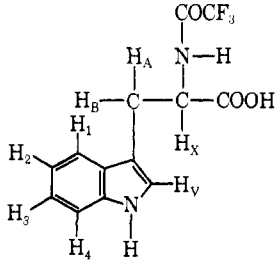
Figure 1. The aromatic proton portion of the pmr spectrum of *N*-trifluoroacetyl-L-tryptophan in the absence (trace A) and presence of α-chymotrypsin. The ratio of concentrations of enzyme to inhibitor was 0.028, 0.053, 0.077, and 0.101 for traces B through E, respectively. Each experimental scan on the left side is 100-Hz wide. The theoretical spectra (right) were calculated using the chemical shifts and coupling constants in Table I and a line-width parameter of 0.5, 1.2, 2.5, 3.5, and 4.0 Hz, respectively.

Table I. Nmr Spectral Parameters of *N*-Trifluoroacetyltryptophan[a]

δ, ppm	J, Hz
A. Aromatic protons[b]	
δ_1 −5.7750	J_1 = 7.77
δ_2 −5.2323	J_{13} = 1.37
δ_3 −5.3111	J_{14} = 0.86
δ_4 −5.5679	J_{23} = 7.09
δ_V −5.297	J_{24} = 1.21
	J_{34} = 7.98
B. Alkyl protons[b]	
δ_A −1.2729	J_{AX} = 8.68
δ_B −1.5373	J_{BX} = 4.70
δ_X −2.6922	J_{AB} = −14.80
	J_{AV} = 0.6
	J_{BV} = 0.8
C. Trifluoromethyl group[c]	
δ_F 0.250	J_{XF} = ∼0.5

[a] Sample was 0.04 *M*, in 0.4 *M* phosphate buffer in 95% D_2O, apparent pH 6.2. [b] Chemical shifts (δ_i, in ppm) were measured relative to 0.005 *M* sodium acetate included in sample as an internal reference. Coupling constants (J_{ij}) are in hertz. The root mean square error estimated by the computer program was ±0.004 ppm for the chemical shifts and ±0.05 Hz for coupling constants. J_{AV} and J_{BV} have estimated uncertainties of ±0.1 Hz. [c] The chemical shift (δ_F) was measured relative to 0.005 *M* sodium trifluoroacetate included as an internal reference. Fluorine experiments were done at 94.1 MHz.

case of the *N*-formyl derivative.[11b] Flourine spectra were taken at 94.1 MHz and exhibited only a single resonance, split into a doublet by spin–spin coupling to the tertiary proton, H_X.



I

When the enzyme α-chymotrypsin is added to samples of the inhibitor a number of spectral changes occur. As was observed with the *N*-formyl derivative the aromatic proton resonances are shifted upfield and broadened considerably as increasing concentrations of protein are introduced into the solutions. These changes are illustrated in Figure 1. In order to obtain a quantitative indication of the extent of enzyme-induced line-width changes, computer-generated theoretical spectra were prepared using various line widths for the shape function and compared visually to the experimental curves until a reasonable match was found. Chemical-shift effects were quantitated by determining the position of the major bands in each multiplet. In both cases, it has been tacitly assumed that the coupling constants are not greatly changed when protein is present in the samples.

The upfield, aliphatic region of the pmr spectra of I showed smaller protein-induced chemical shift effects but larger line-width variations than the aromatic portion. Figure 2 records some typical results; these spectra were analyzed for enzyme-derived changes in the same manner as described above.

For determination of the changes brought about in the fluorine-19 spectrum of I by added enzyme, a trace of sodium trifluoroacetate (∼0.005 *M*) was usually added to provide a chemical-shift and line-width standard. The signal from a capillary of trifluoroacetic acid provided a spectrometer lock signal. It was consistently found by comparing data obtained relative to the internal and external references that the inclusion of protein in the inhibitor solutions results in a downfield shift relative to the external reference that is not due to protein–inhibitor interaction. The effect was present when modified, catalytically inactive enzymes were used and appeared to vary approximately linearly with the concentration of protein present; it is most reasonably assigned to a volume magnetic susceptibility change due to the presence of protein.

