pyridine–DMF mixture (1:1, v/v) was heated to 100° for 15 min. The pale yellow solution was diluted with water and the suspension was stirred at 20° for 1 hr. The solid was collected, washed with water, and dried. The material (0.095 g, 95% recovery) was demonstrated to be homogeneous (system A) with the same R_f as starting material, mp 151–153°.

t-Butyl S,S′-N-Carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycyl-S-trityl-L-cysteinylglycyl-L-valinate (X). A cold suspension of 0.437 g (0.4 mmole) of II (obtained *via* the thiocyanogen reaction) and 0.231 g (0.4 mmole) of IX in a pyridine–DMF mixture (3:2, v/v) was treated with 0.083 g of DCC. The viscous reaction mixture was stirred at −10° for 2 hr and at 25° for 16 hr. Dilution with water afforded a solid which was collected, washed with water, and dissolved in methanol (20 ml). The clear solution was cooled and the small amount of precipitated solid was filtered. The filtrate was evaporated to a residue which crystallized from an acetone–hexane mixture (4:3, v/v, 25 ml) to yield (after drying to constant weight) 0.490 g (74%) of X, homogeneous (system A): mp 228°, $[\alpha]^{30}D$ +17.3° (*c* 0.52, DMF). The amino acid analysis was $CySO_3H_{3.9}$, $Gly_{4.2}$, $Val_{1.0}$, and $Phe_{2.0}$. *Anal.* Calcd for $C_{87}H_{97}N_{11}O_{14}S_4$: C, 63.37; H, 5.93; N, 9.34; S, 7.78; mol wt, 1649. Found: C, 63.29; H, 6.10; N, 9.26; S, 7.56; mol wt, 1642.

S,S′-N-Carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-S-trityl-L-cysteinylglycyl-L-valine (VI). To a solution of 0.137 g (0.083 mmole) of X in 2.5 ml of dry acetic acid was added 0.127 ml (1.0 mmole) of boron trifluoride–diethyl ether. The solution was stirred for 1 hr, poured into 30 ml of ice water, and filtered. The precipitate was washed with water and dried to yield 0.129 (98%) of VI, homogeneous (systems A and B). The analytical sample was prepared by recrystallization from chloroform–hexane (1:2, v/v, 5 ml): 168°, $[\alpha]^{27}D$ +9.1° (*c* 0.5, DMF). *Anal.* Calcd for $C_{88}H_{89}N_{11}O_{14}S_4 \cdot 0.5CHCl_3$: C, 60.68; H, 5.46; N, 9.32; S, 7.76. Found: C, 60.30; 60.50; H, 5.68, 5.75; N, 9.64, 9.76; S, 7.97.

Enzymic Hydrolysis of X. To 6.34 mg (3.845 μmoles) of X was added 2.0 ml of freshly prepared trifluoroacetic acid saturated with hydrogen bromide. The reaction mixture was kept to 40° for 40 min and evaporated *in vacuo* to a yellow residue. The residue was washed by decantation with ether, dried, and dissolved in 1.1 ml of 88% formic acid–methanol. The solution was cooled to −10° and treated with 3.2 ml of a solution of 88% formic acid and 30% hydrogen peroxide (9:1, v/v). The solution was kept at −10° for 4 hr, treated with 0.48 ml of 48% hydrobromic acid, and evaporated *in vacuo* at 40°. The dried residue was dissolved in 4.0 ml of water and aliquots of this solution were used as described below.

A 1.0-ml aliquot was treated with 1.0 ml of concentrated hydrochloric acid and hydrolyzed in a sealed tube at 100° for 24 hr. Automatic amino acid analysis of the residue indicated the following amino acid ratios (corrected for destruction during hydrolysis): $CySO_3H_{3.9}$, $Gly_{4.2}$, $Phe_{2.0}$, and $Val_{1.0}$.

A second 2.0-ml aliquot of the stock solution of deblocked X was incubated with 0.25 ml of aminopeptidase M[20,21] solution (10,000 mEU/ml of water). The pH of the solution was adjusted to 7.75 by the addition of Tris buffer and the solution was covered with a layer of toluene. The reaction mixture was incubated at 40° for 40 hr; the enzyme was denatured by heating for 10 min and the mixture was filtered. The combined filtrate and washings were evaporated to dryness *in vacuo* and the residue was dissolved in 4.0 ml of 0.2 *N* citrate buffer (pH 2.2). Automatic amino acid analysis of a 1.0-ml aliquot of this solution indicated the following ratios of amino acids: $CySO_3H_{4.4}$, $Gly_{4.1}$, $Phe_{2.8}$, and $Val_{1.0}$. No peaks due to cysteic acid peptides were observed in the elution curve.

