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

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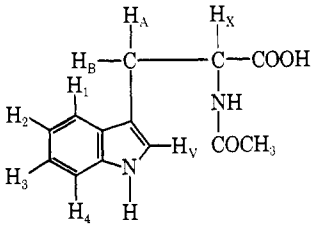
Abstract: The influence of the enzyme α-chymotrypsin on the proton magnetic resonance spectrum of *N*-acetyl-L-tryptophan and the corresponding D isomer has been examined. Substantial line-width and chemical-shift effects are observed; these are discussed with reference to the known structure of the *N*-formyl-L-tryptophan–enzyme complex. In confirmatory experiments, deuterium magnetic resonance spectra of *N*-trideuterioacetyl-L- and -D-tryptophan in the presence of the protein were obtained. Interpretation of the line-broadening effects found in terms of quadrupolar relaxation provides an additional viewpoint on the motions of the inhibitor molecules at the active center of the enzyme.

The interaction of *N*-acetyl-L-tryptophan and *N*-acetyl-D-tryptophan with α-chymotrypsin has been examined by a number of workers using various physical and chemical methods.[2-4] Both materials are potent competitive inhibitors of the enzyme and, as demonstrated by oxygen-18 exchange experiments, the L isomer can act as a pseudosubstrate for the enzyme, in the sense that (in this case) an acyl-enzyme is in equilibrium with the Michaelis complex.[3] We have previously expressed our interest in comparing the complexes formed between the *N*-trifluoroacetyl- and *N*-acetyltryptophans and α-chymotrypsin and a previous paper has described the results of a high-resolution magnetic resonance investigation of the fluorine-containing system.[5] We report herein corresponding experiments with the *N*-acetyl derivatives, including high-resolution deuterium magnetic resonance studies of the *N*-trideuterioacetyl analogs. These results are contrasted and compared to those obtained for the *N*-trifluoroacetylated materials.

Results

The high-resolution proton magnetic resonance spectrum of *N*-acetyltryptophan (I) was recorded at



I

100 MHz utilizing a 0.04 *M* sample in 95% deuterium oxide–water. The solution was buffered at apparent pH 6.2 by 0.4 *M* sodium phosphate. An ABCD pattern and a broadened singlet, assigned to the protons of the indole ring of I, were observed in the spectrum at low applied field while the ABX pattern characteristic of the amino acid side chain and a singlet for the acetyl methyl were found at higher fields. The nontrivial parts of the spectrum were analyzed by employing the Ferguson–Marquardt computer program according to the procedure of Swalen and Reilly.[6,7] The chemical shifts and coupling constants so obtained are listed in Table I and lead to computed theoretical spectra in good agreement with those found experimentally.

Table I. Pmr Spectral Parameters for *N*-Acetyltryptophan[a]

δ, ppm		*J*, Hz	
A. Aromatic protons[b]			
δ_1	−5.7549	J_{12}	= 7.92
δ_2	−5.2319	J_{13}	= 1.24
δ_3	−5.3038	J_{14}	= 0.76
δ_4	−5.5675	J_{23}	= 7.08
δ_V	−5.288	J_{24}	= 0.98
		J_{34}	= 8.25
B. Alkyl protons[b]			
δ_A	−1.1992	J_{AX}	= 7.94
δ_B	−1.4102	J_{BX}	= 4.65
δ_X	−2.5823	J_{AB}	= −14.63
δ_{CH_3}	0.0527	$\lvert J_{AV}\rvert$	= 0.6
		$\lvert J_{BV}\rvert$	= 0.8

[a] Sample was 0.04 *M* in 0.4 *M* phosphate buffer at apparent pH 6.2 (95% D_2O). [b] Chemical shifts (δ_i) were measured relative to 0.005 *M* sodium acetate included in the solution 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 ±0.06 Hz for the coupling constants. J_{AV} and J_{BV} have estimated uncertainties of ±0.1 Hz.

