length was significant over the time required for a scan, OD values were determined by interpolation on a plot of OD_λ *vs.* time. The extinction coefficients used to set the simultaneous equations

Compd (derived from I)	$\epsilon_{365} \times 10^{-4}$	$\epsilon_{300} \times 10^{-4}$	$\epsilon_{260} \times 10^{-4}$
S	1.37	1.41	0.812
N^1-Formyl	0	2.22	0.935
N^{10}-Formyl	0	0.44	1.64

were pH invariant in the range of investigation. In the course of a given reaction, the total molar concentration as calculated above varied by less than 1% up to 90% of reaction.

The computer program used for least-squares fitting is described elsewhere.[13]

The values for buffer pK_a values were taken as the pH of the half-neutralized buffer solution.

Acknowledgment. We are grateful to the National Science Foundation (GB 16789) for support of this investigation.

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

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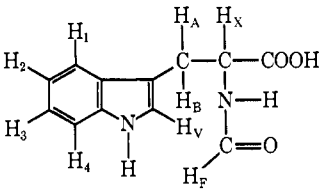
Abstract: High-resolution proton magnetic resonance techniques have been used to study the interaction of *N*-formyl-L-tryptophanate with the proteolytic enzyme α-chymotrypsin and with the catalytically inactive tosyl derivative of this protein at apparent pH 6.2 in deuterium oxide solution. In the presence of the enzyme, marked upfield chemical shifts are observed for the aromatic protons of this inhibitor, as well as with the corresponding D isomer. These shifts are tentatively interpreted in terms of interactions between the tyrosine-228 side chain at the active site of the protein and the inhibitor molecule. Consideration of protein-induced line-broadening effects indicates that both forms of the inhibitor bind tightly to the enzyme while the chemical-shift effects are consistent with the conclusion that the inhibitor–enzyme complex has essentially the same structure in aqueous solution that has been determined for the crystalline form.

Although chemical studies with enzyme model systems can often provide considerable insight into the mechanisms of enzyme catalysis,[2] there is no substitute for the understanding that accurate information about the three-dimensional structure of the enzyme provides. The development of X-ray crystallographic methods for the determination of protein structures has been an all-important step in this regard and, despite sometimes formidable experimental problems, the crystal structures of a number of enzymes have been determined by this method.[3,4] However, the correspondence between the structure of the protein in the crystalline environment and its form in aqueous solution under physiological conditions must be established by additional chemical and spectroscopic experimentation.

Blow and his coworkers have described X-ray crystallographic studies of tosyl-α-chymotrypsin[5] and have also examined the structures of several complexes of chymotrypsin with inhibitors, including *N*-formyl-L-tryptophan.[6] Nuclear magnetic resonance experiments have provided useful information about enzyme–inhibitor complexes in solution for several systems[7] and, in view of the wide interest in α-chymotrypsin and the availability of the above-mentioned crystallographic results, we decided to examine the solution-state complex formed between this enzyme and *N*-formyltryptophan by these high-resolution nmr techniques. The results of this investigation are described below.

Results

The spectrum of this inhibitor as a 51 m*M* solution in D_2O (apparent pH 6.2) obtained at 100 MHz is shown in Figures 1 and 2. The spectrum is similar to that found for tryptophan,[8] with an ABCD pattern for the aromatic protons (H_1–H_4) and a broad singlet for the vinyl proton (H_V) appearing at low field and the



AB part of two sets of ABX signals apparent at higher fields. It is clear from Figure 2 that two different ABX systems are present in the spectrum, probably due to

(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. A preliminary report has appeared: *Biochem. Biophys. Res. Commun.*, **40**, 1149 (1970).

(2) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," W. A. Benjamin, New York, N. Y., 1966.

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(6) T. A. Steitz, R. Henderson, and D. M. Blow, *ibid.*, **46**, 337 (1969).

(7) (a) G. C. K. Roberts and O. Jardetzky, *Advan. Protein Chem.*, **24**, 447 (1970); (b) M. A. Raftery and F. W. Dahlquist, *Fortschr. Chem. Org. Naturst.*, **27**, 341 (1969).

(8) J. T. Gerig, *J. Amer. Chem. Soc.*, **90**, 2681 (1968).

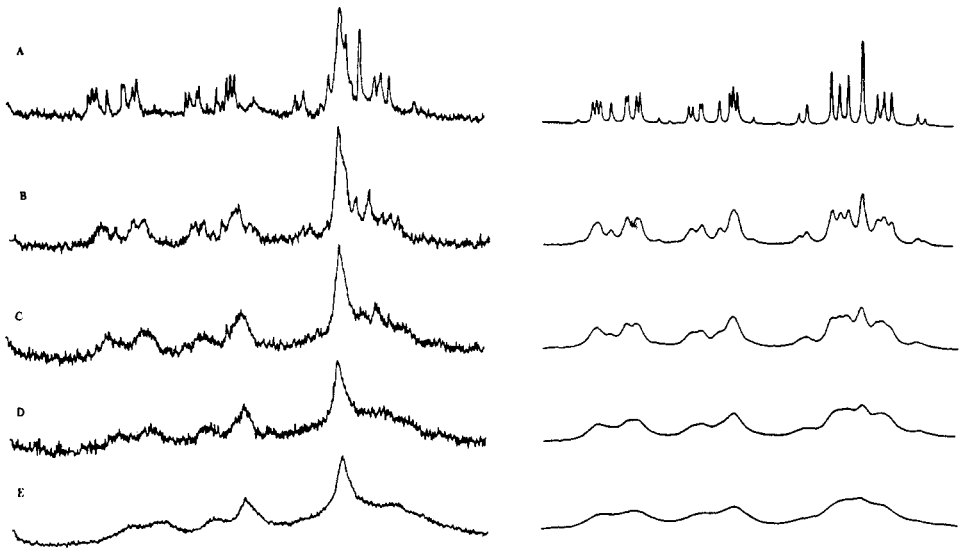


Figure 1. The effect of enzyme on the aromatic portion of the pmr spectrum of *N*-formyl-L-tryptophan. Each experimental curve is 100-Hz wide. The computed line shapes were generated using line widths of 0.4, 1.5, 2.5, 3.5, and 4.5 Hz, for enzyme–inhibitor concentration ratios of 0, 0.025, 0.05, 0.077, and 0.104, respectively.