Acknowledgment. We are grateful to Mr. A. J. Dennis for the molecular weight determinations and to Mrs. Mary Pendergraft for the amino acid analysis. The initial preparation of the C-terminal tripeptide was performed by Mr. J. T. Sparrow.

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(21) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).

Nuclear Magnetic Resonance Studies of the Interaction of Tryptophan with α-Chymotrypsin

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Abstract: The Michaelis complex formed by the interaction of tryptophan with α-chymotrypsin has been investigated by high-resolution nmr techniques. Appreciable line broadening is observed with both the D and L isomers of the amino acid. The D form appears to be more tightly bound to the enzyme than the L isomer. It is concluded that the binding is tight enough to imbue the bound tryptophan molecule with the same over-all rotational characteristics that characterize the motion of the enzyme in solution. Experiments with chemically modified enzymes suggest that tryptophan interacts with only one site on the enzyme and that this site is at or near the active site.

As a result of a great deal of experimental effort the mechanism of α-chymotrypsin-catalyzed reactions is largely understood.[1] Minimally, the reaction (eq 1) involves a preequilibrium between substrate (S) and enzyme (E) to give an enzyme–substrate complex (ES) which reacts to give an acylated enzyme (ES′) that is derived by transfer of the acyl portion of the substrate to a serine hydroxyl on the enzyme. Deacylation of the enzyme essentially completes the reaction although a third equilibrium involving the complexation of the product acyl group with the enzyme in a manner analogous to the formation of ES may intervene between the deacylation step and the regeneration of the enzyme.[2]

A large number of substrates and inhibitors of α-chymotrypsin-catalyzed reactions have been examined and the factors responsible for the specificity of the

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \xrightarrow{k_3} \text{products} \qquad (1)$$

(1) For reviews, see (a) M. L. Bender and F. J. Kézdy *Ann. Rev. Biochem.*, **34**, 49 (1965); (b) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. I, W. A. Benjamin, Inc., New York, N. Y., 1960, p 212; (c) P. Desnuelle, *Enzymes*, **4**, 93 (1960).

(2) M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2582 (1962).

enzyme have been elucidated.[3] In large measure the selectivity of this enzyme, as is probably true with most enzymes, is due to the manner in which the substrate is attracted and held to the enzyme in the equilibrium steps of eq 1.

Several elegant studies by Jardetzky and coworkers[4] and other groups[5] make it clear that nmr can be of real value in studies of the binding interactions between small molecules and large biopolymers. This is so because, in favorable cases, the rate of exchange between the bound and unbound states of the small molecule is fast. Under these conditions the observed nmr spectrum of the small species is an averaged one; the parameters which characterize the averaged spectrum are weighted means of the parameters which characterize the spectrum of the bound and unbound states with the contribution of each being weighted by the appropriate mole fraction. Thus, one can determine the spectrum of the bound molecule if the equilibrium constant relating the bound and unbound states and the spectrum of the material in the unbound state are known.

The rates involved in the formation of the Michaelis complex, ES, probably approach those expected for a diffusion-controlled process and, as a result, it should be possible to examine the ES complex by the type of nmr experiment described above. Recent reports have described the study of the interaction of substrate-like molecules with the enzymes lysozyme,[6,7] yeast alcohol dehydrogenase,[8] and α-chymotrypsin[9] by this technique.

The appearance of high-resolution nmr spectra depends on three classes of parameters: the chemical shifts of the nuclei, the scalar spin–spin coupling interactions between nuclei, and the rates of various nuclear relaxation processes. In principle, all of these parameters could be altered when the molecule under examination is in the presence of an enzyme. Previous workers have noted dramatic changes in chemical shift[7,9] and relaxation rates[8] that were ascribed to the expected influence of the enzyme. Variations in the relaxation rates, $1/T_1$ and $1/T_2$, are of particular interest since these rates are sensitive to the freedom of molecular motion.[10] One might thus expect a correlation between the strength of enzyme–substrate binding and the nuclear relaxation rates of the substrate as it is bound to the enzyme.

As a first step in the exploration of this possibility, we have studied the interaction between tryptophan and α-chymotrypsin. This amino acid was chosen since it is known that substrates based on amino acids with aromatic side chains bind well to this enzyme[11] and that tryptophan itself is a fairly effective reversible inhibitor of the enzymatic reaction.[12] The results of our study are described here.