The values for these spectral parameters are generally quite similar to those found for the *N*-formyl[8] and *N*-trifluoroacetyl[5] derivatives and provide a strong indication that the acetates are conformationally similar to these other derivatives. There was no evidence in the proton spectrum of I for a second conformational isomer such as was found with the *N*-formyl derivative.[8] Inclusion of protein in the solutions results in appreciable line broadening and, in the case of the aromatic nuclei, significant upfield chemical shifts. In quantitating these protein-induced spectral effects, we

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

(2) (a) R. A. Wallace, A. N. Kurtz, and C. Niemann, *Biochemistry*, **2**, 824 (1963); (b) A. Yapel, Ph.D. Thesis, University of Minnesota, Minneapolis, Minn., 1967.

(3) F. J. Kežđy, G. E. Clement, and M. L. Bender, *J. Amer. Chem. Soc.*, **86**, 3690 (1964).

(4) (a) D. D. F. Shiao and J. M. Sturtevant, *Biochemistry*, **8**, 4910 (1969); (b) D. D. F. Shiao, *ibid.*, **9**, 1083 (1970).

(5) J. T. Gerig and R. A. Rimerman, *J. Amer. Chem. Soc.*, **94**, 7549 (1972).

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(7) J. D. Swalen and C. A. Reilly, *ibid.*, **37**, 21 (1962).

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have assumed that the interaction of the inhibitors with the enzyme may be represented by an equilibrium of the type shown below. Here E represents the enzyme, I the inhibitor, and EI an enzyme–inhibitor complex,

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

presumably analogous to the Michaelis complex that is formed during the reaction of substrates with the enzyme. It has been noted previously that when the exchange rate constants k_1 and k_{-1} are sufficiently large and the equilibrium constant K_I ($= k_1/k_{-1}$) sufficiently small relative to the initial concentration of the inhibitor (I_0), eq 2 can be an adequate description of enzyme-induced changes in nmr parameters of interest.[5,8] The symbol x represents the measured

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

change in a given parameter relative to its value when protein is absent, χ corresponds to its value within the enzyme–inhibitor complex, and E_0 is the initial value of the enzyme concentration. The interpretation of the parameter χ in terms of specific protein inhibitor interactions depends upon the association behavior of the enzyme and upon the binding properties of each oligomeric form.[8]

The proton-chemical-shift effects induced by native α-chymotrypsin were estimated by determining the shift in positions of the major peaks in a multiplet assigned to a given nucleus while the changes in line width were evaluated by comparing computer-generated theoretical curves to the experimental ones until a good match was found. The line-width and chemical-shift data were fit to eq 2 by a least-squares procedure;[9] the resulting slopes are recorded in Table II.

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

Proton	Chemical shifts, ppm[b] *N*-Acetyl-L-tryptophan	*N*-Acetyl-D-tryptophan
H_1	0.69 ± 0.04	0.39 ± 0.04
H_2	0.74 ± 0.08	0.61 ± 0.04
H_3	0.51 ± 0.04	0.72 ± 0.08
H_4	0.19 ± 0.04	0.37 ± 0.04
H_V	−0.08 ± 0.03	0.0 ± 0.02
H_A	−0.03 ± 0.04	0.37 ± 0.03
H_B	0.21 ± 0.02	0.0 ± 0.05
CH_3	0.17 ± 0.01	−0.13 ± 0.02
	Line widths, Hz	
H_1–H_4	31 ± 2	39 ± 4
H_A, H_B	41 ± 5	53 ± 5
CH_3	10 ± 1	8 ± 1

[a] Observed at 100 MHz. Sample temperature was 34° at pD 6.6, 0.04 *M* phosphate buffer. [b] Negative sign indicates a downfield shift.