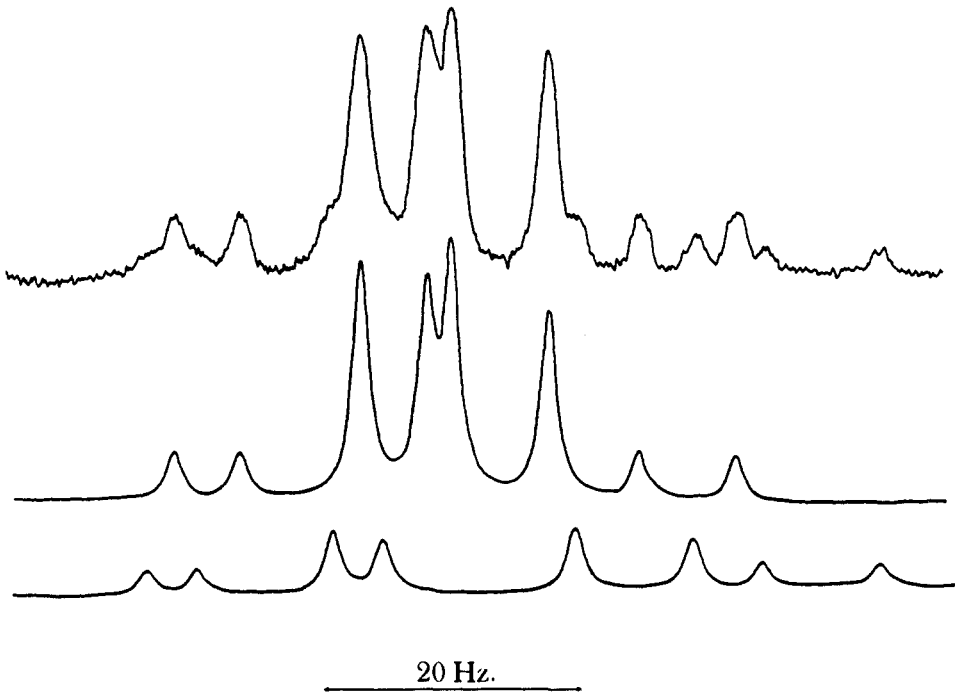


Figure 2. The AB part of the pmr spectrum of *N*-formyl-L-tryptophan.

cis and trans isomerism at the amide bond.[9] The minor spectrum contributes about 22% of the total intensity observed in this region of the spectrum. All spectra were analyzed for coupling constants and chemical shifts by utilizing the Ferguson–Marquardt computer program;[10] the resulting data are collected in Table I. There was no evidence of the minor conformational isomer in the aromatic region of the spectrum and it has been assumed that the same spectral parameters characterize these signals for both forms.

(9) L. A. LaPlanche and M. T. Rogers, *J. Amer. Chem. Soc.*, **86**, 337 (1964).
(10) R. C. Ferguson and D. W. Marquardt, *J. Chem. Phys.*, **41**, 2087 (1964).

A number of spectral changes are observed when the enzyme, α-chymotrypsin, is present in the inhibitor solutions. The aromatic signals are shifted upfield as increasing quantities of protein are included and, as indicated in Figure 1, the spectral lines broaden considerably. The chemical-shift effect for a given aromatic proton at each concentration of protein was estimated by considering the effect on the major lines in the submultiplet assigned to that nucleus. This procedure is based on the assumption that the coupling constants will not change detectably when the inhibitor molecule is bound to the enzyme. The line-broadening effects at higher protein concentrations made it difficult to determine shift effects with accuracy, and the pos-

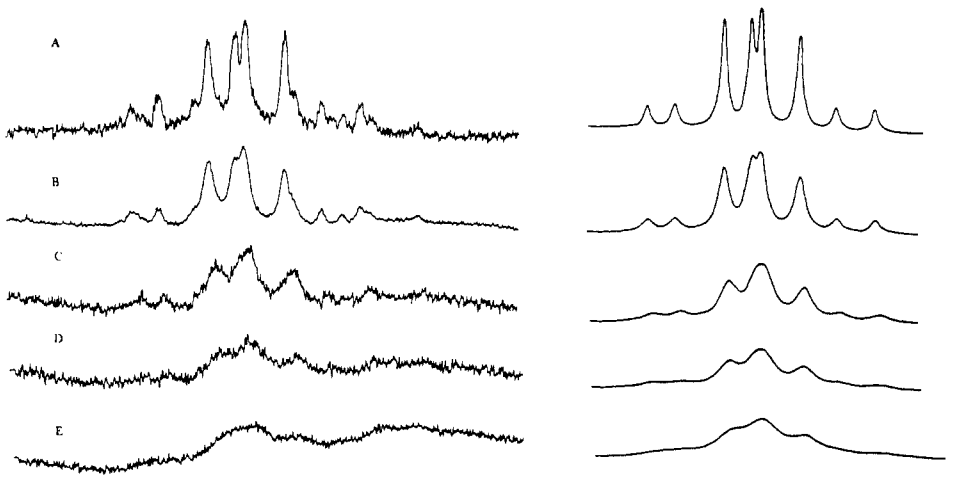


Figure 3. The effect of enzyme on the AB section of *N*-formyl-L-tryptophan. Each experimental trace is 100-Hz wide. The computed line shapes include only the contribution of the major isomer and were generated with line widths of 1.6, 2.3, 3.5, 5.0, and 7.0 Hz., respectively, for the same samples as used for the spectra in Figure 1.

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

	δ, ppm	J, Hz
A.	Aromatic protons[b]	
	δ_1 −5.7778	J_{12} = 8.13
	δ_2 −5.2300	J_{13} = 1.08
	δ_3 −5.3085	J_{14} = 0.85
	δ_4 −5.5705	J_{23} = 7.12
	δ_V −5.307	J_{24} = 0.91
		J_{34} = 8.35
B.	Alkyl protons	
	Major Minor	Major Minor
	δ_A −1.2398, −1.1230	J_{AX} = 7.85, 9.52
	δ_B −1.4186, −1.4601	J_{BX} = 5.12, 4.16
	δ_X −2.6602, −2.2923	J_{AB} = −14.88, −14.81
C.	Formyl proton	
	δ_F −6.016	$J_{X,F}$ = 0.9 ± 0.1, ~1

[a] Sample was 0.04 *M* in 0.4 *M* phosphate buffer, pD = 6.6. [b] Chemical shifts (δ_i) were relative to 0.005 *M* sodium acetate dissolved in sample. The root mean square error estimated for the chemical shifts by the computer program was ±0.0002 ppm and, for the coupling constants, ±0.02 Hz (aromatic protons) and ±0.03 Hz (aliphatic hydrogens).

sible errors in these estimates probably vary from 0.2 to 0.5 Hz, depending on protein concentration.

In order to quantitate the line-broadening effect, a series of computer generated theoretical curves was prepared which differed only in the line width of the Lorentzian function used to simulate a given transition in the theoretical spectrum. These were compared visually to the experimental curves until a reasonable match was obtained. Figure 1 illustrates the correspondence that could be obtained. Since each line in the spectrum in principle can have a different line width,[11] this procedure can only be approximate, but it is believed that the differential line broadening determined in this way is accurate to at least 1.0 Hz.

Although the chemical-shift effects are smaller in the alkyl portion of the spectrum (Figure 3), the line-broadening influence of the enzyme is more potent in this part of the spectrum. In analyzing this effect, the contribution of the minor rotational isomer was neglected and the computed curves in Figure 2 are derived only from data for the major form.