Experimental Section

D-Tryptophan, L-tryptophan, and bovine α-chymotrypsin (three-times recrystallized, salt free) were obtained from Mann Research Laboratories. Diisopropylphosphorylchymotrypsin was obtained from Worthington Biochemicals Corp. Deuterium oxide (99.8%) was obtained from Stohler Isotope Chemicals.

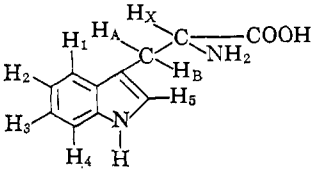
Solutions were prepared gravimetrically taking 25,000 as the molecular weight of the enzyme and using 5% water in deuterium oxide as the solvent. The apparent pH of the sample, as measured with a glass electrode, was adjusted to 6.6–6.9 with small amounts of 0.1 *N* NaOD in deuterium oxide. Small changes in pH in this range did not appreciably affect the spectra.

Spectra were obtained with a Varian Associates HA100 nmr spectrometer equipped with a Varian C1024 time-averaging computer. About 50 scans, collected at a scan rate of 2 Hz/sec, were averaged for each spectrum. The water peak of the spectrum was used as an internal lock signal for field-frequency stabilization. The ambient probe temperature was approximately 36°. The resolution of the instrument was checked by running samples of tryptophan before and after enzyme-containing samples were run. The average of the line widths for these control spectra was taken as the natural or instrumental line width.

In cases where the spectra were so broadened that it was difficult to define the width of an individual line, the line widths were estimated by comparison of the appearance of the experimental curves to a series of computer-generated line shapes until a reasonable match was found. The computed curves were prepared with the assumption that the spectral lines were Lorentzian in shape and did not change their relative positions in the presence of the enzyme. At the present stage of development, this procedure is not highly accurate and the errors in the resulting line widths may be as large as 1 Hz. The conclusions drawn are not greatly affected by an error of this magnitude, however.

Results

As a prelude to the study of tryptophan in the presence of α-chymotrypsin, an analysis of the proton magnetic resonance spectrum of the amino acid was carried out by the Swalen–Reilly iterative procedure as modified by Ferguson and Marquardt.[13,14] The labeling scheme shown below was used and the resulting chemical



shift and spin–spin coupling parameters are listed in Table I. The analysis of the ABX pattern arising from

Table I. Nmr Parameters for Tryptophan[a]

A. Chemical Shifts (ppm from internal water)[b]	
$\nu_1 = -3.030 \pm 0.001$	$\nu_5 = -2.586$
$\nu_2 = -2.482 \pm 0.001$	$\nu_A = 0.679 \pm 0.002$
$\nu_3 = -2.565 \pm 0.001$	$\nu_B = 1.272 \pm 0.001$
$\nu_4 = -2.827 \pm 0.001$	$\nu_X = 1.455 \pm 0.002$
B. Coupling Constants (Hz)[b]	
$J_{12} = 8.11 \pm 0.1$	$J_{24} = 1.02 \pm 0.1$
$J_{13} = 0.75 \pm 0.1$	$J_{34} = 8.74 \pm 0.1$
$J_{14} = 0.65 \pm 0.1$	$J_{AB} = -15.6 \pm 0.3$
$J_{23} = 7.51 \pm 0.2$	$J_{BX} = 5.0 \pm 0.2$
$J_{AX} = 8.3 \pm 0.2$	

[a] Sample was 0.045 *M* L-tryptophan in 95% deuterium oxide at an apparent pH of 6.7. [b] Root-mean-squared errors as calculated by the Ferguson–Marquardt program.[14]

(3) G. E. Hein and C. Niemann, *J. Am. Chem. Soc.*, 84, 4495 (1962).
(4) J. J. Fisher and O. Jardetzky, *ibid.*, 87, 3237 (1965).
(5) D. J. Blears and S. S. Danyluk, *Biopolymers*, 5, 535 (1967).
(6) E. W. Thomas, *Biochem. Biophys. Res. Commun.*, 24, 611 (1966).
(7) M. A. Raftery, California Institute of Technology, private communication.
(8) D. P. Hollis, *Biochemistry*, 6, 2080 (1967).
(9) T. McL. Spotswood, J. M. Evans, and J. H. Richards, *J. Am. Chem. Soc.*, 89, 5054 (1967).
(10) O. Jardetzky, *Advan. Chem. Phys.*, 7, 499 (1964).
(11) For a review of the mode of binding to chymotrypsin, see B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," John Wiley and Sons, Inc., New York, N. Y., 1967, Chapter 3.
(12) R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, 77, 3369 (1955).
(13) J. D. Swalen and C. A. Reilly, *J. Chem. Phys.*, 37, 21 (1960).
(14) R. C. Ferguson and D. M. Marquardt, *ibid.*, 41, 2087 (1964).