There are crystallographic[10] and other indications[11] that organic molecules with hydrophobic side chains may bind at several locations on the surface of α-chymotrypsin and our previous nmr studies tend to bear out these suggestions of nonactive site binding.[5,8] In order to provide a correction for influences that binding of the *N*-acetyltryptophan inhibitors to sites on the protein other than the active center might exert on the nmr spectral behavior, we repeated the above experiments with serine-195-tosylchymotrypsin. This protein features an aromatic ring permanently bound to the serine-195 residue at the active site of the enzyme through a sulfonate ester bridge. In the crystalline state, the toluenesulfonic acid moiety occupies the same hydrophobic pocket as that utilized by the aromatic ring of the inhibitor *N*-formyl-L-tryptophan when this latter molecule binds to the enzyme.[10] We assumed that the tosyl enzyme (by a mutual exclusion effect) would not permit binding of I to the active center but that secondary binding sites on the protein surface would not be appreciably affected in this derivative. Smaller and less distinctive chemical-shift and line-broadening effects were noted when the tosyl enzyme was used. The slopes of plots of x *vs.* E_0/I_0 for these experiments are recorded in Table III; it should be emphasized that these slopes do not necessarily correspond to the chemical-shift or line-width effect associated with a particular locus on the enzyme but rather represent some kind of averaged effect that is the base line from which active-site derived nmr effects should be measured.

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

Proton	Chemical shifts, ppm *N*-Acetyl-L-tryptophan	*N*-Acetyl-D-tryptophan
H_1	0.22 ± 0.05	0.11 ± 0.06
H_2	0.23 ± 0.09	0.11 ± 0.04
H_3	0.19 ± 0.07	0.15 ± 0.04
H_4	0.17 ± 0.08	0.12 ± 0.05
H_V	−0.03 ± 0.07	−0.07 ± 0.05
H_A	−0.01 ± 0.06	−0.02 ± 0.01
H_B	0.01 ± 0.05	−0.02 ± 0.02
CH_3	0.03 ± 0.01	−0.01 ± 0.01
	Line widths, Hz	
H_1–H_4	12 ± 1	6 ± 1
H_A, H_B	21 ± 2	16 ± 2
CH_3	3 ± 1	3 ± 1

[a] At 34°, pD 6.6, 0.4 *M* phosphate buffer. Observing radio frequency was 100 MHz.

As will be discussed below, our line-broadening data can lead to certain conclusions regarding the freedom of inhibitor motion at the active site of the enzyme provided that a mechanism for nuclear relaxation is assumed. In order to substantiate the conclusions reached, we examined the spectral behavior of the deuterium resonance from the D and L forms of I in which the acetyl methyl group has been replaced by a trideuteriomethyl group. Since the gyromagnetic ratio of deuterium is about one-seventh that of protium, no enzyme-induced chemical-shift effects were detected and any exchange-rate contribution to the protein-induced line widths should therefore be negligible. However, significant line-width variations were observed; for the deuterium spectroscopy experiments the concentration of protein was held essentially constant so that the line broadenings found cannot reasonably

(9) G. W. Snedecor, "Statistical Methods," The Iowa State University Press, Ames, Iowa, 1956, p 123.
(10) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, **46**, 337 (1969).
(11) A. N. Glazer, *J. Biol. Chem.*, **242**, 4528 (1967).