If one represents the binding of *N*-formyltryptophan to the enzyme by the simple equilibrium shown in eq 1, then the change in an nmr parameter (chemical

$$EI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E + I \tag{1}$$

shift or line width) relative to its value in the absence of protein is given by

$$x = \frac{[EI]}{I_0}\chi_{EI} \tag{2}$$

where x is the observed change, I_0 is the total concentration of inhibitor, and χ_{EI} is the value of the nmr parameter under consideration within the enzyme–inhibitor complex.[8,12] The concentration of the complex, [EI], can be computed from the relation

$$[EI] = \frac{(E_0 + I_0 + K_I) \pm [(E_0 + I_0 + K_I)^2 - 4E_0I_0]^{1/2}}{2} \tag{3}$$

as noted by Sykes.[13] Here E_0 represents the total enzyme concentration. When the inhibitor is a good one K_I is small relative to I_0 or E_0 and eq 3 reduces to $[EI] \approx E_0$ so that eq 2 can be written

$$x \cong \frac{E_0}{I_0}\chi_{EI} \tag{4}$$

Under these conditions a plot of x *vs.* the ratio of the enzyme and inhibitor concentrations should be linear, should pass through the origin, and should have a slope approximately equal to χ_{EI}. Available precedent suggests that K_I for the inhibitors used in this work should be 4 m*M* or less[14,15] and calculations using eq 3 show that for the protein and inhibitor concentrations used in this work, values of K_I up to 4 m*M* lead

(11) R. M. Lynden-Bell, *Proc. Roy. Soc., Ser. A*, **286**, 337 (1965).

(12) F. W. Dahlquist and M. A. Raftery, *Biochemistry*, 7, 3269 (1968).
(13) B. D. Sykes, *J. Amer. Chem. Soc.*, **91**, 949 (1969).
(14) D. D. F. Shiao, *Biochemistry*, **9**, 1083 (1970).
(15) J. T. Gerig and R. A. Rimerman, *J. Amer. Chem. Soc.*, **94**, 7558, 7565 (1972).

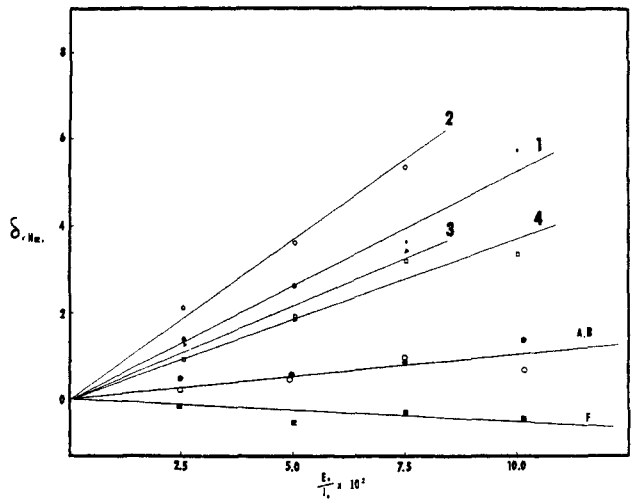


Figure 4. A plot of the observed enzyme-induced, chemical-shift effect *vs.* the enzyme–inhibitor concentration ratio.

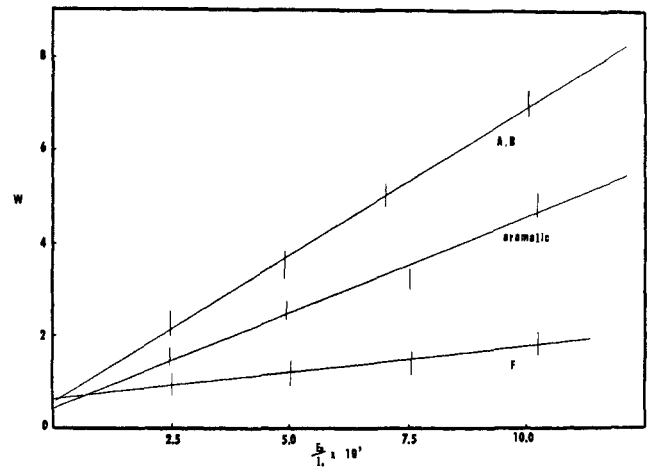


Figure 5. A plot of the observed enzyme-induced, line-width changes *vs.* the chymotrypsin–N-formyl-L-tryptophan concentration ratio.

to errors in the approximate eq 4 of less than 10%. It is doubtful that our data for enzyme-induced chemical-shift and line-width changes are more accurate than this and we have chosen to treat our experimental results according to eq 4.

Figure 4 records chemical-shift data for the various proton resonances of *N*-formyl-L-tryptophan in the presence of α-chymotrypsin when plotted according to eq 4 while Figure 5 summarizes the line-width changes observed with this system. In most cases, the observed effects are linear with E_0/I_0, within experimental error. The empirical chemical-shift and line-width parameters (χ_{EI}) for the protons of this inhibitor in the enzyme-bound form are collected in Table II;

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

Proton	Chemical shifts[b] *N*-Formyl-L-tryptophan	Chemical shifts[b] *N*-Formyl-D-tryptophan
H_1	0.52 ± 0.03	0.45 ± 0.04
H_2	0.71 ± 0.04	0.68 ± 0.06
H_3	0.48 ± 0.05	0.68 ± 0.07
H_4	0.31 ± 0.03	0.50 ± 0.04
H_V	0.0	0.09 ± 0.03
H_A	0.04 ± 0.02	0.23 ± 0.02
H_B	0.06 ± 0.02	−0.09 ± 0.01
H_F	−0.06 ± 0.02	−0.09 ± 0.01
	Line widths[c]	
H_1–H_4	40 ± 1	51 ± 4
H_A,H_B	47 ± 4	50 ± 5
H_F	10 ± 3	5 ± 1

[a] At 34°, pD 6.6, 0.4 *M* phosphate buffer. Slopes were calculated by least squares; the estimated standard deviation is appended to each value. *Cf.* G. W. Snedecor, "Statistical Methods," Iowa State University Press, Ames, Iowa, 1956, p 123. [b] In ppm. No sign indicates an upfield shift while the negative sign denotes a downfield shift. [c] In hertz.

included there are the corresponding data for the D isomer.

Establishing a quantitative relationship between the observed effects on the pmr spectrum of the inhibitor and the concentrations of protein and inhibitor present in solution is complicated by the known propensity of α-chymotrypsin to oligomerize under conditions of high protein and electrolyte concentration such as those used in this work.[16,17] Various models which take protein polymerization into account can be used to define the empirical parameters χ_{EI} and will be discussed below.

We have presumed that the major part of chemical-shift and line-width effects observed in these experiments is due to the binding of the inhibitor to the active center of the enzyme. However, it is known that hydrophobic dye molecules bind to this protein at several locations other than the active site.[18] Moreover, in the crystallographic study of the *N*-formyl-L-tryptophan–α-chymotrypsin complex, four binding sites other than the active center were found,[6] although these sites were interstitial in nature and were assumed to be artifacts of the crystalline state. In order to correct the nmr data described above for effects that might derive from binding to sites on the protein other than the active center, we have carried out a series of experiments with the serine-195 tosyl ester of α-chymotrypsin.[5,19] In the crystalline state, the toluenesulfonic acid moiety of this derivative occupies the same hydrophobic pocket that is taken up by the aromatic ring of the inhibitor, *N*-formyl-L-tryptophan. We have assumed that the native enzyme and its tosylated derivative are essentially identical conformationally in solution and that, because of the presence of the tosyl ring at the active center, binding of the inhibitor to the region near the active site will be greatly diminished while binding to other, secondary sites will not be perturbed. It was hoped, therefore, that the results with the tosyl enzyme would provide a reasonable base line from which to measure nmr effects that could be associated directly with the active center of the native enzyme. Overall, the spectral effects (Table III) seen with this enzyme derivative are smaller than those observed with the native material, but, nonetheless, they indicate an appreciable contribution of secondary

(16) M. S. N. Rao and G. Kegeles, *J. Amer. Chem. Soc.*, 80, 5724 (1958).
(17) K. C. Aune and S. N. Timasheff, *Biochemistry*, 10, 1609, 1617 (1971).
(18) A. N. Glazer, *J. Biol. Chem.*, 242, 4528 (1967).
(19) (a) D. E. Fahrney and A. M. Gold, *J. Amer. Chem. Soc.*, 85, 997 (1963); (b) A. M. Gold, *Biochemistry*, 4, 897 (1965).