In analyzing the proton chemical-shift and line-width data, we have assumed that the interaction of I

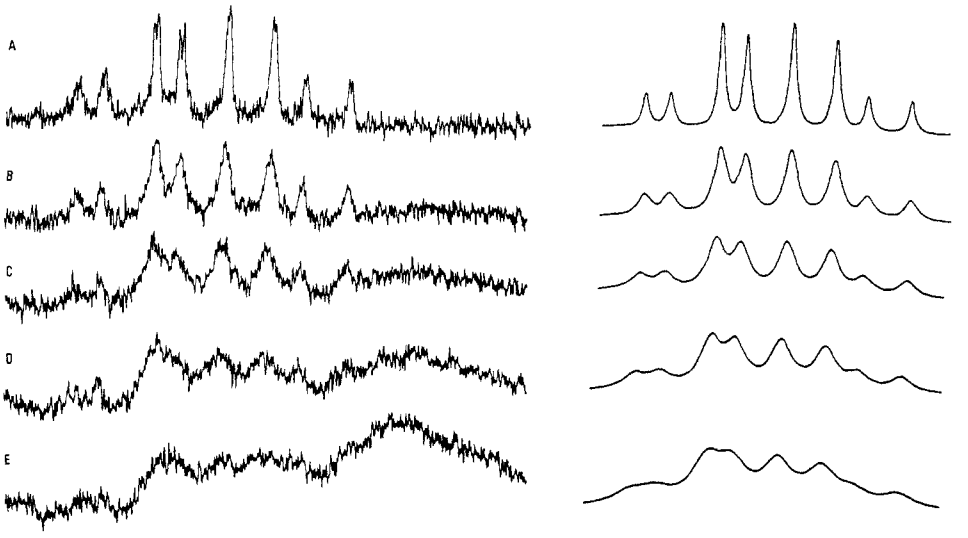


Figure 2. The AB (alkyl) part of the pmr spectrum of *N*-trifluoroacetyl-L-tryptophan under the same conditions as those used for the spectra reported in Figure 1. The line-width parameters for the theoretical spectra were 1.5, 2.8, 4.0, 5.0, and 7.0 Hz, respectively.

with α-chymotrypsin can be represented by the simple equilibrium

$$E + I \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} EI \qquad (1)$$

where E, I, and EI refer to the enzyme, the inhibitor, and the enzyme–inhibitor complex, respectively. If the initial concentration of I is large enough, the dissociation constant (K_I) characteristic of eq 1 small enough, and the exchange rates fast enough, then the effect of the association of I with the enzyme is described by

$$x = \frac{E_0}{I_0}\chi \qquad (2)$$

where x refers to the observed change in an nmr parameter relative to its value in the absence of protein, χ, its value in the EI complex relative to that found in free solution, and the subscripts indicate the initial concentration of the reactants.[11b] Within the limitations listed above, this empirical equation is useful for treating data that are scattered by random experimental errors in that a plot of x *vs.* E_0/I_0 is linear with a slope equal to χ and an intercept of zero. Calculations showed that for the present experiments the assumption of enzyme saturation by the inhibitor led to an error of less than 5% in χ if K_I = 1 m*M*. The proton chemical-shift and line-width effects induced by the native enzyme, measured as described above, were fit to eq 2 by a least-squares procedure to afford the data listed in Table II. In order to assess the importance of inhibitor binding to loci on the enzyme other than the active center, similar experiments were made with the serine-195 tosyl ester of chymotrypsin;[13] these data are collected in Table III. Spot checks made with chromatographically purified tosyl enzyme showed insignificant differences between data obtained with this material and with unpurified protein.[14]

(13) (a) D. E. Fahrney and A. M. Gold, *J. Amer. Chem. Soc.*, **85**, 997 (1963); (b) A. M. Gold, *Biochemistry*, **4**, 897 (1965).

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Table II. Pmr Spectral Changes Induced by Native α-Chymotrypsin[a]

Proton	Chemical shifts, ppm[b] L isomer	Chemical shifts, ppm[b] D isomer
H_1	0.57 ± 0.02	0.42 ± 0.02
H_2	0.67 ± 0.02	0.82 ± 0.05
H_3	0.55 ± 0.01	0.80 ± 0.05
H_4	0.34 ± 0.01	0.40 ± 0.01
H_V	0.02 ± 0.01	0.08 ± 0.02
H_A	−0.12 ± 0.02	0.15 ± 0.02
H_B	0.01 ± 0.01	−0.07 ± 0.01
	Line widths, Hz	
H_1–H_4	36 ± 2	40 ± 2
H_A,H_B	49 ± 3	56 ± 6

[a] At 34°, pD 6.6, 0.4 *M* phosphate buffer. Data were fit to eq 2 by least squares; the estimated standard deviation of a slope follows each value. [b] A negative sign indicates a downfield shift.