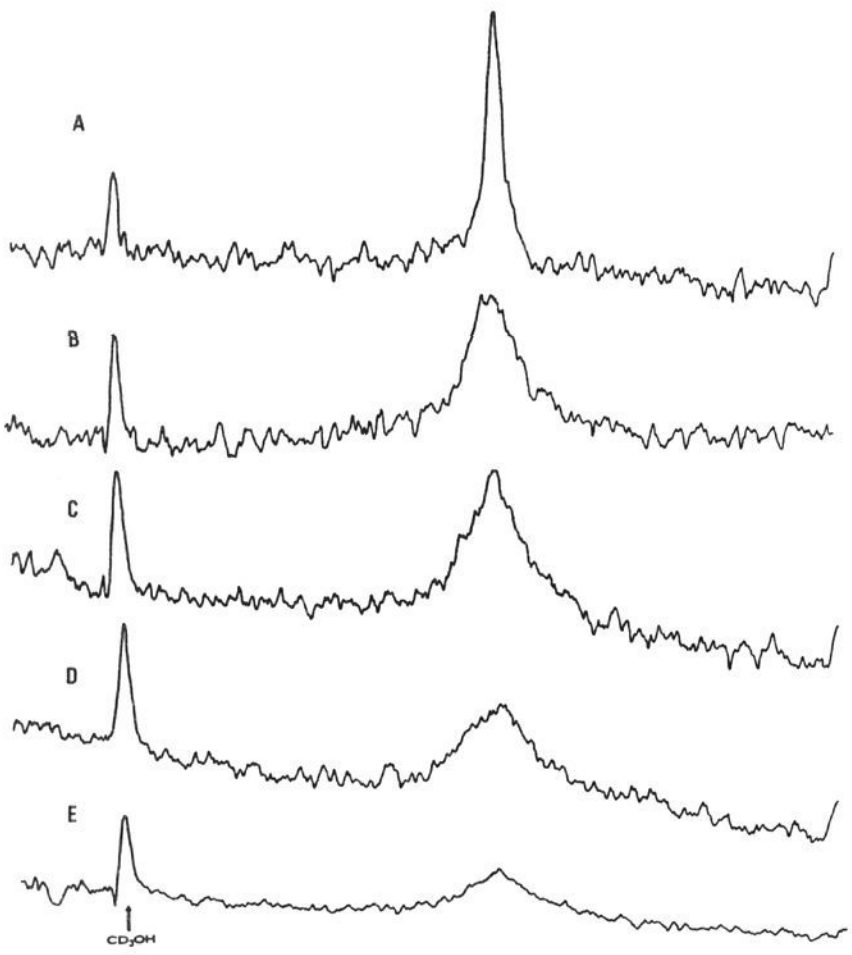

Figure 1. The influence of native α-chymotrypsin on the deuterium resonance of *N*-trideuterioacetyl-D-tryptophan at 15.4 MHz. The top curve was obtained with the absence of protein while in traces B–E the enzyme:inhibitor ratios were 0.021, 0.029, 0.043, and 0.052, respectively. The concentration of enzyme was maintained at ~2 m*M* throughout the series. Deuteriomethanol (CD_3OH) was included in the samples at a concentration of 20 m*M* to provide a reference signal. Each experimental spectrum is 50-Hz wide.

be attributed to changes in solution viscosity, but rather, must reflect directly changes in the relaxation of the deuterium nuclei when the inhibitor molecules bind to the enzyme. Typical deuterium spectra are shown in Figure 1. The deuterium line-width data were treated according to eq 2 and the resulting line widths for the CD_3 group in the enzyme–inhibitor complexes are presented in Table IV.

Table IV. Dmr Line-Width Changes Induced by Native and Tosylated α-Chymotrypsins[a]

	L isomer[b]	D isomer[b]
Native enzyme	59 ± 3	72 ± 5
Tosyl enzyme	14 ± 2	14 ± 3

[a] Samples were made up in a solvent consisting of 95% H_2O, 5% D_2O, and 0.02 *M* CD_3OD, which served as an internal line-width standard. The observing radio frequency was 15.4 MHz, and the sample temperature 34°. [b] Line-width parameters (χ) in hertz.

Discussion

An important aspect of this work is the correspondence between the nmr spectral behavior of *N*-trifluoroacetates of tryptophan in the presence of α-chymotrypsin and the *N*-acetyl compounds. Should the substitution of a CF_3 group for the CH_3 group of the acetates bring about a significant change in the structure of the corresponding enzyme–inhibitor complexes, one would expect to find appreciable and, hopefully, diagnostic changes in the nmr chemical-shift and line-width parameters derived for these complexes by the methods

