NUCLEAR MAGNETIC RESONANCE STUDIES OF α-CHYMOTRYPSIN

I. NMR STUDIES OF THE BINDING OF SMALL MOLECULE INHIBITORS TO α-CHYMOTRYPSIN

II. NMR STUDIES OF THE INTERACTION OF N-TFA-D-TRYPTOPHAN SEMICARBAZIDE WITH α-CHYMOTRYPSIN

III. ^{13}C-NMR STUDIES OF METHYLATED α-CHYMOTRYPSIN

IV. NMR STUDIES OF ACYLATED CHYMOTRYPSINS

Thesis by

Kenneth Lee Gammon

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1973

(Submitted August 9, 1972)

ACKNOWLEDGMENT

Firstly, I wish to thank Professor J. H. Richards for his guidance and friendship during the course of this work. His patience with my own personal indecision is especially acknowledged.

I also wish to thank all the undergraduates, graduate students, and post docs who have been associated with the Richards' research group during the past three years for their helpful discussions and friendship. A special thanks is due to M. W. Hunkapiller for very useful discussions concerning this work and for typing this thesis.

The help which I have received from Steven Smallcombe is most gratefully acknowledged. His collaboration on Part I of this thesis and instrumental help throughout this work have been of invaluable assistance to me.

I acknowledge the National Science Foundation (Predoctoral Fellowship, 1969-1970), the National Institutes of Health (Predoctoral Traineeship, 1970-1972), and the California Institute of Technology (Supplementary Teaching Assistantship, 1969-1972) for financial support.

ABSTRACT

Part I

Magnetic resonance studies of the interaction of N-trifluoroacetyl-D-(and L-)p-fluorophenylalanine and N-trifluoroacetyl-D-(and L-)tryptophan with α-chymotrypsin have been carried out at pH 5.0-8.0. The effect of enzyme oligomerization and competitive inhibition have been quantitatively accounted for. The trifluoroacetyl group of the D-isomer of both inhibitors is directed toward the active site of the enzyme, while that of the L-isomers is directed toward Ser 214. The aromatic side chain of all inhibitors resides in the hydrophobic specificity pocket of the enzyme. Ionization of a group on the free enzyme with a pK_a of 6.6 (presumably His 57) leads to a sharp decrease in binding affinity of the enzyme for anionic inhibitor molecules.

Part II

Nuclear magnetic resonance studies of the binding of N-trifluoroacetyl-D-tryptophan semicarbazide at pH 5.0-9.0 have been carried out. Ionization of a group on the free enzyme (tentatively assigned to His 40) causes a nine-fold increase in the enzyme-inhibitor dissociation constant. Neutralization of His 57

affects only the chemical shift of the bound inhibitor molecule.

Part III

Carbon-13 nuclear magnetic resonance studies of methylated α-chymotrypsin have been carried out. Selective enrichment of the modifying methyl group allows assignment of this resonance. The pH-dependent shift of the methyl group has been measured and indicates a pK_a of 6.75. The direction of the shift upon ionization (30 Hz downfield) indicates that this ionization is accompanied by a conformational change.

ABSTRACTS OF PROPOSITIONS

Proposition I

A spectroscopic study of the reactive intermediate formed between copper (II) salts and diazoketones is proposed.

Proposition II

A study of the nature of the methylene species involved in the reaction of diazomethane and ketene with alkylsilanes is proposed.

Proposition III

The preparation of trypsin with a modifying methyl group on the active site histidine residue is proposed. ^{13}C-nuclear magnetic resonance studies of this protein are also proposed.

Proposition IV

Nuclear magnetic resonance binding studies of N-TFA-D-p-fluorophenylalanine semicarbazide and α-chymotrypsin are proposed to further study the effect of the ionization with pK_a of ~ 6.1 on binding of inhibitors to α-chymotrypsin.

Proposition V

Studies of counterion binding to micelles by the technique of ultrasonic absorption are proposed.

TABLE OF CONTENTS

PART I.

PART IV.