Table III. Pmr Spectral Changes Induced by Tosyl-α-chymotrypsin[a]

Proton	Chemical shifts, ppm L isomer	Chemical shifts, ppm D isomer
H_1	0.24 ± 0.06	0.21 ± 0.02
H_2	0.27 ± 0.03	0.25 ± 0.01
H_3	0.31 ± 0.03	0.31 ± 0.01
H_4	0.22 ± 0.01	0.24 ± 0.02
H_V	0.01 ± 0.03	0.03 ± 0.03
H_A	0.01 ± 0.04	−0.03 ± 0.03
H_B	0.04 ± 0.05	0.05 ± 0.02
	Line widths, Hz	
H_1–H_4	13 ± 1	12 ± 1
H_A,H_B	21 ± 5	20 ± 6

[a] At 34°, pD 6.6, 0.4 *M* phosphate buffer.

Because the nmr signal is virtually a singlet and is not as extensively broadened by the enzyme, somewhat more data were collected to document the influence of protein binding on the fluorine-19 signals from the trifluoromethyl group of I. In treating the fluorine shift

data, use was made of the expression

$$x = \frac{[EI]}{I_0}\chi \qquad (3)$$

where [EI] is given by an expression that is dependent upon the particular model for enzyme polymerization and enzyme–inhibitor interaction chosen. As will be discussed below two models were considered in this work: (1) the formation of enzyme dimers and trimers occurs with the monomer, dimer, and trimer of the protein capable of binding one, two, or three molecules of inhibitor at sites which exert the same chemical shift effect or (2) enzyme dimerization and trimerization occur but only the monomer is capable of binding the inhibitor. Equation 3 still is predicted on a fast-exchange situation. A computer program was devised to fit eq 3 to the fluorine-shift data; in all cases it was found that the chemical-shift data were much better fit by this procedure if the correlation line was not forced through zero. As indicated in Figure 3, with both the D and L isomers of I in the presence of native and tosylated chymotrypsin, it was found that the intercept (extrapolated chemical shift effect, χ, when $E_0 = 0$) was about -1 Hz. These coincidences seemed to be too frequent to be fortuitous and suggest that a protein may rather nonspecifically induce small fluorine chemical-shift effects simply by being present in the aqueous medium. In preliminary experiments with I and other proteins and nonionic detergents, we have observed small fluorine chemical-shift effects of a similar magnitude and in the same direction. It is possible that introduction of a large, solvent-reordering macromolecule produces a small but measurable solvation effect on fluorine chemical shifts sufficient to explain this observation.

The equilibrium constants obtained from analysis of the chemical-shift data were used in determining the enzyme-derived effects on the fluorine line widths. The resulting line-width data, as well as data for the chemical-shift effects, are collected in Table IV.

Table IV. Fluorine Magnetic Resonance Changes Induced by Native and Tosylchymotrypsin[a]

	Native	Tosyl
N-Trifluoroacetyl-L-tryptophan		
Chemical shift, ppm	-0.034 ± 0.05	-0.025 ± 0.01
Line width, Hz	1.4 ± 0.1	0.6 ± 1
K_I, m*M*	*b*	*c*
N-Trifluoroacetyl-D-tryptophan		
Chemical shift, ppm	-1.03 ± 0.01	-0.08 ± 0.05
Line width, Hz	20 ± 4	0.6 ± 1
K_I, m*M*	0.9	*c*

[a] At 34°, pH 6.6 in water, 0.4 *M* phosphate buffer. [b] Not considered reliable because of the small range of chemical shift effects found; values of K_I from 0.1 m*M* to 2 m*M* gave essentially the same value for the chemical shift. [c] Not enough data were obtained to reliably evaluate.