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

Proton	Chemical shifts[b] N-Formyl-L-tryptophan[c]	Chemical shifts[b] Formyl-D-tryptophan
H_1	0.19 ± 0.01 (0.15)	0.09 ± 0.04
H_2	0.26 ± 0.01 (0.21)	0.19 ± 0.02
H_3	0.26 ± 0.03 (0.16)	0.22 ± 0.05
H_4	0.21 ± 0.02 (0.15)	0.18 ± 0.05
H_V	0.03 ± 0.02 (−0.03)	0.01 ± 0.03
H_A	−0.04 ± 0.02 (−0.05)	−0.03 ± 0.05
H_B	−0.03 ± 0.05 (−0.08)	−0.05 ± 0.07
H_F	−0.04 ± 0.01 (−0.13)	−0.01 ± 0.05
	Line widths[d]	
H_1–H_4	19 ± 2 (19)	11 ± 2
H_A,H_B	13 ± 2 (15)	15 ± 3
H_F	3 ± 1 (3)	2 ± 1

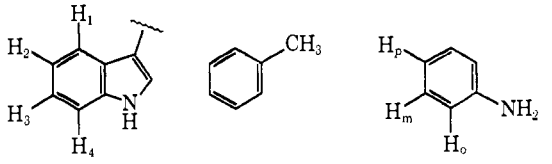
[a] At 33°, pD 6.6, 0.4 *M* phosphate buffer. Estimated standard deviation appended. [b] In ppm. [c] The parenthesized value given was obtained with chromatographically purified tosyl enzyme. [d] In hertz.

site binding to the total effects produced by the native enzyme. Use of chromatographically purified tosyl enzyme produced data that were not significantly different from those observed with the unpurified material.[20] We, therefore, assume that low molecular weight polypeptides present in the native enzyme and, presumably in the unpurified tosyl enzyme, do not contribute to the nmr effects reported above.

Discussion

Several important points must be considered before one can make conclusions from the data presented above. These are discussed in turn below.

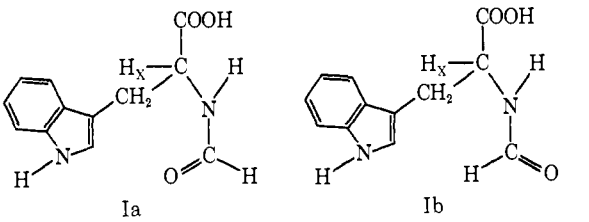
Nmr Spectral Assignment and Conformation of the Inhibitor. The coupling constant pattern observed for the aromatic ABCD spectrum of *N*-formyltryptophan serves only to identify the relative position of the four nuclei and does not directly specify which of the four chemical shifts may be assigned to a given nucleus. In order to make this assignment the indole ring of I



I

was considered to represent a substituted benzene ring. Alkyl substituents such as methyl do not exert a pronounced effect on the chemical shifts of the adjacent aromatic protons. However, with amino or *N*-alkylamino substituents, the aromatic protons ortho and para to the amino function are shifted upfield substantially more (~0.5 ppm) than those at the meta position.[21] On this basis we expect that H_2 and H_4 of the inhibitor molecule will resonate at higher fields than H_1 or H_3. With this assumption and the observed coupling constants, the assignment of the multiplet at lowest field to H_1 can confidently be made with the other aromatic chemical shifts assigned as indicated in Table I.

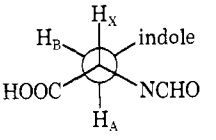
The appearance of evidence for two rotational isomers in the pmr spectrum of *N*-formyltryptophan is not unexpected.[9] An important question pertains to which of the two possible rotamers (Ia or Ib) is present in greatest abundance. It has been observed in simple *N*-alkylformamides that the resonance position of protons on *N*-alkyl substituents cis to the amide carbonyl appears at lower applied magnetic field than when the substituent is trans.[9] This situation holds both for the neat liquids and in deuterium oxide solutions. The protons H_X in the molecules used in this work are in a molecular environment analogous to the *N*-methyl protons in *N*-methylformamide and since H_X for the most abundant isomer of I resonates at lower field than does the corresponding signal for the less abundant isomer, we suggest that the cis form of the inhibitor (Ia) is the prevalent (~80%) conformational isomer at the amide group. The X-ray crystallographic work indicates that Ia is the form that binds to the enzyme.[6]



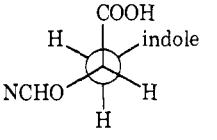
Ia

Ib

Conformational isomerism is also possible about the C_α–C_β bond giving rise to three possible staggered rotamers for I. The magnitudes of the coupling constants (J_{AX}, J_{BX}) observed for the alkyl portion of the spectrum are consonant with the proposition that either Ic or Id is the dominant species in solution. It might be noted in this regard that an X-ray study of glycyl-L-tryptophan provides evidence that the rotational isomer corresponding to Ic is the one found in the crystalline state of this dipeptide.[22] It is reasonable, therefore, to expect Ic to be the dominant solution form of the inhibitor.[23] No large coupling constant changes are apparent when the pmr spectrum of I is observed in the presence of the enzyme and we tentatively take this to mean that the most probable isomer (Ic) in solution is also the one that is bound most firmly to the protein. This is admittedly not a strong argument since appreciable coupling constant changes would probably not be expected until the enzyme-induced, line-broadening effects are large enough to prohibit measurements of these changes with any accuracy.



Ic



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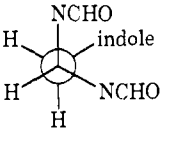

Ie

Exchange Kinetics. Any interpretation of the data in Tables II and III will depend upon the rate of exchange

(20) A. Yapel, M. Han, R. Lumry, A. Rosenberg, and D. F. Shiao, *J. Amer. Chem. Soc.*, **88**, 2573 (1966).

(21) J. N. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," Pergamon Press, New York, N. Y., 1966, p 750.

(22) R. A. Pasternack, *Acta Crystallogr.*, **9**, 341 (1956).

(23) Cavanaugh has concluded that the rotamer of the tryptophan anion analogous to Ic is the major rotational isomer in solution.[24] The amino group and formamido group should be somewhat similar sterically so that it is not unreasonable to expect similar conformational preferences in I and tryptophan.