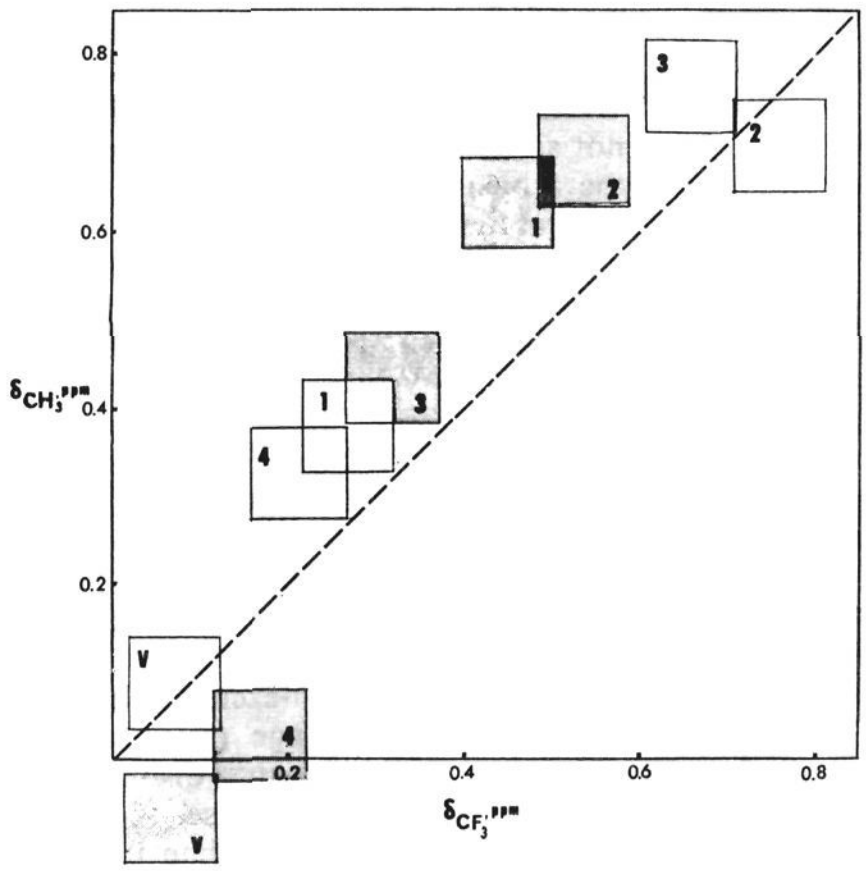

Figure 2. Correlation of the enzyme-induced chemical shifts on the indole protons of the *N*-acetyltryptophans (δ_{CH_3}) and the *N*-trifluoroacetyltryptophans (δ_{CF_3}). Data for the L isomers are represented by the shaded blocks. The dotted line corresponds to a perfect correlation.

described above. Figure 2 is a plot of the enzyme-induced chemical-shift changes at the indole positions observed for the *N*-acetyltryptophan–enzyme complexes *vs.* the corresponding shift effects for the *N*-trifluoroacetates. The shift values have been corrected for the effects of secondary site binding by use of the data obtained with the tosyl enzyme and have also been corrected for the number of active sites per gram of commercial protein.[12] If the enzyme–inhibitor complexes formed with the CH_3- and CF_3-substituted compound were identical insofar as the orientation of the indole ring of the inhibitor within the hydrophobic pocket of the active site is concerned, one might expect the shift data to lie along the dotted line of Figure 2. Most of the points are, in fact, above this line intended to indicate a perfect correlation, although the slope of the line through the points is essentially parallel to the anticipated line. The chemical-shift values for the *N*-acetyl complexes thus appear to be consistently too large by about 0.1 ppm. While this difference is within the probable error of these determinations, it seems unlikely that the chemical-shift effects for both the D and L isomers of the N-acetylated materials would be consistently in error by the same amount. The structures of the enzyme–acetyltryptophan complexes must be different for the two antipodes of the inhibitors so that replacement of the acetyl methyl by a CF_3 group would be expected to have different effects on the chemical-shift patterns for the D isomer relative to the L forms. Our feeling is that some systematic effect, either an undetected experimental artifact or some nonspecific solvation effect, is responsible for the apparent constant differences between the aromatic chemical-shift data

(12) M. L. Bender, N. L. Begué-Cantón, R. L. Blakely, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, Jr., T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Amer. Chem. Soc.*, **88**, 5890 (1966).

for the acetates and trifluoroacetates, and we have reached the tentative conclusion that, fundamentally, the structures of the corresponding inhibitor–enzyme complexes are not significantly different as regards the orientation of the aromatic indole ring relative to the tyrosine-228 residue at the active center.