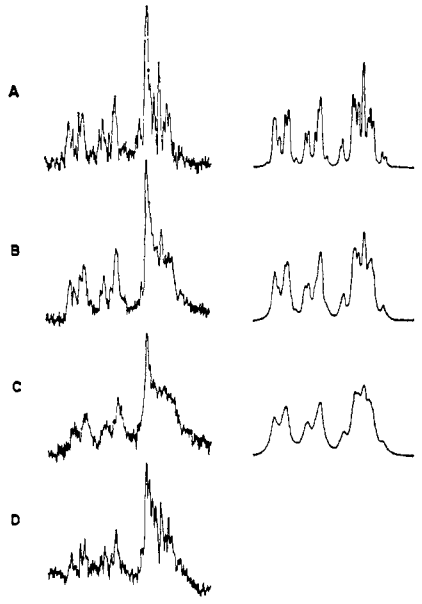


Figure 1. Observed and calculated aromatic pmr spectra of (A) 44.5 mM L-tryptophan, (B) 46.0 mM tryptophan and 1.2 mM α-chymotrypsin, (C) 44.5 mM L-tryptophan and 2.3 mM α-chymotrypsin, and (D) 44.5 mM L-tryptophan and 3.8 mM alkylated α-chymotrypsin in 95% D_2O. The left side of each spectrum is at -3.20 ppm from internal water; the spectra are 100 Hz wide. The computed spectra in the right column use line widths of 1.4, 2.5, and 4.0 Hz, respectively.

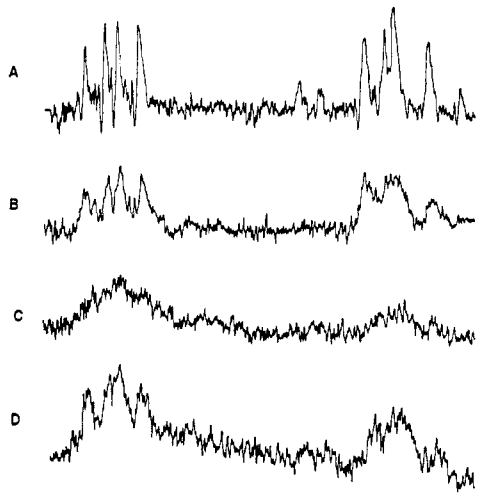


Figure 2. Observed pmr spectra of the ABX region of (A) 39.8 mM D-tryptophan, (B) 49.5 mM D-tryptophan and 1.1 mM α-chymotrypsin, (C) 39.8 mM D-tryptophan and 2.6 mM α-chymotrypsin, and (D) 39.8 mM D-tryptophan and 3.3 mM DFP-chymotrypsin in 95% D_2O. The left side of each spectrum is at 0.50 ppm from internal water; the spectra are 100 Hz wide. The upfield wing of the AB resonance pattern is not shown.

the alkyl portion of the molecule was straightforward.[15] Somewhat more difficulty was experienced in the analysis of the aromatic region of the spectrum; the parameters quoted lead to a calculated spectrum in moderate agreement with the experimental spectrum as is shown in Figure 1. Over-all, the spin coupling parameters are not extraordinary for a system of this type and are accurate enough to serve to identify each of the aromatic protons.[16]

The proton magnetic resonance spectrum of tryptophan undergoes striking changes when the amino acid is in the presence of native α-chymotrypsin. As illustrated in Figure 1, the aromatic portion of the spectrum becomes progressively broader and more poorly defined as the concentration of enzyme relative to the concentration of L-tryptophan is increased. A similar but more pronounced effect is observed in the alkyl portion of the spectrum, as is shown in Figure 2. At a given ratio of enzyme to amino acid concentrations, the spectrum of the D antipode appears to be slightly broader than that of the L isomer.