PROPOSITIONS

LIST OF TABLES

LIST OF FIGURES

PART I

Nuclear Magnetic Resonance Studies of the Binding of Small Molecule Inhibitors to α-Chymotrypsin

INTRODUCTION

In the past several years the technique of nuclear magnetic resonance (nmr) has been increasingly used in the field of enzymology (1-4). Although some of these studies have involved observation of nmr signals of a protein molecule (5), most have monitored nmr signals from a smaller molecule interacting with the protein (2, 6-8). From such investigations information has been obtained about the chemical shifts and relaxation times of the small molecule when bound to the enzyme. These parameters have been interpreted in terms of the thermodynamics and stereochemistry of binding.

Potentially nmr is capable of providing very detailed information about the nature of the small molecule-protein interaction since individual nuclei can be monitored. Proper interpretation of these interactions should lead to precise information about the environment which the small molecule experiences when bound to the protein. If it is possible to observe the nmr parameters of the small molecule in the bound state, then it is reasonably straightforward to extract accurate chemical shift and relaxation data. This is not possible with systems in fast exchange since only an average state somewhere between the bound and unbound forms is directly observable, and the bound state parameters are obtainable only by extrapolation.

Several papers (2, 9-15) have appeared on the interaction of small molecules with the proteolytic enzyme α-chymotrypsin, a digestive enzyme found in the small intestine of higher animals. These studies have attempted to quantitatively determine the binding affinity of several aromatic amino acids with α-chymotrypsin. The chemical shift and relaxation parameters of various nuclei of the small molecule in the bound complex have been measured and interpreted in terms of the structure of the complex.

The fast exchange situation exists in all α-chymotrypsin systems studied thus far (2, 9-15). Therefore, the desired information about the enzyme-small molecule complex must be obtained by extrapolation. This turns out to be a rather difficult task and must be done carefully and with a minimum number of assumptions and mathematical approximations.

Unfortunately most of the work reported so far (2, 9-15) does not involve the proper care in extrapolating the bound nmr parameters, and erroneous and inconsistent results have been reported. For example, none of the previous nmr studies have even attempted to quantitatively consider the effect of enzyme oligomerization on the analysis of α-chymotrypsin systems. It has long been known that α-chymotrypsin forms polymeric species in solution (16, 17), and recently quantitative studies on the extent of such aggregation have been made (18-21). From these studies it is very apparent that oligomerization is an extremely important

phenomenon especially at the high concentrations (~ 2 mM) used in most nmr studies. In this work oligomerization has been quantitatively included, and constants for self-association equilibria have been determined from the nmr data. Neglect of this perturbation when analyzing α-chymotrypsin equilibria cannot possibly lead to quantitatively correct values of binding constants and nmr parameters for the protein-small molecule complex.

In the fast exchange situation, the nmr parameters which can be experimentally observed are the weighted average of the values for the two sites. If reliable information about the enzyme-inhibitor complex is to be obtained, it is important that a reasonable fraction of the inhibitor be in the bound form. Otherwise, a dangerously long extrapolation is necessary to obtain information about the enzyme-inhibitor complex.

Previously reported nmr work with α-chymotrypsin (2, 9-15) has involved the use of rather high concentrations of the small molecule. This was done because of the inherently low signal to noise sensitivity of the nmr technique and because the mathematical analysis is simpler if the enzyme is saturated with inhibitor molecules. As a result of these high inhibitor molecule concentrations, the concentration ratio of inhibitor to enzyme is generally quite high. This makes it very difficult to extract reliable information about the bound form of the small molecule. In the present work, a much wider range of relative concentrations has been used, and usually direct observation of nmr parameters at least half way

between the solution and bound values has been possible. This should make the extrapolation to the bound form more reliable than in other studies with α-chymotrypsin.