Discussion

The coupling constants and chemical shifts found for *N*-trifluoroacetyltryptophan are quite similar to those previously reported for *N*-formyltryptophan;[11b] this similarity provides good evidence that the two molecules are conformationally nearly identical. The long-range coupling between the trifluoromethyl group and the tertiary proton is not unprecedented,[15] but there does not appear to be enough information available to permit unambiguous interpretation of the magnitude of this coupling constant in terms of preferred rotational isomers at the trifluoroacetamide bond. It is likely that the rotamer Ia would be the favored one on steric grounds and we tentatively conclude that structure Ia represents the majority of the *N*-trifluoroacetyltryptophan molecules in solution under the conditions of these experiments.

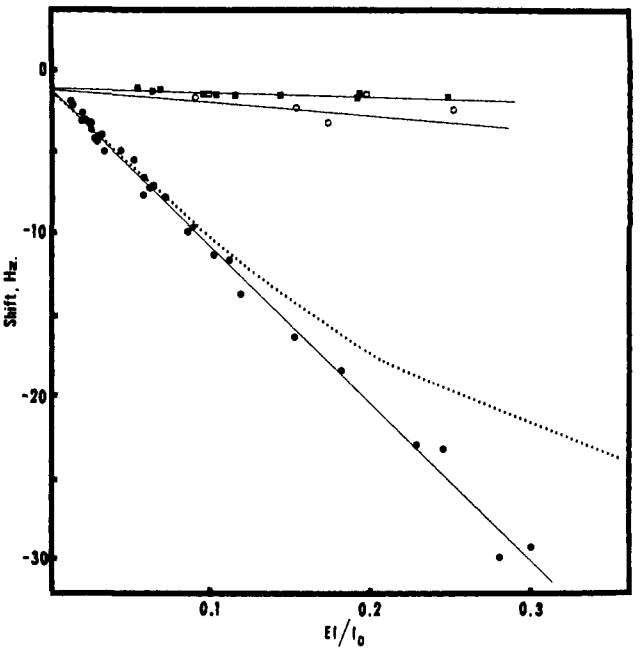
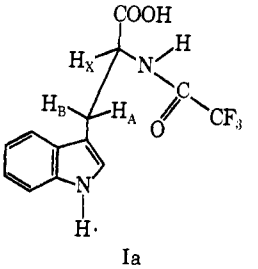


Figure 3. Fluorine-19 chemical-shift data for *N*-trifluoroacetyl-L-tryptophan in the presence of native α-chymotrypsin (solid squares) and tosylchymotrypsin (open squares) and for *N*-trifluoroacetyl-D-tryptophan with the native (solid circles) and tosyl (open circles) forms of the enzyme. A binding constant of 0.9 m*M* was used to estimate the EI concentration for the D isomer while a value of 0.5 m*M* was used for the L material. The polymerization of α-chymotrypsin was assumed to be described by the dimerization and trimerization constants of Rao and Kegeles.[20] The solid curves are those obtained by assuming that only the enzyme monomer binds the inhibitor. The dotted line represents a plot of observed shift *vs.* mole fraction of total binding sites computed under the assumption that all oligomeric forms of the enzyme bind the inhibitor equivalently. The abscissa is either the mole fraction or monomeric enzyme–inhibitor complex or the mole fraction of total (equivalent) binding sites on all enzyme forms.



Ia

In order for our treatment of the enzyme-induced effects on the nmr parameters of I to have validity it is

(15) (a) Y. A. Cheburkov, *et al.*, *Dokl. Akad. Nauk SSSR*, **169**, 128 (1966); (b) M. T. Rogers and J. C. Woodbrey, *J. Phys. Chem.*, **66**, 540 (1962).

necessary that the exchange of I between the enzyme-bound and free states be sufficiently rapid to average the corresponding parameters for both states. Preliminary pulsed fluorine nmr experiments indicate that k_1 for the equilibration involving the D isomer of I is at least 7×10^3 sec^{-1},[16] a value that is not unreasonable in view of previous stopped-flow and temperature-jump experiments with similarly constituted molecules.[17,18] For fast-exchange averaging of chemical shifts to obtain, the rate constants for the exchange process must be about an order of magnitude greater than $2\pi\nu$, where ν represents the chemical shift difference (in hertz) between bound and free states.[11b,19] The observed exchange rate for the D isomer fulfills this condition.