These observations must be due to an interaction between the enzyme and the amino acid and, as such, can be expressed more quantitatively. If one assumes that a single equilibrium constant (K_T) is sufficient to describe the experimental situation, it can be shown that

$$ET = \frac{T_t E_t}{K_T + T_t} \tag{2}$$

where ET is the concentration of enzyme–tryptophan complex, T_t and E_t are the total concentrations of tryptophan and enzyme, respectivly, and T and E are the concentrations of each species at equilibrium. The equilibrium constant is defined by

$$K_T = \frac{E \cdot T}{ET} \tag{3}$$

In the derivation of eq 2 the assumption has been made that the concentration of the complex ET is small compared to T_t. The observed line width, $w_{1/2}$, is given by

$$w_{1/2} = \frac{ET}{T_t} w_{ET} + \frac{T_t - ET}{T_t} w_{free} \tag{4}$$

where w_{ET} is the line width at half-height of the tryptophan in the enzyme–substrate complex and w_{free} is the line width in the absence of enzyme. If the assumption regarding the relative concentrations of T_t and ET is again invoked, eq 4 reduces to

$$\delta w = w_{1/2} - w_{free} = \frac{E_t}{T_t - K_T} w_{ET} \tag{5}$$

where the concentration of ET has been replaced by its equivalent as defined by eq 3 and δw is defined as the line width in excess of the natural line width.

Data were obtained for the AB, X, and aromatic resonances of D- and L-tryptophan in the presence of α-chymotrypsin. Appropriate control experiments allowed evaluation of the extent of line broadening, δw, and the resulting data were analyzed by plotting $E_t/(T + K_T)$ against δw for several assumed values of K_T. Typical results of this procedure are shown in Figure 3. As can be seen, the slopes (w_{ET}) are not very sensitive to the choice of K_T when the value of this constant lies in the range 3–12 mM. It has been assumed that K_T for the D isomer is 6 mM and that for the L isomer is 12 mM.[13] This procedure leads to approximate line widths for the various resonances of the spectrum of tryptophan as it is bound to the enzyme; these are listed in Table II.

(15) J. D. Roberts, "An Introduction to the Analysis of Spin-Spin Splitting in Nuclear Magnetic Resonance," W. A. Benjamin, Inc., New York, N. Y., 1962, p 71.

(16) A. A. Bothner-By, *Advan. Magnetic Resonance*, **1**, 304 (1965).

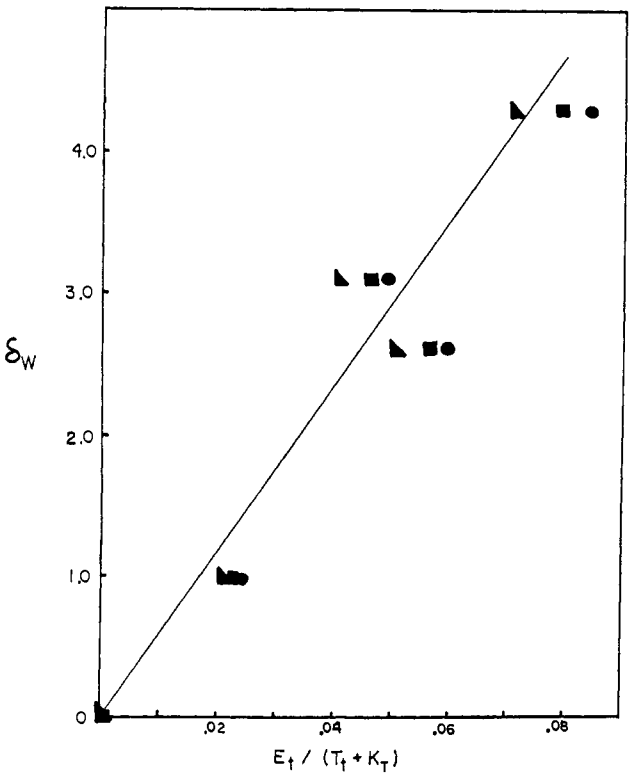


Figure 3. A plot of δw for the AB resonances of L-tryptophan *vs.* $E_t/(T_t + K_T)$ for several values of K_T: ▲, assumed $K_T = 12$; ■, assumed $K_T = 6$; ●, assumed $K_T = 3$.

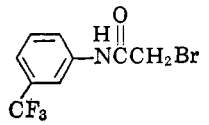
As can be seen in Figure 1, there is an apparent upfield shift of the aromatic proton signals of tryptophan relative to the vinyl singlet when the amino acid is in the presence of the enzyme. This effect is small and we have not tried to evaluate it quantitatively at this time.