As in some previous studies (2, 9, 10), fluorine labeled amino acids have been used in this work to monitor the interaction between α-chymotrypsin and amino acids. There are several advantages to using fluorine instead of some other nucleus. Fluorine shows about the same signal to noise sensitivity as do protons (22), and its spectrum can be obtained in a reasonable amount of time. Other possible nuclei such as carbon-13 are much less sensitive (23) and place severe time limitations on data collection. Fluorine has two advantages over protons. First, there is a large protein background in the proton spectrum of an inhibitor being studied. Except at very high concentrations of inhibitor relative to protein, the small molecule resonances may be totally lost in the background. Fluorine signals on the other hand are easily detected since there is no fluorine nmr background. Secondly, fluorine exhibits a much larger chemical shift range than protons and is more sensitive to environmental changes caused by the enzyme upon binding (22).

Fluorine, of course, has the disadvantage of being an unnatural perturbation to the aromatic amino acid-α-chymotrypsin system. It is found, however, that fluorine labeled compounds behave similarly to more natural amino acid derivatives (2, 9-15) and seem

to act as expected from what is known about α-chymotrypsin-small molecule interactions. The ease with which fluorine spectra can be obtained (relative to other nuclei)seems to compensate for the possibility of introducing unnatural perturbations with the fluorine label.

α-Chymotrypsin was used in this nmr study for several reasons. The three-dimensional structure of the molecule has recently been determined by X-ray crystallography techniques (24). This is of great benefit when trying to correlate chemical shifts of the bound inhibitor molecule to environmental changes caused by the enzyme. A great deal of chemical work has been done on α-chymotrypsin and much is known about various ionizing groups on the molecule (25). Determination of nmr parameters and binding constants as a function of pH should lead to information about conformational changes caused by these ionizations. Part I of this thesis reports chemical shift and dissociation constants for the complex between α-chymotrypsin and several anionic amino acids as a function of pH. Part II deals with neutral inhibitors which have been reported to show significantly different behavior as a function of pH (26). Results obtained are discussed in terms of binding modes and conformational changes produced by ionizations of enzymic groups.

THEORY AND MATHEMATICAL ANALYSIS

Exchange Rates for the Interaction of Small Molecules with α-Chymotrypsin

If the nmr spectrum of a nucleus of an inhibitor molecule which is exchanging between solution and an enzyme binding site is observed, two limiting cases are possible (27). (i) If the inhibitor is in slow exchange, two peaks would be observed, one for the bound inhibitor and one for the inhibitor free in solution. (ii) If a condition of fast exchange exists, only one peak would be observed; and the observed shift would be a population weighted average of the shifts in solution and bound to the enzyme. More precisely, if τ_A is the lifetime of the inhibitor in the site on which it has the shorter lifetime, then fast exchange requires that

$$\tau_A < \sqrt{2}/2\pi\Delta_{AB} \qquad (1)$$

where Δ_{AB} is the chemical shift difference (in Hertz) between the solution and enzymic environments. If this inequality does not hold, then a slow exchange condition exists. In a condition of slow exchange it is easy to measure the chemical shift of the inhibitor nucleus when bound to the enzyme since it can be experimentally observed; however, it is not possible to measure the binding constant for the inhibitor since the two observed resonances will

not show a concentration dependence (except for changes in solution diamagnetic susceptibility). If, however, the inhibitor is in fast exchange, it is possible, with the proper analysis, to obtain both the binding constant and the chemical shift of the inhibitor nucleus when bound to the enzyme.

Since a solution of α-chymotrypsin with the inhibitors studied here exhibits only one nmr peak for any given nucleus, a condition of fast exchange exists. From eq 1 it can be seen that fast exchange requires that (assuming a reasonable value for Δ_{AB} of 100 Hz)

$$\tau_A < \sqrt{2}/2\pi\Delta_{AB} = 2.3 \times 10^{-3} \text{ sec.}$$

Converting this to a dissociation constant for the protein-inhibitor complex yields

$$k_{off} = 1/\tau_A > 4.4 \times 10^2 \text{ sec}^{-1}.$$

Other workers measuring rates for small molecule interactions with α-chymotrypsin obtained a value of $k_{off} = 3.5 \times 10^3$ sec^{-1} for N-TFA-D-tryptophan (28). Thus, the fast exchange condition is met. In all data analysis that follows it is assumed that the small molecule being observed is in fast exchange with the protein molecule.