A primary consideration when applying magnetic resonance spectroscopy in experiments of the type described above is the extent to which the "fast exchange" approximation is valid. If the dissociation rate constant k_1 is at least three times larger than $2\pi\delta\nu$, where $\delta\nu$ is the chemical-shift difference between the enzyme-bound and free solution environments for a nucleus on the inhibitor and also greater than πw, where w is the line width in the enzyme–inhibitor complex, then the observed chemical shifts of the inhibitor resonances should be approximately the appropriately averaged signals expected in the rapid-exchange limit.[8,13] Yapel has measured k_1 for exchange of *N*-acetyl-D-tryptophan at pH 8 and 20° by a dye displacement technique and finds a value of 4×10^2 sec^{-1}.[2b] The same experiments indicate a value less than 0.5 sec^{-1} for the L isomer, a rate constant that seems anomalously low, when results obtained in similar systems are considered.[5,8] If the magnitudes of our enzyme-induced chemical-shift and line-broadening effects are approximately correct, one would require that k_1 for the D isomer be greater than $\sim 5 \times 10^2$ sec^{-1}, an estimate obtained by considering the largest chemical-shift effect ($\sim$0.8 ppm at H_3) in the inhibitor molecule. At the temperature of our experiments (34°) one would expect a rate constant perhaps double that which Yapel found at 20° so that it would appear to a good approximation that the data obtained for *N*-acetyl-D-tryptophan are valid in the fast-exchange limit.

The situation is less clear for the L enantiomer of the inhibitor. Although one might expect some complications from the formation of an acyl-enzyme intermediate in this case, the exchange rates for acylation–deacylation should be rapid ($k_{-1} \approx 300$ sec^{-1}) at the pH used in this study.[3] The very slow exchange rate indicated by Yapel's value for k_1 would result in virtually no detectable effect of the enzyme on the spectrum of *N*-acetyl-L-tryptophan, in contradistinction to what is observed experimentally. Although the source of this discrepancy is not clear, because the experimental results for *N*-trifluoroacetyl-L-tryptophan and *N*-acetyl-L-tryptophan are so similar, it is believed that the results reported above with the acetylated L isomer also derive from a fast-exchange situation.

The line-broadening effects observed when I is in the presence of α-chymotrypsin can, in principle, reveal details of the motion of the inhibitor as it is bound to the enzyme, if line-width changes due to chemical exchange can be neglected. The line-width data obtained when the tosylated enzyme is used should provide a correction for the relaxation effects of the enzyme on the inhibitor at the secondary binding sites and also for any influence solution viscosity may have on these effects. Presuming that nuclear–nuclear dipolar relaxation is the dominant influence on the line widths of the nuclei of the inhibitor in its complexed form, and neglecting any relaxation effect of the protons of the enzyme on the inhibitor, the correlation time, τ_c^i, for a given proton of the inhibitor may be estimated with the aid of eq 3.[14]

$$\tau_c^i = \frac{\pi w_i}{C\sum_j r_{i,j}^{-6}} \qquad (3)$$

Here w_i is the line width found for the nucleus in the enzyme–inhibitor complex, $r_{i,j}$ is the distance between nucleus i and nucleus j measured in ångströms, and C is a constant, having a value of 8.85×10^{11} when both i and j refer to protons. This equation is strictly valid only in the extreme narrowing approximation ($\omega\tau_c^i \ll 1$) where ω is the spectrometer operating frequency, but because transverse relaxation times are not very sensitive to ω, it should provide a reasonable estimate even as this approximation begins to break down.[15] Using the internuclear distances computed from the X-ray data for the *N*-formyl-L-tryptophan molecule given previously,[8] one can estimate the correlation times, τ_c, for the various parts of the inhibitor molecule using the line-width data presented in Tables II and III. The results of these calculations are given in Table V. It should be emphasized that the values of τ_c obtained in this way for the aromatic protons of the inhibitor may be considerably in error since the rate of exchange between the environments available to these nuclei may not be fast enough to give a time "fast exchange" averaging of line widths. The enzyme-induced chemical-shift effects are much smaller at protons H_A and H_B and the acetyl group so that complications in interpretation due to exchange effects are negligible in these cases.