Exchange broadening in addition to enzyme-induced relaxation effects can contribute to the line-width effects observed. It has been shown that, to a first approximation, the slope of a plot of the observed line-width effect (W_{obsd}) against the mole fraction of enzyme-bond inhibitor (P_{EI}) is

$$W_{obsd} = W_{EI} + \frac{4\pi\Delta^2}{k_1} \quad (4)$$

At $\Delta = 97$ Hz, the exchange portion of the line broadening is computed to be about 17 Hz, a value close to the fluorine line-width effect observed. Exchange contributions to the proton line widths should be of this order of magnitude for the aromatic protons of the D inhibitor where chemical shift effects are large but should have a negligible effect on the total line widths for the alkyl protons where the enzyme-induced shifts are much smaller. It seems reasonable to assume that the exchange rates for the L isomer will be similar to those found for the D form of *N*-trifluoroacetyltryptophan, so that similar line-broadening effects on the proton signals are expected for both optical antipodes in the presence of the enzyme.

An important consideration in interpreting the results described above is the extent to which the protein is oligomerized under the conditions of these experiments. Rao and Kegeles have examined the polymerization of α-chymotrypsin at pH 6.2 in 0.2 *M* phosphate buffer at temperatures between 20 and 25°.[20] These experimental conditions are close to those used in the present work (pH 6.6, 0.4 *M* phosphate, 34°) and we have presumed that the dimerization and trimerization equilibrium constants reported by these authors are reasonable approximations to those which would characterize the present system. It has previously been shown that two limiting models for the protein–inhibitor interaction can account for the results of nmr experiments under conditions of large inhibitor concentration relative to the concentration of protein.[11b] These models—either (a) enzyme polymerization but binding only to the monomer or (b) equivalent binding of the inhibitor to all oligomeric forms of the enzyme—become experimentally indistinguishable under these conditions. At low concentrations of inhibitor relative to protein a choice between these possibilities can in principle be made by nmr spectroscopic experiments of the type reported here. In practice, the distinction would be difficult by proton spectroscopy because the many resonances from the protein would obscure those of the inhibitor. The fluorine nmr experiments described herein do not have this disadvantage, and the effects of enzyme association were explored by this means.

The algebraic expressions relating the concentrations of various enzyme–inhibitor complexes to the initial protein and inhibitor concentrations for both models for the effect of protein oligomerization were solved by iterative computer techniques[11b] assuming $K_D = 9.6 \times 10^{-4}$ and $K_T = 2.9 \times 10^{-4}$, values for the dimerization and trimerization constants found in the Rao–Kegeles system. Plots of observed chemical shift *vs.* the mole fraction of monomeric enzyme–inhibitor complex (assuming the inhibitor binds only to the monomer) were linear within experimental error (Figure 3). However, plots of observed shift against the mole fraction of total binding sites, computed with the assumption that the inhibitor binds to one, two, or three identical sites on the monomeric, dimeric, and trimeric forms of the enzyme, respectively, were decidedly nonlinear (Figure 3). Although these results strongly suggest that *N*-trifluoroacetyl-D-tryptophan binds only to the monomeric form of α-chymotrypsin, it should be kept in mind that this conclusion rests on the degree to which the equilibrium constants for protein association have been correctly approximated. It might also be possible to fit the chemical-shift data to models based on the assumption that binding sites of monomer or oligomers of the enzyme are characterized by separate, unique chemical-shift parameters and equilibrium constants. Up to ten parameters for enzyme-induced shifts and six binding constants could be independently varied to achieve this fit which would, of course, be superlative, but which would have doubtful physical significance. We have assumed in the discussion that follows that the proton chemical-shift and line-width data reported above are characteristic of the monomeric enzyme–inhibitor complexes.

If the presence of the tosyl group effectively excludes the D or L forms of the inhibitor from the aromatic pocket at the active site,[21] then the nmr results with the tosyl enzyme described above indicate that there are additional sites of protein–inhibitor interaction. The proton chemical-shift and line-broadening changes induced by these sites are smaller than those which, by difference, can be attributed to the active site and are close to the same effects observed with the *N*-formyl derivatives.[11b] These data provide no insight into how many secondary binding sites are involved *nor* what are their individually characteristic spectral properties. It is expected that the difference between the chemical-shift and line-width changes induced by the native enzyme and the corresponding effects that arise when the tosyl protein is used can be related, to a good approximation, to the effects due solely to interaction at the catalytic center of the protein.[22]

(16) J. T. Gerig and A. D. Stock, work in progress; Professor J. H. Richards has informed us that his group has determined this rate constant under similar conditions and obtained a value of $\sim 4 \times 10^3$ sec^{-1} (private communication).