Analysis of the Interaction of One Inhibitor Molecule with Monomeric Enzyme

As previously mentioned, in a fast exchange situation the observed chemical shift will be a population weighted average of the possible environments (27). For the two site case (solution and enzyme) the observed shift is given by the following expression.

$$\delta_{obs} = \frac{[I]}{[I_o]} \Delta_{solution} + \frac{[EI]}{[I_o]} \Delta_{enzyme} \qquad (2)$$

where δ_{obs} is the experimentally observed chemical shift, $[I]$ is the concentration of free inhibitor molecule, $[I_o]$ is the total inhibitor concentration, $[EI]$ is the enzyme-inhibitor complex concentration, and $\Delta_{solution}$ and Δ_{enzyme} are the chemical shift of the observed nucleus in solution and when bound to the enzyme. If chemical shifts are measured relative to the solution environment, $\Delta_{solution} = 0$ and eq 2 reduces to

$$\delta_{obs} = \frac{[EI]}{[I_o]} \Delta \qquad (3)$$

where $\Delta = \Delta_{enzyme}$.

It should be remembered that if eq 3 is to hold, measurements must be made relative to the shift of the inhibitor molecule in a solution containing enzyme and not just a buffer solution.

This is due to the change in diamagnetic susceptibility of bulk water when enzyme is added. This problem is most easily handled by using an internal reference from which measurements can be made. Such a reference experiences the same changes in diamagnetic susceptibility as the inhibitor molecule and thus corrects for such changes.

If the only equilibrium involved in a solution of enzyme and inhibitor is given by eq 4, then the analysis of concentration

$$EI \rightleftharpoons E + I$$

$$K_I = \frac{[E][I]}{[EI]} \tag{4}$$

dependent chemical shifts is direct. In this case both enzyme and inhibitor molecules exist in only two forms and the following material balance equations can be written.

$$[E_o] = [EI] + [E]$$
$$[I_o] = [EI] + [I]$$

$[E]$ and $[E_o]$ are the free enzyme concentration and the total enzyme concentration, respectively. Substituting these expressions into eq 4 yields

$$K_I = \frac{[E_o - EI][I_o - EI]}{[EI]} .$$

This expression may be rearranged to give the following formula for [EI].

$$[EI] = \frac{[E_o] + [I_o] + K_I - (\{[E_o] + [I_o] + K_I\}^2 - 4[E_o][I_o])^{\frac{1}{2}}}{2} \quad (5)$$

The other quadratic root with the plus sign has no physical significance since it gives values of [EI] which are greater than $[E_o]$ or $[I_o]$ and therefore are not physically possible.

In many systems eq 4 adequately describes the equilibria involved. In the present work the situation is complicated by dimerization and the presence of more than one inhibitor molecule. It is however much easier to understand the analysis of the more complicated case if the simple case presented above is discussed first.

If a value of K_I is known or assumed, then eq 5 can be used to calculate [EI] from the experimentally measureable quantities $[E_o]$ and $[I_o]$. With [EI], it is easy to calculate $[EI]/[I_o]$, a quantity which has a linear relationship with δ_{obs} from eq 3. Thus, a plot of δ_{obs} versus $[EI]/[I_o]$ (determined for the $[E_o]$ and $[I_o]$ values at which δ_{obs} was obtained) should in theory be linear, and any deviation from linearity reflects an error in K_I (assuming some other equilibrium has not been overlooked). By determining what value of K_I gives the best straight line fit to

eq 3, one can then determine the correct K_I for the system.