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

Nucleus	$w_{1/2}$, corrected[b] D	L	$\sum_j r_{i,j}^{-6}$[c]	$\tau_c \times 10^8$ sec D	L
H_1	44	25	0.0053	2.9	1.7
H_2[d]	(44)	(25)	0.0070	2.2	1.3
H_4[d]	(44)	(25)	0.0080	2.0	1.1
H_4	44	25	0.0033	4.7	2.7
H_A	49	27	0.0558	0.3	0.2
H_B	49	27	0.0556	0.3	0.2
CH_3	7	8	0.0618[e]	0.04	0.05
CD_3	77	60		0.06	0.05

[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 to correct for the number of titratable active sites. [c] Taken from ref 8, Table V. [d] Line widths for these nuclei were assumed to be approximately the same as those for H_1 and H_4. [e] The H–H distance in the methyl group was taken to be 1.78 Å.

The correlation times τ_c described above are based upon a simple model for nuclear relaxation that includes a strong dependence on the geometrical arrangement of the nuclei in the inhibitor. It also neglects any contributions to proton relaxation made by interaction of the inhibitor with protons on the enzyme. Quadrupolar relaxation should be the dominant influence on the line-width effects observed with the trideuterated forms of I. Deuterium line-width effects will not be entangled with exchange contributions, geometrical dependencies, or additional dipolar interactions. In the extreme narrowing approximation, the transverse relaxation time for deuterium is given by eq 4.[16]

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

(14) O. Jardetzky, *Advan. Chem. Phys.*, 7, 512 (1964).

(15) A. Carrington and A. D. McLachlan, "Introduction to Magnetic Resonance," Harper and Row, New York, N. Y., 1967, p 192.

(16) A. Abragam, "Principles of Nuclear Magnetism," Oxford University Press, London, 1961, p 313 *et seq.*

$$\frac{1}{T_2} \approx \frac{1}{T_1} = \frac{3\pi^2}{2}\left(\frac{e^2qQ}{h}\right)^2 \tau_c^i \quad (4)$$

Since

$$w_i = \frac{1}{\pi T_2} \quad (5)$$

the correlation time for deuterium nuclei can be estimated from the observed line-broadening effects with the aid of eq 6. The symbols within the parentheses in

$$\tau_c^i = \frac{2w_i}{3\pi\left(\frac{e^2qQ}{h}\right)^2} \quad (6)$$

expressions 4 and 6 define the quadrupole coupling constant for the nuclei under consideration and collectively have a value somewhat characteristic of the nature of the chemical bond that holds the deuterium atoms to a molecule. It appears that a reasonable value for the quadrupolar coupling constant of a CD_3 is 165 kHz[17-19] and, by assuming that this value is appropriate for the trideuteriomethyl group of I as it resides at the active site of α-chymotrypsin, one can compute the correlation times for the CD_3 group shown in Table V. In spite of the semiquantitative nature of these correlation time calculations, the agreement between values obtained by considering the dipolar relaxation of the CH_3 groups or the quadrupolar relaxation of the CD_3 groups is surprisingly good and indicates the essential correctness of the approaches used in their estimation.[20]

Precise interpretation of the chemical-shift and linewidth effects on the *N*-acetyltryptophans induced by α-chymotrypsin depends upon the model chosen to represent the association of this enzyme and the manner in which the inhibitors interact with each polymeric form of the enzyme. Two possible models are suggested by the work of others. Sarfare, *et al.*, have shown that hydrocinnamate interacts equally well with one site on the monomeric form of α-chymotrypsin, two sites on the dimer and three sites on the trimer.[23] In contrast, Faller and La Fond[24] have produced evidence that proflavine binds only to the monomeric form of the protein. If one assumes that the binding sites on the putative oligomeric complexes are characterized by the same inhibitor binding constant and exert the same chemical shift effect, it can be shown that the two models predict virtually identical dependences of observed nmr effects (χ) on the $E_0:I_0$ concentration ratio when the value of this ratio is small.[8] It is only at larger values of $E_0:I_0$ that a distinction can be made. Fluorine-19 experiments with *N*-trifluoroacetyl-D-tryptophan are most consistent with this inhibitor binding only to the enzyme monomer. Given the similarity between the acetyl and trifluoroacetyl derivatives, it is reasonable to expect a similar situation to hold in the present work. It is also worthy of mention that attempts to prepare crystalline samples of the *N*-acetyltryptophan–α-chymotrypsin complexes have been unsuccessful; in the crystalline state the inhibitor would be forced to bind with the dimeric form of the enzyme.[25]