In order to help elucidate the site of interaction between this amino acid and α-chymotrypsin, similar experiments were performed with two chemically modified enzymes. Experiments with diisopropylphosphorylchymotrypsin showed a marked diminution of the line-broadening effect (Figure 2, trace D). For example, at an enzyme–tryptophan concentration ratio of 0.083, the observed line broadening of the AB portion of the spectrum was 2.1 Hz for D-tryptophan in the presence of the phosphorylated enzyme. One calculates from the data in Table II that the effect at this concentration ratio with the native enzyme would be 6.2 Hz. Thus, binding, as evidenced by the linewidth effect, is considerably diminished by phosphorylation of the enzyme. Secondly, experiments were performed with methionine-S-(N-3-trifluoromethylphenyl)-carbamylmethylsulfonium bromide-192-α-chymotrypsin, obtained by treating the enzyme with N-bromoacetyl-3-trifluoromethylaniline (I).[17] Schramm and Lawson have

Table II. Line Widths of Tryptophan Bound to α-Chymotrypsin

Line	w_{ET}, Hz[a] L-Tryptophan	w_{ET}, Hz[a] D-Tryptophan	Ratio D/L
AB, alkyl	60	85	1.4
X, alkyl	20	33	1.6
Aromatic	28	43	1.5

[a] Estimated error, ±15%.

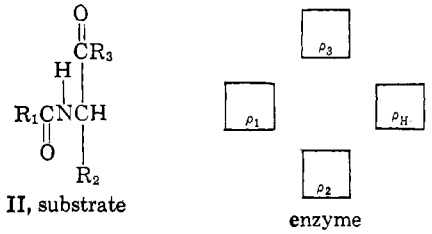


I

observed that a variety of N-bromoacetyl aromatic amines alkylate the methionine-192 residue of α-chymotrypsin and we presume that this modified enzyme is alkylated at this position also.[18] When this enzyme is used in the nmr experiments, the line-broadening effect completely disappears (Figure 1, trace D) and both the aromatic and alkyl portions of the tryptophan spectrum appear essentially normal. This experiment also demonstrates that the observed line-broadening effects are not due to changes in the macroscopic viscosity of the solvent.

Discussion

It seems most profitable to interpret these observations in terms of the ρ-area theory of substrate binding to chymotrypsin. This theory was developed by Niemann and coworkers[3] and postulates selective interactions between portions of the substrate molecule and complementary areas on the enzymic surface. It suggests that binding of the generalized substrate II



II, substrate enzyme

to the enzyme will be strongest when it is possible for each of the R_n groups shown to interact with a corresponding binding area (ρ_n). The ρ_2 area is apparently an area where strong hydrophobic interactions with the side chain (R_2) lead to productive binding and it is likely that during the reaction of substrates based on the aromatic amino acids, the aromatic side chain is bound at this site. It has been suggested that the methionine-192 residue of chymotrypsin is resident in the ρ_1 binding area and that alkylation of this amino acid with molecules such as N-bromoacetyl-3-trifluoromethylaniline (I) will place the aromatic ring of the alkylating agent in the ρ_2 site.[19,20] As a result, the binding of aromatic amino acid substrates to enzymes so alkylated is greatly diminished.[18,20]

The observation that the pmr spectrum of tryptophan is normal in the presence of this modified enzyme suggests that the only point of interaction between α-chymotrypsin and this amino acid is specifically the ρ_2 binding area. This conclusion is substantiated by the observation that placement of a diisopropylphosphoryl

(17) E. W. Bittner and J. T. Gerig, unpublished work. The preparation and characterization of this enzyme will be described in a subsequent paper.

(18) H. Schamm and W. B. Lawson, *Z. Physiol. Chem.*, 332, 97 (1963).

(19) Reference 12, pp 136–139.