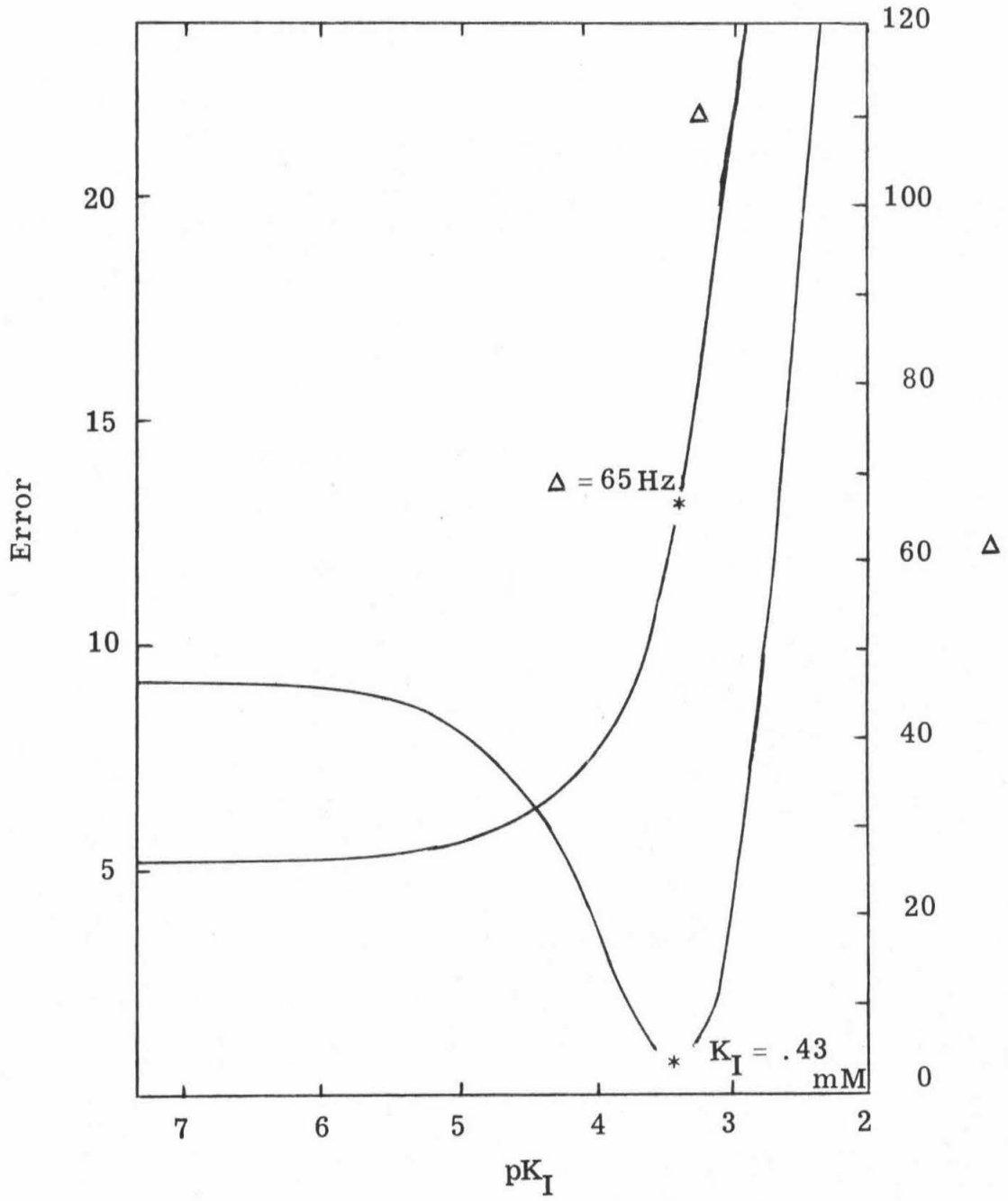
The analysis of a simple system is carried out in just this manner. A value of K_I is assumed, and for each such value a plot of $[EI]/[I_o]$ versus experimentally observed values for δ_{obs} is made. The best straight line through the points is determined using a least squares treatment. Next, the errors between the actual δ_{obs} and those values of δ calculated from the line are summed. The value of this sum is a measure of how well the assumed K_I defines the system being studied. With a new value of K_I, the process is repeated. K_I values are scanned through a reasonable range, and the K_I which gives the smallest least squares error is taken as correct. The slope of the line corresponding to this value of K_I can be seen to be equal to Δ from eq 3. Thus, with this technique it is possible to determine the binding constant, K_I, for the inhibitor being studied and the chemical shift, Δ, of the monitored nucleus of the molecule when bound to the enzyme.