(24) J. R. Cavanaugh, *J. Amer. Chem. Soc.*, **92**, 1488 (1970).

of the inhibitor molecule between the free-solution state and the enzyme-bound state or states; at issue is the validity of the "fast-exchange" assumption made in the derivation of eq 2 as it applies to both chemical-shift and line-width effects. Swift and Connick have shown that for exchange between two sites when one component is in large concentration excess over the other, the resonance position of the major line is shifted an amount δ given by

$$\delta = \frac{P_{EI}k_1^2\Delta}{(\pi W_{EI} + k_1)^2 + (2\pi\Delta)^2} \quad (5)$$

where P_{EI} refers to the mole fraction of EI complex and W_{EI} and Δ are the line width and chemical shift of a given inhibitor resonance.[25] If the dissociation rate constant, k_1, is much larger than πW_{EI} or $2\pi\Delta$, then eq 5 reduces in form to eq 2. For line widths the corresponding equation is

$$\Delta W_{obsd} = P_{EI}k_1\frac{\pi W_{ES}^2 + W_{ES}k_1 + 4\pi\Delta^2}{(\pi W_{ES} + k_1)^2 + (2\pi\Delta)^2} \quad (6)$$

where ΔW_{obsd} is the observed line-width change in the major resonance.[25] For fast-exchange averaging of line widths the conditions $k_1 \gg \pi W_{ES}^2$ and $k_1 \gg 4\pi\Delta^2$ must be met; under these conditions eq 6 reduces to eq 2.

The extent to which k_1 must be greater than $2\pi\Delta$ to bring about a fast-exchange averaging of chemical shifts was investigated by computer simulation of high-resolution line shapes.[26] Taking the highest protein concentration used in this work and using a chemical shift of 60 Hz and a line width of 30–40 Hz for the enzyme–inhibitor complex, it was found by simulation that $k_1 = 1 \times 10^3$ ($\cong 3 \times 2\pi\Delta$) led to an error of $\sim$0.4 Hz in the position of the averaged resonance, while for $k_1 = 2 \times 10^3$ or larger, the error was 0.1 Hz or less. Dissociation rate constants greater than 10^4 sec^{-1} resulted in averaged line widths that were exchange broadened by less than 0.2 Hz in these simulations.

We do not have direct evidence regarding the rate of exchange in this system as yet, but the results obtained by several other investigators are instructive. Working with the *N*-acryloyl derivative of tryptophan Hess and coworkers found that the rate constant for dissociation of the enzyme–inhibitor complex formed between α-chymotrypsin and this material was 2.7×10^3 sec^{-1} at pH 7.4 and 15°.[27] For *N*-acetyl-D-tryptophan the rate constant measured by a dye displacement technique was 4×10^2 sec^{-1} at pH 8 and 20°, although the same rate constant for the L isomer is claimed to be three orders of magnitude smaller than for the D isomer.[28] Work in our laboratory with Carr–Purcell[29] pulsed nmr experiments indicates that this dissociation rate constant for trifluoroacetyl-D-tryptophan at pH 6.6 is greater than 7×10^3 sec^{-1}.[30] The dissociation rate constant for the proflavine–chymotrypsin complex is 1.7×10^3 sec^{-1} at pH 6.2 and 22.5° in 0.2 *M* phosphate buffer, conditions roughly analogous to those used in this work.[31] Considering these data, a reasonable estimate for the dissociation rate constant, k_1, in equilibrium 1 at pH 6.6 and 34° is 2×10^3 sec^{-1} when the inhibitor I is *N*-formyl-L-tryptophan.

If the assumed magnitude for k_1 is correct then both the conditions derived from the Swift and Connick equations and the line-shape simulation experiments suggest that our chemical shift data indeed are derived from a fast-exchange situation. The situation is less clear for the line-width parameters and an appreciable line-width variation due to chemical exchange may be present. When exchange is not quite rapid enough to give complete averaging of the nmr signals from the free and bound inhibitor, the observed line-width effect is given by[32]

$$\Delta W_{obsd} = P_{EI}W_{EI} + \frac{(P_{EI} - 2P_{EI}^2 + P_{EI}^3)4\pi\Delta^2}{k_1} \quad (7)$$

Assuming as we have before that $P_{EI} \simeq E_0/I_0$, the slope of a plot of ΔW_{obsd} against E_0/I_0 will be the first derivative of eq 7 or

$$\text{slope} \cong W_{EI} + \frac{4\pi\Delta^2}{k_1} \quad (8)$$

where higher order terms in P_{EI} have been neglected since P_{EI} is small. The chemical-exchange contribution to the observed slopes of these plots can be estimated to be about 23 Hz if $k_1 = 2 \times 10^3$ and $\Delta = 60$ Hz.[33] Calculations of this type serve to warn that possibly up to one-half of the observed line widths for the aromatic proton resonances of the *N*-formyltryptophan complexes can be due to exchange effects. However, for the alkyl protons, the enzyme-induced, chemical-shift effects are small and exchange must make a negligible contribution to the observed line-width effects in these cases.

Oligomerization of the Enzyme. The self-association of α-chymotrypsin has been widely studied by a variety of physical and chemical techniques.[16,17,31] Below pH 6, the enzyme exists predominantly as monomeric and dimeric species and high ionic strengths appear to enhance dimer formation.[17] At pH 6.2 in 0.2 *M* phosphate buffer Rao and Kegeles found that α-chymotrypsin is extensively dimerized and trimerized[16] while Tinoco has found that at neutral pH ionic strengths greater than 0.1 inhibit the polymerization of this enzyme.[34] The influence of inhibitor binding on oligomerization of α-chymotrypsin has also been investigated. Sarfare, *et al.*, have presented evidence that hydrocinnamic acid binds equally well to the monomeric or oligomeric forms of the enzyme.[35] More recently, Faller and La Fond have suggested that the dye proflavin binds well only to the monomeric protein.[31] Kinetic data gener-

(25) T. J. Swift and R. E. Connick, *J. Chem. Phys.*, 37, 307 (1962).
(26) C. S. Johnson, Jr., *Advan. Magn. Resonance*, 1, 33 (1965).
(27) G. P. Hess, J. McConn, E. Ku, and G. McConkey, *Phil. Trans. Roy. Soc. London, Ser. B*, 257, 89 (1970).
(28) A. Yapel, Ph.D. Dissertation, University of Minnesota, 1967.
(29) (a) H. Y. Carr and E. M. Purcell, *Phys. Rev.*, 94, 630 (1954); (b) H. S. Gutowsky, R. L. Vold, and E. J. Wells, *J. Chem. Phys.*, 43, 4107 (1965); (c) A. Allerhand and E. Thiele, *ibid.*, 45, 902 (1966).
(30) J. T. Gerig and A. D. Stock, work in progress, reported in preliminary form at the Second Enzyme Mechanism Conference, Santa Barbara, Calif., Dec 27–29, 1970. Professor J. H. Richards has informed us that his group has measured this rate constant under similar conditions and obtained a value of 4×10^3 sec^{-1} (private communication).
(31) L. D. Faller and R. E. La Fond, *Biochemistry*, 10, 1033 (1971).
(32) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., New York, N. Y., 1959, p 222.
(33) High-resolution line-shape simulations indicate that eq 7 overestimates the exchange contribution to the observed line width by 40–50% at $k_1 = 2 \times 10^3$; the exchange effect quoted above can be regarded as an upper limit.
(34) I. Tinoco, *Arch. Biochem. Biophys.*, 68, 367 (1957).
(35) P. S. Sarfare, G. Kegeles, and S. J. Kwon-Rhee, *Biochemistry*, 5, 1389 (1966).

ally seem to indicate that only the complexes with the monomeric form of the protein can go on to give product.[36] In order to gain insight into the possible effects of protein association on the experiments reported herein, the following series of equilibria for the binding of the inhibitor (I) to α-chymotrypsin were considered.