The expected rotational correlation time for the enzyme under conditions of these experiments is about 1.4×10^{-8} sec if the protein is monomeric or approximately 2.8×10^{-8} sec if the enzyme is appreciably dimerized.[8] The observed correlation times for the aromatic ring protons of the *N*-acetyltryptophans, although uncertain to a degree, lie between these two anticipated limits and suggest that molecular motion of this part of the inhibitor molecule within the enzyme–inhibitor complex is strongly restricted. This would be expected if the indole ring is firmly wedged into the hydrophobic binding locus at the active site. Considerably greater freedom of motion is indicated for the alkyl side chain of the amino acid while the movement of the *N*-acetyl methyl group at the active center is restricted only slightly. The experimental observations are consistent with the proposed structure for the inhibitor–protein complex since these last mentioned nuclei project progressively farther away from the protein surface and should, therefore, experience less interaction with the enzyme and greater freedom of motion.

In summary, these results indicate that the complexes of *N*-acetyl-D- and -L-tryptophan with α-chymotrypsin are very similar to the corresponding complexes formed with the *N*-trifluoroacetyl derivatives of tryptophan. The large fluorine-chemical-shift effect observed with with D-CF_3 compound appears to be the result of unique influences from the enzyme; these influences are not reflected in the acetyl proton shifts of the D-CH_3 compound.

Experimental Section

***N*-Acetyl-L-tryptophan** (Lot U3447, Mann Research Laboratories) **and N-acetyl-D-tryptophan** (Lot K5474, Cyclo Chemical Co.) were obtained commercially and used as received.

***N*-Trideuterioacetyl-L- and -D-tryptophan** were prepared by a procedure modeled after the mixed carbonic anhydride method of Vaughan and Osato using acetic-d_4 acid (Stohler Isotope Chemicals).[26] Repeated recrystallization from 20% ethanol–water afforded a pale purple sample of the L isomer, mp 178–179° (lit. 180–181°), $[\alpha]^{26}D$ +25.2° (*c* 1, 1 *N* NaOH). Mass spectral analysis at 110° and an ionizing voltage of 70 eV indicated that approximately 98% of the material consisted of the d_3 compound. A pale purple sample of the D isomer was also prepared, mp 179–180° and $[\alpha]^{26}D$ −26.2° (*c* 1, 1 *N* NaOH), which was shown by mass spectral data to be approximately 97% of the d_3 form.

The enzyme samples, buffer solutions, and instrumental techniques were identical with those employed in previously reported investigations.[5,8]

Acknowledgments. This work was supported by the National Science Foundation (Grant GP-24161). R. A. R. acknowledges the support of a predoctoral fellowship from the National Institute of General Medical Sciences.

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(18) J. A. Glasel, *J. Amer. Chem. Soc.*, **91**, 4569 (1969).

(19) There seems to be some confusion over the use of h or $\hbar$ ($= h/2\pi$) in defining the quadrupole coupling constant for CD_3. Powell, *et al.*, use the symbol h and quote a value of 165 kHz for the constant in $C_6H_5CD_3$ while Glasel uses the symbol $\hbar$ in quoting Phillips' work but gives the same numerical value. We have used Phillips' notation in writing eq 4 and in our calculations.

(20) There are available more sophisticated theoretical treatments of dipolar[21] and quadrupolar[22] relaxation which take into account motions of the protein and, separately, the effect of rotation of the methyl group about its threefold symmetry axis. We hope to apply these theories when we have on hand more accurate T_1 and T_2 relaxation time data and the frequency dependence of these quantities.

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