(17) G. P. Hess, J. McConn, E. Ku, and G. McConkey, *Phil. Trans. Roy. Soc. London, Ser. B*, 237, 89 (1970).

(18) A. Yapel, Ph.D. Dissertation, University of Minnesota (1967).

(19) T. J. Swift and R. E. Connick, *J. Chem. Phys.*, 37, 307 (1962).

(20) M. S. N. Rao and G. Kegeles, *J. Amer. Chem. Soc.*, 80, 5724 (1958).

(21) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 46, 337 (1969).

(22) The interpretation ignores any complication that may be introduced by self-association of tosylchymotrypsin.[23]

The appreciable line-broadening effects on the pmr signals of I that arise when it interacts with the protein probably result principally from intramolecular nuclear dipolar relaxation processes. In this event, the effect of these mechanisms on the observed line width of a signal, w_i, is given by

$$w_i = \frac{C\tau_c{}^i}{\pi}\sum_j r_{i,j}{}^{-6} \qquad (5)$$

where C is a constant characteristic of the interacting nuclei and has the value 88.5×10^{10} for proton–proton relaxation and 69.6×10^{10} for fluorine–fluorine interactions, $\tau_c{}^i$ is a correlation time for nucleus i, and r_{ij} is the distance between nucleus i and nucleus j measured in ångström units.[24] Assuming that the coordinates of the hydrogen atoms in I are the same as those in *N*-formyltryptophan,[11b] one can estimate the correlation times, $\tau_c{}^i$, for the various parts of the inhibitor molecule using the line-width data from Tables II and III. The results of this calculation are given in Table V. When

Table V. Computation of Correlation Times for α-Chymotrypsin–Trifluoroacetyltryptophan Complexes[a]

Nucleus	$w_{1/2}$, corr[b] D	L	$\sum_{ij}\frac{1}{r_{ij}{}^6}$[c]	$\tau_c{}^i \times 10^8$ sec D	L
H_1	28	31	0.0053	1.9	2.1
H_2[d]	(28)	(31)	0.0070	1.4	1.6
H_3[d]	(28)	(31)	0.0080	1.2	1.4
H_4	28	31	0.0033	3.0	3.3
H_A	36	37	0.0558	0.2	0.2
H_B	36	37	0.0556	0.2	0.2
CF_3	5.9	1.1	0.0191[e]		0.03

[a] At pD 6.6 in 0.4 *M* phosphate buffer at 34°. [b] The difference between the native and tosyl enzyme effects multiplied by 100/75 as a correction for the number of titratable active sites. [c] Taken from Table V, ref 10. [d] Line widths assumed to be approximately the same as those for H_1 and H_4. [e] The F–F distance was taken as 2.17 Å; *cf.* A. L. Andreassen, D. Zebelman, and S. H. Bauer, *J. Amer. Chem. Soc.*, **93,** 1148 (1971).

the inherent accuracy of these calculations is considered, the results come out in good agreement with the corresponding values for the *N*-formyltryptophan–enzyme complexes. While recognizing that the uncertainty with regard to the exchange contribution to the line-width effects in the case of the aromatic proton may introduce an error of up to a factor of two, the correlation times estimated for the aromatic rings of both forms of I are close to those expected from Brownian motion of the enzyme. This situation would be anticipated if this region of the inhibitor molecule is bound tightly to the protein.[11] Additional freedom of motion in the alkyl portion of I is indicated by the decreased correlation times for these nuclei. In a qualitative sense, these results then are consistent with the idea derived from crystallographic studies[21] that the aromatic ring occupies a snug, hydrophobic pocket on the enzyme surface while the nuclei nearer the amino acid side chain are more exposed to solvent and, therefore, have greater freedom of motion.