$$E_2 \overset{K_D}{\rightleftharpoons} 2E \quad (9)$$

$$E_3 \overset{K_T}{\rightleftharpoons} E_2 + E \quad (10)$$

$$E_1I \rightleftharpoons E + I \quad (11)$$

$$E_2I \rightleftharpoons E_2 + I \quad (12)$$

$$E_2I_2 \rightleftharpoons E_2I + I \quad (13)$$

$$E_3I \rightleftharpoons E_3 + I \quad (14)$$

$$E_3I_2 \rightleftharpoons E_3I + I \quad (15)$$

$$E_3I_3 \rightleftharpoons E_3I_2 + I \quad (16)$$

The first two equations represent formation of enzyme dimers (E_2) and trimers (E_3) in accord with the equilibrium constants K_D and K_T. Subsequent steps correspond to binding of an inhibitor (I) to the monomeric (E) and oligomeric forms of the enzyme, assuming that the dimer has two strong binding sites and the trimer, three. Accepting that each binding locus can exert an observable effect on the pmr spectrum of the inhibitor requires the modification of eq 2 to

$$x = \frac{EI}{I_0}\chi_{EI} + \frac{E_2I}{I_0}\chi_{E_2I} + \frac{E_2I_2}{I_0}\chi_{E_2I_2} + \frac{E_3I}{I_0}\chi_{E_3I} + \frac{E_3I_2}{I_0}\chi_{E_3I_2} + \frac{E_3I_3}{I_0}\chi_{E_3I_3} \quad (17)$$

in which we have assumed that the enzyme-induced effects (χ) for all binding sites in a given type of complex are identical. To keep the number of unknown parameters within manageable bounds it was also assumed that the dissociation constants for the various complexes (eq 9–14) are equal and that K_D and K_T are 9.6×10^{-4} and 2.9×10^{-4}, respectively, as found by Rao and Kegeles under similar but not identical conditions.[12] A computer was employed to iteratively solve the resulting equations. These solutions showed that for the range of initial enzyme and inhibitor concentrations employed in this work and at dissociation constants 5 mM or less, the predominant enzyme–inhibitor complexes are EI, E_2I_2, and E_3I_3. Figure 6 shows the computed variation of the concentrations of these species over the range of initial protein concentrations used.

The concentrations of monomeric or trimeric complex do not individually correlate well with the observed chemical-shift effects although plots of the mole fraction of E_2I_2 (the dimeric complex) against the observed shifts are reasonably linear. However, the slopes of these plots imply that the enzyme-induced, chemical-shift effects must be 1–4 ppm—values that are unprecedentedly large. In order to fit this model to our data one must assume either (1) that χ_{EI}, $\chi_{E_2I_2}$, and $\chi_{E_3I_3}$ for chemical shifts are either of the same sign and similar magnitude or (2) that χ_{EI} and $\chi_{E_3I_3}$ are of opposite signs and together act to mitigate the influence of $\chi_{E_2I_2}$ in eq 15. The most reasonable conclusion within the framework of this postulate seems to us to be that the nmr spectral responses induced by the three types of binding sites are similar if not identical.

(36) (a) R. B. Martin and C. Niemann, *J. Amer. Chem. Soc.*, 80, 1473 (1958); (b) F. J. Kezdy and M. L. Bender, *Biochemistry*, 4, 104 (1965); (c) L. D. Faller, *J. Biol. Chem.*, 246, 7446 (1971).

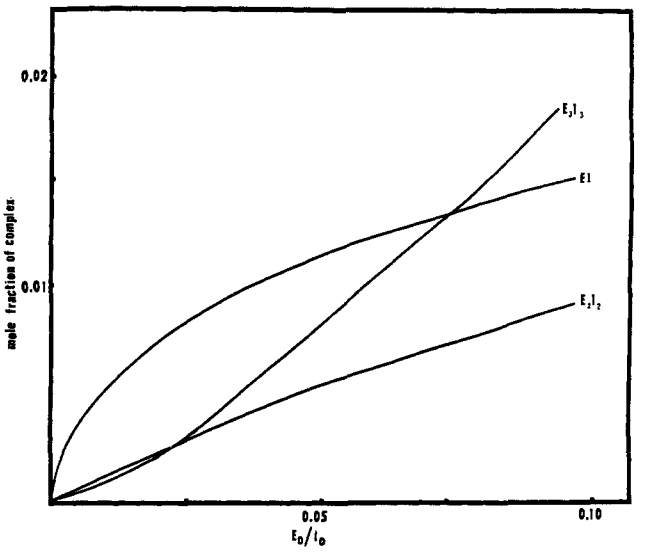

Figure 6. Variation of the mole fraction of EI, E_2I_2, and E_3I_3 complexes with $E_0:I_0$ concentration ratio. Values were calculated assuming $K_I = 2 \times 10^{-3}$ M, $K_D = 9.6 \times 10^{-4}$ M, and $K_T = 2.9 \times 10^{-4}$ M by iterative solution of eq 9–16 in the text.

Another description of the binding of *N*-formyltryptophan to α-chymotrypsin can be based upon the model of Faller and La Fond for the proflavine–chymotrypsin interaction. In this case one can assume that only the monomeric protein is able to bind the inhibitor; equilibria 9–11 would describe this situation. Computer solution of these equations shows that at low $E_0:I_0$ concentration ratios the mole fraction of monomeric enzyme–inhibitor complex increases linearly with $E_0:I_0$ and is essentialy equivalent to the mole fraction of total binding sites computed according to the alternative model discussed above (Figure 7). Therefore, plots of observed enzyme-induced nmr effects (chemical shifts or line-width variations) against $E_0:I_0$ for the range of values used in this work would have nearly the same slope irrespective of whether the inhibitor binds only to the monomeric enzyme or equivalently to all oligomeric forms of the enzyme. Our chemical-shift and line-width data cannot, therefore, be used to distinguish between the possible binding situations described above for interaction of *N*-formyl-L-tryptophan with α-chymotrypsin. Experiments at high $E_0:I_0$ concentration ratios would be useful in deciding between these possibilities; we have not yet attempted such work because of the long periods of signal averaging that would be required to give usable results and because resonances from the protein would complicate the spectrum greatly.