(20) F. J. Kezdy, J. Fedor, and M. L. Bender, *J. Am. Chem. Soc.*, 89, 1009 (1967).

group at the serine-195 position diminishes but does not eliminate the interaction between enzyme and amino acid. This serine residue is presumed to be in the ρ_3 binding region but it is not unreasonable to expect that the alkyl groups of the phosphorus ester will protect the nearby ρ_2 area to some extent. Our results with both modified enzymes are only suggestive at this stage and we cannot rule out the possibility that both of these materials are so conformationally different from the native enzyme that the normal hydrophobic binding site is covered or otherwise modified so as to be no longer accessible to a tryptophan molecule. There is ample evidence from optical rotatory dispersion data that the conformation of chymotrypsin is altered by diisopropylphosphorylation but the effect of this change on substrate binding is not clear from previous work.[21]

The greater line widths observed for D-tryptophan in the bound state indicate that the interaction with enzyme is somewhat stronger for this isomer. This conclusion is consistent with the observation that the D forms of several tryptophan-like molecules are more potent inhibitors of the chymotryptic reaction than are the L isomers.[12,22] Richards and coworkers have found that the fluorine-19 resonances of N-acetyl-D-*p*-fluorophenylalanine are considerably shifted downfield relative to the L isomer when the compound is in the presence of α-chymotrypsin.[9] The origin of this effect and the differential line-broadening effect noted here are both probably related to the chirality or stereoselectivity of the active site of chymotrypsin.

If instrumental effects are subtracted, a major contribution to the relaxation rate, $1/T_2$, of a given nucleus arises from dipolar interactions with neighboring nuclei. If it is assumed that the relative motion of the interacting nuclei is rapid enough to average their relative orientations, this contribution to the relaxation rate is given by

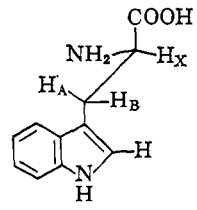
$$\left(\frac{1}{T_2}\right)_i \cong \frac{3}{2}\nu^4\hbar^2\sum_j \frac{\tau_{ij}}{\langle r_{ij}\rangle^6} \qquad (6)$$

where ν is the gyromagnetic ratio for protons, $\langle r_{ij}\rangle$ is the average distance between nuclei i and j, and τ_{ij} is a constant called the correlation time which can be taken as a crude measure of the time during which two spins maintain a given orientation relative to one another.[23] If the molecular structure is such that the nuclei are maintained in a single relative orientation, one time constant is sufficient to describe the motion and eq 6 reduces to

$$\left(\frac{1}{T_2}\right) \cong C\tau_c\sum \frac{1}{\langle r_{ij}\rangle^6} \qquad (7)$$

where C is now used to represent the constants of the previous equation and τ_c is the correlation time.

The spin coupling constants of the alkyl region of the tryptophan pmr spectrum do not appear to change when enzyme is added to the sample. (It is difficult, however, to estimate these constants at high enzyme concentrations where the lines are very broad.) This may be taken as tentative evidence that the internuclear distances (r_{ij}) for this part of the molecule are not greatly perturbed by complexation with α-chymotrypsin. The internuclear distances of the aromatic ring system are, of course, fixed by the nature of the system. As an approximation, it is therefore assumed that a model of the uncomplexed amino acid can be used to determine internuclear distances in the enzyme–tryptophan complex. Space-filling models of the tryptophan were used to study the possible conformations of the molecule, and primarily on this basis, the conformation below would seem to be the most energetically favorable.[24]



In this conformation the AB protons lie somewhat below the plane defined by the indole ring while the X proton is above the plane. The relative magnitudes of J_{AX} and J_{BX} are consistent with the conformation of the side-chain shown. Internuclear distances were estimated from this model and the quantities $\Sigma 1/\langle r_{ij}\rangle^6$ were calculated. The ratios of these quantities are compared in Table III with the experimental relaxation rates observed for the tryptophan–enzyme complex.

Table III. Calculation of Relaxation Rates in Tryptophan

Proton	$\frac{1}{\langle r_{ij}\rangle^6} \times 10^2$	Ratio[a]	Obsd ratio[b] D	Obsd ratio[b] L
1	0.0073	1.7		
2	0.0042	1.0		
3	0.0042	1.0	1.0	1.0
4	0.0021	0.5[c]		
5	0.0042	1.0[c]		
A	0.0104	2.5	2.0	2.1
B	0.0080	1.9		
X	0.0030	0.7[c]	0.8	0.7

[a] Relative to H_3. [b] Calculated from the data in Table II. The experimental line broadening (δw) is related to the relaxation rate by $1/T_2 = \pi\delta w$.[10] [c] Nitrogen-14 quadrapolar effects were not taken into account but may significantly change the relaxation rates for these signals.

The agreement of the calculated and observed relaxation rate ratios is good considering the assumptions that have been made, and one is forced to conclude that a single correlation time is sufficient to describe the behavior of tryptophan in its complex with α-chymotrypsin. By applying eq 7, it is calculated that τ_c for complexed D-tryptophan is 3.6×10^{-8} sec while τ_c for the L-tryptophan complex is 2.3×10^{-8}.