The suggestion that the optical isomers of *N*-trifluoroacetyltryptophan bind to chymotrypsin in a

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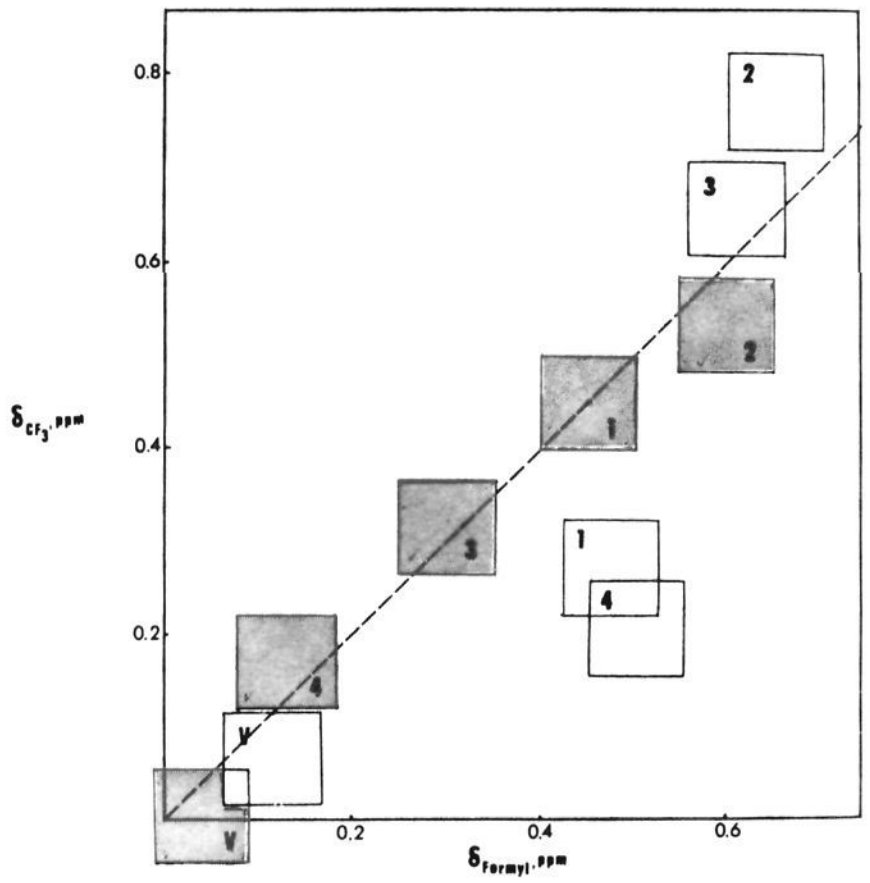

Figure 4. A correlation of the corrected enzyme-induced, chemical-shift effects at the indole protons of the *N*-formyltryptophans and *N*-trifluoroacetyltryptophans. The squares are 0.1 ppm on a side. The shaded blocks represent data for the corresponding L isomers; the dotted line defines a perfect correlation.

manner very similar to that found for the corresponding *N*-formyl compounds can be additionally illuminated by the comparison of the proton chemical-shift effects. In Figure 4, a plot of the corrected chemical-shift effects[25] for the complexes formed with corresponding isomers of the *N*-formyl and *N*-trifluoroacetyl derivatives of tryptophan are plotted against each other. The shaded areas represent data for the L isomers while the open circles are for the D forms. It is seen that the chemical shifts for the aromatic protons of the two L isomers are in good agreement, considering the substantial error that accompanies each chemical-shift determination.[26] This agreement suggests rather strongly that the *N*-formyl-L-tryptophan complex and the *N*-trifluoroacetyl-L-tryptophan complex have very similar structures in solution, at least as regards the orientation of the indole ring of each substance. We have previously described evidence that the structure of the formyl-substituted L-tryptophan–enzyme complex is similar in solution to its known form in the crystalline state,[11b,21] so that we conclude that trifluoroacetylation does not, in this case, change the structure of the enzyme–inhibitor complex markedly.

Even with a generous allowance for experimental error, the lack of strong correlation for the aromatic proton shifts in the two D isomers would seem to signal the possibility that the aromatic rings of these inhibitors are bound in somewhat different ways within the hydrophobic binding site.

(25) Obtained by multiplying the difference between the native enzyme and tosyl enzyme shifts of the complex by a factor to take into account the number of titratable active sites present (~75%).

(26) Inclusion of the equilibrium constant explicitly in computing the enzyme-induced effects would change the data used here but since the binding constants for both the formyl and trifluoroacetyl substituted compounds should be similar in value, the correction that would be applied to both sets of data would be approximately the same and, thus, not change the basic conclusions drawn from Figure 4.