Enzyme-Induced Relaxation Effects. It is likely that the line widths for the pmr signals of the inhibitor in the enzyme–inhibitor complex are determined by nuclear dipole–dipole interactions.[37] Neglecting the influence of chemical-shift differences, the effect of these interactions is given within the extreme narrowing approximation by $w_i = (1/\pi T_{2,i}) = (C\tau i/\pi)\Sigma_j \cdot$

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

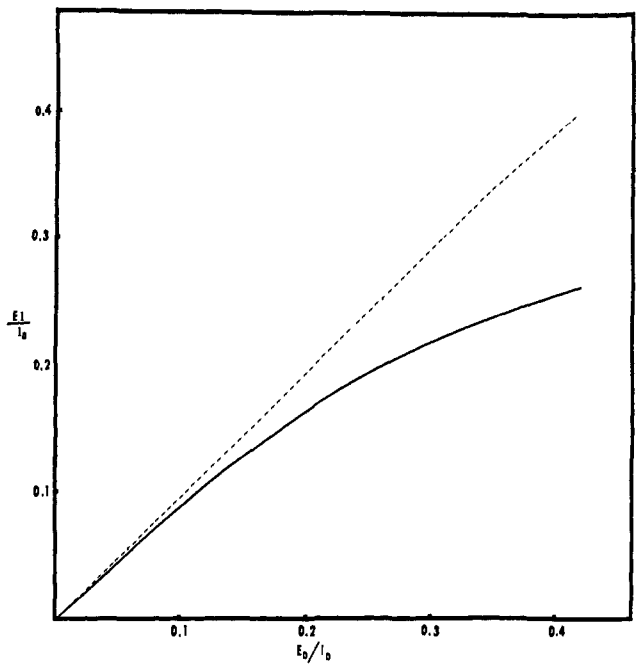

Figure 7. The mole fraction of monomeric enzyme–inhibitor complex (EI/I_0) calculated assuming oligomerization of the enzyme and $K_1 = 2 \times 10^{-3}$ M, $K_D = 9.6 \times 10^{-4}$ M, and $K_T = 2.9 \times 10^{-4}$ M as a function of $E_0:I_0$ concentration ratio (solid line). The dotted line represents the mole fraction of total binding sites computed with the assumption that enzyme monomer, dimer, and trimer have one, two, and three equivalent binding sites, respectively.

r_{ij}^{-6}, where w_i is the nmr line width for nucleus i, $T_{2,i}$ is the corresponding transverse relaxation time, r_{ij} is the internuclear distance between nucleus i and nucleus j, C is a constant, which has the value 88.5 $\times 10^{10}$ when both nuclei i and j are protons, and τ_i is a correlation time characteristic of the relative motion of these nuclei. We have computed the correlation times, τ_i, for the various hydrogen atoms of *N*-formyltryptophan in the enzyme–inhibitor complex using the (corrected) line-width data presented above and the atomic coordinates estimated for these nuclei using the available data for the crystalline enzyme–inhibitor complex,[6] as collected in Table IV. The results of these calculations, given in Table V, indicate

Table IV. Estimated Hydrogen Atom Coordinates of *N*-Formyl-L-tryptophan[a]

Atom	x	y	z
H_1	17.8	−2.1	7.3
H_2	19.9	−3.7	8.2
H_3	21.7	−4.4	6.7
H_4	21.9	−4.2	4.1
H_V	18.0	−0.8	2.3
H_A	16.8	0.6	5.6
H_B	15.8	−0.6	6.1
H_X	15.8	0.0	3.3

[a] Given in the same coordinate system as used in Table II of ref 6.

that the correlation time for the alkyl part of the L inhibitor is about 0.3 $\times 10^{-8}$ sec while the average value for the aromatic protons is about 1.9 $\times 10^{-8}$ sec. Values for the D inhibitor are similar, although somewhat larger in the latter case. If we assume that the

Table V. Computation of Correlation Times for EI Complexes[a]

Nucleus	$w^{1/2}$, corr[b] D	$w^{1/2}$, corr[b] L	$\sum_{i,j} \frac{1}{r_{ij}^6}$	$\tau_c \times 10^8$ sec D	$\tau_c \times 10^8$ sec L
H_A	47	45	0.0558	0.3	0.3
H_B	47	45	0.0556	0.3	0.3
H_1	53	28	0.0053	3.6	1.9
H_2[c]	(53)	(28)	0.0070	2.7	1.4
H_3[c]	(53)	(28)	0.0080	2.4	1.2
H_4[d]	53	28	0.0033	5.7	3.0

[a] At pD 6.6 in 0.4 M phosphate buffer. [b] The difference between the native and tosyl enzymes corrected for the number of titrated active sites; exchange effects have not been taken into account nor have interactions with protons of the protein been considered. [c] Line widths assumed to be the same as those for H_1 and H_2. [d] Nuclear relaxation at H_4 will likely be affected by the ^{14}N quadrupole of the indole ring as well as N–H solvent exchange processes at this position; these effects were not included in the calculation.

indole ring of the inhibitor is bound tightly enough to the enzyme so that the correlation time of the inhibitor and enzyme are identical, it would be anticipated that $\tau_c = \sim 1.4 \times 10^{-8}$ sec if the enzyme is monomeric or $\tau = \sim 2.8 \times 10^{-8}$ sec if the protein is present as the dimer.[38] As noted above, the large chemical-shift effects found for the aromatic protons of the inhibitor could lead to an appreciable exchange-rate contribution to the observed line width so that the correlation time estimated for the aromatic protons of the inhibitor within the enzyme-bound complex may be too large by a factor of two or so. It is also possible that dipolar interactions between protons bound to the protein and those of the inhibitor can contribute to the observed relaxation effects. Consideration of a model of the solid-state structure of this complex suggests that interactions between the side chains of valine-213, serine-190, and glycines-216 and -226 might contribute to the relaxation of the aromatic nuclei of the inhibitor.[40] However, the distances between the inhibitor protons and the protons of these amino acids are generally in the range 2.5–3.5 Å so that these contributions to relaxation should not exceed 10% of the observed effect. Even after correction for these contributions to relaxation of the aromatic protons of I, the correlation times estimated above suggest that the indole rings of both enantiomers of *N*-formyltryptophan are bound rather tightly to the enzyme while the alkyl portion of the molecule enjoys some molecular freedom in addition to that defined by the motion of the enzyme as a whole. The differences in τ_c found for the D and L isomer could easily be the result of differences in exchange rate, however.

Chemical-Shift Effects. The relatively small enzyme-induced chemical-shift effects at the alkyl (A, B),

(38) These correlation times were computed using the relation $\tau_c = (\eta V/kT)$, where η is the viscosity of the solution at the temperature T, k is Boltzmann's constant and V is the molecular volume.[32] The viscosity of the protein-containing solution was estimated from Einstein's equation;[39] these estimates agree well with experimental viscosities (R. A. Rimerman, preliminary experiments). The molecular volume of the protein molecule in solution was assumed to be the same as that given by the molecular dimensions in the crystalline state (45 Å × 38 Å × 35 Å); the molecular volume of the dimer was assumed to be twice that of the monomer.

(39) R. B. Martin, "Introduction to Biophysical Chemistry," McGraw-Hill, New York, N. Y., 1964, p 166.

(40) Recently reported nuclear Overhauser effects indicate an interaction between alkyl amino acid side chains of chymotrypsin and tryptophan within the enzyme–inhibitor complex; A. A. Bothner-By, P. Balarum, and G. Gassend, Paper 1.1, 13th Experimental Nmr Conference, Pacific Grove, Calif., May 1, 1972.

vinyl (H_V), and formyl (H_F) protons of these inhibitors are probably too imprecise at this stage to justify detailed interpretation. However, the resonance positions of the four aromatic protons (H_1–H_4) are substantially shifted to higher fields in the enzyme–inhibitor complex. We note that in the crystalline state the aromatic side chain of the tyrosine-228 residue lies moderately close to the tryptophan ring. Using the available atomic coordinates for the *N*-formyl-L-tryptophan complex,[6,41] it was possible to estimate the aromatic ring current effect of this tyrosine residue assuming that its substituted aromatic ring would exert the same influence as a simple benzene ring.[42] The results of these calculations are summarized in Table VI and contrasted therein to the experimental shifts.