If one considers the enzyme to be a sphere of radius a moving through a medium of viscosity η, the rotational correlation time can be estimated from eq 8.[25]

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$$\tau_c = \frac{4\pi\eta a^3}{3kT} \quad (8)$$

A recent crystallographic study of chymotrypsin at 2-Å resolution shows that the over-all dimensions of the molecule are approximately 45 × 35 × 38 Å.[26] If the mean of these dimensions (39 Å) is taken as the effective radius of the enzyme in solution, the rotational correlation time for the enzyme as a whole is estimated to be 4×10^{-8} sec. The agreement between the calculated rotational correlation time of the enzyme molecule as a whole and the correlation time of tryptophan in the tryptophan–enzyme complex as determined by nmr is striking when the approximations involved are considered. This observation indicates that tryptophan interacts so strongly with the enzyme that the rotational motions of the amino acid are essentially identical with those of the enzyme and provides support for the proposition that chymotrypsin binds substrates tightly enough to seriously curtail molecular motion in the substrate.[27] It remains for further experimentation to more precisely define the details of this interaction.

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Communications to the Editor

The Borohydride-Catalyzed Reaction of Diborane with Epoxides. The Anti-Markovnikov Opening of Trisubstituted Epoxides

Sir:

Diborane is an interesting reducing agent with unusual characteristics.[1] However, in contrast to the simplicity of most other reactions, the reaction of diborane with epoxides is quite complex.[2] Thus, the reaction of 1,2-butylene oxide with diborane in tetrahydrofuran at 25° is a relatively slow reaction, requiring approximately 48 hr to proceed to the utilization of one "hydride" per mole of epoxide. However, analysis of the reaction mixture reveals the presence of only 48% of butanol (4% 1- and 96% 2-). The reaction is even more complex with trisubstituted epoxides, such as 1-methylcyclohexene oxide. In such cases the reaction proceeds with the utilization of 2 moles of hydride per mole of epoxide, with 1 mole of hydrogen being evolved. Only trace amounts of the simple alcohols are found in the reaction mixture. The major product is evidently an organoborane which is oxidized with alkaline hydrogen peroxide to the isomeric 2-hydroxymethylcyclohexanols.

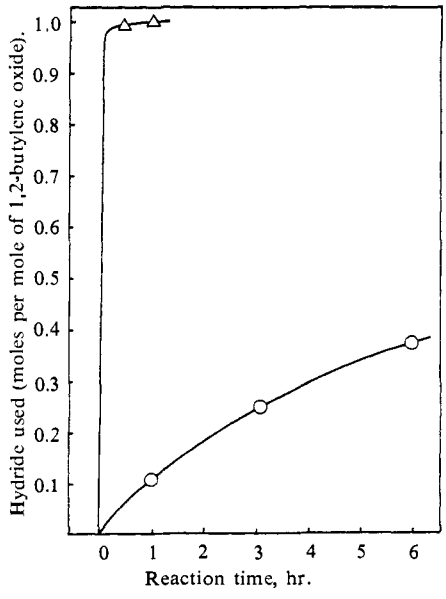
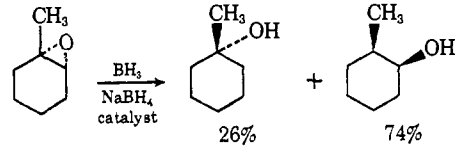


Figure 1. The reaction of borane (0.33 *M*) with 1,2-butylene oxide (0.25 *M*) in the presence (△) and absence (○) of sodium borohydride (0.036 *M*).

The presence of even minor amounts of sodium or lithium borohydride has a major effect on both the speed of the reaction and its course. In Figure 1 are shown the curves for the reaction of 1,2-butylene oxide with diborane in the presence and absence of a small quantity of sodium borohydride.[3] Not only is the rate affected, but the yield in 1 hr rises to 95% 2-butanol.

In the case of trisubstituted epoxides, such as 1-methylcyclohexene oxide, the rate of the reaction is also increased. Moreover, the course of the reaction is modified tremendously. One mole of hydride per mole of compound is utilized, hydrogen is not evolved, and there is observed a predominant anti-Markovnikov opening of the epoxide ring (1).



(1)

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(3) The solubility of sodium borohydride in 0.33 *M* THF–BH_3 was determined to be 0.036 *M* at 25°.