There is a substantial difference in the chemical shift effect found for the fluorine resonances of the two enantiomeric enzyme–inhibitor complexes of I. The trifluoromethyl group of the complex formed with the L isomer appears to be in a very nearly magnetically neutral environment, with the result that only a small downfield shift is observed. In agreement with previous work[8] a large downfield shift is found with *N*-trifluoroacetyl-D-tryptophan, the observed effect being more than thirty times larger than that exhibited by the L isomer. If the complex formed with the trifluoroacetyl derivative of L-tryptophan is basically the same as that found for the corresponding *N*-formyl compound, then the trifluoromethyl group which replaces the formyl hydrogen of the latter compound would point away from the active site of the enzyme, toward free solution. (In a natural polypeptide substrate of α-chymotrypsin, the polyamide chain must project in the same manner.) There is very little interaction between the trifluoromethyl group and the protein in this case and the solvation of the fluorine nuclei should be similar to that found in aqueous solution. A small fluorine-19 chemical shift effect for the L compound would, therefore, be expected since the environment of the trifluoromethyl group is not altered appreciably upon binding to the enzyme. The freedom of molecular motion indicated by the small correlation time, τ_c, for this group is also consistent with the proposition that it is oriented well away from the protein surface and can undergo rapid molecular motion, probably by rotation about the carbon–carbon single bond which holds the CF_3 group to the inhibitor.

When the configuration at the asymmetric carbon atom of the inhibitor is changed to afford the D isomer, significantly different enzyme–trifluoromethyl group interactions must be present. Presuming that the indole ring occupies the hydrophobic pocket at the active site in a manner similar to that found with the L isomers and that there is no major change in rotamer population at the C_α–C_β bond, space-filling models indicate appreciable protein–trifluoromethyl interactions regardless of the orientation chosen for the indole ring relative to the amino acid side chain. These interactions may lead to an appreciable change in the solvation of the trifluoromethyl group relative to what it experiences in free solution. However, chemical shifts of trifluoromethyl groups attached to detergent molecules move substantially upfield ($\sim$1.2 ppm) when the trifluoromethyl group is incorporated into the relatively water-free, hydrocarbon-like interior of a micelle upon formation of these species;[27] these environments are thought to resemble in a gross way those found at the active sites of enzymes.[28] Other types of enzyme–fluorine interaction such as hydrogen-bonding or ring-current effects would seem, therefore, to be necessary to rationalize the observed downfield shifts with the D isomer.

Our conclusions, then, are that the *N*-trifluoroacetyl-L-tryptophan–enzyme complex is quite similar to that found for the corresponding *N*-formyl derivative in the solid state,[11b,21] but that the D isomer is structured in an appreciably different way. Neither our nmr data nor our understanding of the structure of this second complex is sufficiently precise at this time to allow confident speculation in detail about the nature of this complex nor how its structure should be reflected in the nmr parameters that can be observed.

Experimental Section

***N*-Trifluoroacetyl-L- -and D-tryptophan** were prepared by the method of Tschesche and Jenssen.[29] After several recrystallizations from toluene, a final crystallization was initiated by cooling the supernatant to 0°, after which fine white needles, mp 161–163° (lit.[30] 160°), were deposited. The L isomer had $[\alpha]^{25}D$ −1.8° (*c* 2, methanol) while the rotation for the D isomer was $[\alpha]^{25}D$ +1.8° (*c* 2, methanol). Rotations were determined with a Bendix Model 1169 polarimeter.

The procedures and precautions for obtaining pmr spectra at 100 MHz were identical with those used for investigation of the *N*-formyltryptophan complexes.[11] Fluorine nmr spectra were obtained on the same instrument tuned for observation at 94.1 MHz by essentially the same techniques. A sealed capillary containing trifluoroacetic acid was used to provide a lock signal in this case, and samples were allowed to equilibrate at least 15 min in the probe before observations were initiated. A trace of sodium trifluoroacetate (0.005 *M*, K and K Laboratories) was used as an internal reference. In these experiments the range of initial enzyme concentrations was 0.06–2.8 m*M* while the initial inhibitor concentrations were between 0.5 and 60 m*M*.

Computer simulations of nmr line shapes were performed with an IBM 360/75 computer interfaced to a Houston plotter.

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