Table VI. Chemical-Shift Effects in *N*-Formyltryptophan Complexes

Nucleus	ρ^a	z^a	Δ, calcd[b]	Δ^L, corr[c]	Δ^D, corr[c]
H_1	2.0	4.1	0.16	0.45	0.48
H_2	1.7	2.7	0.36	0.60	0.65
H_3	2.7	3.7	0.10	0.30	0.61
H_4	3.4	5.3	0.0	0.13	0.50

[a] Coordinates of proton relative to the center of the tyrosine ring according to the convention of Johnson and Bovey,[41] in units of the radius of the benzene ring. [b] Calculated chemical shift effect in the enzyme–L-inhibitor complex in ppm. [c] Observed chemical shift effects in ppm, taken from data in Tables II and III and corrected for 75% enzyme activity.

Quantitative comparison of these observed and calculated chemical-shift effects is uncertain for several reasons. First, the coordinates of the atoms of the inhibitor and the protein can be in error by ±0.5 Å so that there may be appreciable errors in the various distances utilized in the ring current calculations.[6] (These uncertainties are also present in the relaxation–correlation time calculations described above.) In addition, the quantitative validity of the Johnson–Bovey treatment of ring current effects for nuclei above the mean plane of the aromatic π system remains to be demonstrated. It is possible that the carbonyl groups of the peptide functions about the inhibitor in the active site can make appreciable contributions to the observed shielding effects, although, again, the quantitative aspects of the various models for anisotropic shielding by the carbonyl group have not been verified, especially at large distances. (Indeed, the question of which is the best model has not been settled.[43]) We have neglected these contributions. Finally, there may well be a differential solvent effect on the chemical shifts of the inhibitor molecule as it is transferred from a polar, aqueous milieu to what is probably a nonpolar, hydrocarbon-like environment at the enzymic active site.[6,44] This effect should be relatively nonspecific as regards the position of the protons on the indole ring of the inhibitor; an influence of a nonspecific nature is indicated by the tendency of all of the observed values in Table VI to be larger than the computed values by a constant amount (~0.2 ppm). When these uncertainties are considered, the agreement between calculated and observed enzyme-induced chemicals shifts for the enzyme–L-inhibitor complex is reasonable enough to be consistent with the proposition that the structure of this complex in solution resembles the structure in the solid state.

Taken together, the pmr experiments with the *N*-formyl-L-tryptophan complex described above can be interpreted easily in terms of the structure of this complex in the crystalline state. The quantitative aspects of the observed nuclear relaxation and chemical shift effects of the enzyme on the inhibitor molecules are convincingly accounted for by the geometrical parameters of the solid-state structure and provide evidence that the solution-state and solid-state structures of the *N*-formyl-L-tryptophan–α-chymotrypsin complex are quite similar, if not identical. The nmr results indicate that the D isomer binds slightly more tightly to the enzyme and in a somewhat different manner than the L isomer of the inhibitor.

Experimental Section

***N*-Formyl-L- and -D-tryptophan** were prepared by minor modification of the procedures of Kanoaka, *et al.*,[46] and Dalgliesh.[47] To L- or D-tryptophan (0.1 mol, Mann Research Laboratories) and 40 ml of 98–100% formic acid was added 0.1 mol of acetic anhydride with stirring. The mixture was stirred until all solid material dissolved and then let stand without stirring for 0.5 hr. Water (200 ml) was added, and the mixture was rapidly stirred for 1 hr. The solid so produced was recrystallized from 20% ethanol–water to constant optical rotation. The L isomer (mp 122–127°) had a rotation $[\alpha]^{26}D$ +44.5° (*c* 1, 95% ethanol). The data for the D isomer were mp 145–146° and $[\alpha]^{26}D$ −44.6 (*c* 1, 95% ethanol). Excepting the literature value for the rotation, $[\alpha]^{20}D$ 48 ± 1° (*c* 1, 95% ethanol),[48] these materials are at least 96% optically pure.

α-Chymotrypsin (three times recrystallized, salt free) was obtained from Worthington Biochemicals Corp., Lot No. CDI 8LK.

Deuterium oxide (99.8%) was obtained from Stohler Isotope Chemicals or International Chemical and Nuclear Corp.

Tosylchymotrypsin was prepared according to the method of Sigler, *et al.*[49] The resulting protein was shown to be >98% catalytically inactive by an assay involving the hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide.[50] Chromatographed tosylchymotrypsin was obtained following the technique of Yapel, *et al.*,[20] and employed a 3.5 cm × 100 cm column of Sephadex G-25 prepared and calibrated by Dr. L. Aronson.

A phosphate buffer (0.4 *M* in ~95% D_2O) was used throughout this work The buffer solution was prepared by adding 4.3 g of $NaH_2PO_4 \cdot H_2O$ and 1.2 g of $Na_2HPO_4 \cdot 7H_2O$ to 100 g of deuterium oxide and adjusting the apparent pH (glass electrode) to 6.2 with concentrated phosphoric acid or sodium hydroxide solutions. The pD of the solution was computed according to the equation pD = pH (glass electrode) + 0.4;[51] no account was taken of the isotopic composition of the solvent and it was assumed that 95% D_2O would behave to a good approximation the same as 100% D_2O. The apparent pH of the samples used in the nmr experiments was checked after the inhibitor and enzyme had been added and readjusted if necessary. Sodium acetate (0.005 *M*) was used as an internal reference. Samples were run the same day that they were prepared.

Pmr spectra were obtained with a Varian Associates HA-100 spectrometer operating at 100 MHz. The residual HOD peak was

(41) J. J. Birktoft, B. W. Matthews, and D. M. Blow, *Biochem. Biophys. Res. Commun.*, 36, 131 (1969).

(42) C. S. Johnson and F. A. Bovey, *J. Chem. Phys.*, **29**, 1012 (1958). The table of shielding contributions given in ref 15, p 595, was used.

(43) (a) J. A. Pople, *J. Chem. Phys.*, 37, 60 (1962); (b) J. W. ApSimon, W. G. Craig, P. V. Demarco, D. W. Mathieson, A. K. G. Nasser, L. Saunders, and W. B. Whalley, *Chem. Commun.*, 754 (1966).

(44) Solubilization of aromatic molecules in micellar systems usually results in an upfield shift of the pmr signals from the aromatic ring.[45]

(45) (a) J. C. Ericksson, *Acta Chem. Scand.*, **17**, 1478 (1963); (b) T. Nakagawa and K. Tori, *Kolloid-Z. Z. Polym.*, **194**, 143 (1964); (c) J. C. Ericksson and G. Gillberg, *Acta Chem. Scand.*, **20**, 2019 (1966).

(46) Y. Kanoaka, E. Sato, and Y. Ban, *Chem. Pharm. Bull.*, **15**, 101 (1967).

(47) C. E. Dalgliesh, *J. Chem. Soc.*, 137 (1952).

(48) British Patent 745,097; *cf. Chem. Abstr.*, **51**, 9701f (1957).

(49) P. B. Sigler, B. A. Jeffrey, B. W. Matthews, and D. M. Blow, *J. Mol. Biol.*, **15**, 175 (1966).

(50) B. R. Baker and J. A. Hurlburt, *J. Med. Chem.*, **10**, 1129 (1967).

(51) T. H. Fife and T. C. Bruice, *J. Phys. Chem.*, 65, 1079 (1961).

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

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