The value of K_I can be mathematically refined to whatever tolerance the experimental accuracy dictates by iterating between K_I values which are less and less different; however, the best measure of the reliability of the K_I value chosen is determined from a plot of K_I versus error. If such a plot shows a sharp minimum indicating that only a small range of K_I values fit the experimental data, the selected K_I is quite reliable.

Figure 1 shows such a plot obtained in this work. It can be seen that a well defined minimum is obtained indicating that the K_I value determined is reliable. Figure 1 also emphasizes an important point which should be kept in mind when doing work of this type. It can be seen that for very strong binding ($K_I \ll [E_o]$) the error is extremely insensitive to the assumed value of K_I. This would make it extremely difficult to pick a reliable binding constant. It is possible, however, to determine Δ very accurately since Δ is not very dependent on K_I in the strong binding region. In the limit of weak binding ($K_I \gg [E_o]$), this type of experiment is essentially useless since when $K_I \gg [E_o]$ it is not possible to experimentally observe a situation in which a large portion of the inhibitor is bound to the enzyme. This makes extrapolation to the information desired about the bound form of the inhibitor very dangerous. For these reasons, it is desirable to use enzyme concentrations which are in the same range as the binding constant to be measured ($K_I/10 \leq [E_o] \leq 10K_I$).

Since in general enzymes are not particularly soluble, measurement of binding constants greater than 5×10^{-2} M is very difficult. At the other extreme, it is difficult to measure binding constants much smaller than 10^{-5} M. Such measurements would require the use of rather low enzyme concentrations, and consequently the inhibitor concentrations would be kept very low to

FIGURE 1. Dependence of Least-Square Error and Δ on pK_I at pH 6.5 Using $K_D = 2.0 \times 10^{-4}$ M



insure that $[EI]/[I_o]$ was large enough for a reasonable extrapolation. Since even for the most sensitive nuclei (fluorine and hydrogen) concentrations below 5×10^{-5} M are difficult to observe in a reasonable amount of time, an instrumental sensitivity problem would develop.

The inhibitors of α-chymotrypsin used in this experiment have effective binding constants on the order of 10^{-3} M, and an enzyme concentration of $\sim 2 \times 10^{-3}$ M was used. Thus, reliable values of K_I and Δ are obtainable. Furthermore, it was possible to vary $[I_o]$ over a range of $\sim 10[E_o]$ to $\sim [E_o]/2$ so that experimental points over a wide range of $[EI]/[I_o]$ values were taken. This should make plots of δ_{obs} versus $[EI]/[I_o]$ much more reliable than those obtained in other works (2, 9-15) which did not use a wide concentration range.

Extension of Analysis to Include Enzyme Oligomerization

The above analysis can be extended to include systems which are not adequately described by eq 4 due to the presence of other equilibria. For the present purposes, this will include the effects of enzyme oligomerization and a second inhibitor molecule competing for enzyme binding sites. The first perturbation is a particular consequence of the fact that this study involved α-chymotrypsin, an enzyme which exhibits strong self-association (16-21). Fortunately, this effect is not found in all enzyme systems, and

mathematical analysis to include this phenomenon is not generally required. The effect of a second inhibitor is a more generally useful concept since, as will be shown, the binding constant of inhibitors which are not themselves easily observed by nmr techniques can be measured by the effect of this inhibitor on another inhibitor which can be monitored by nmr. This could prove to be a useful method of determining binding constants for a variety of enzyme systems.

First, the effect of enzyme oligomerization on the analysis of nmr data will be considered. It will be assumed that only dimerization occurs and that higher polymeric forms are not present. If higher degrees of association do occur, the dimerization constant measured can be considered to be an effective oligomerization constant which is reliable over the range of enzyme concentrations used for measuring the constant. Enzyme concentration dependent nmr studies in the present case indicated that oligomerization was indeed a serious perturbation and that proper analysis of the data absolutely required the explicit inclusion of such oligomerization in the mathematical analysis.

Enzyme dimerization can be described by the equilibrium given in eq 6

$$\begin{aligned} EE &\rightleftharpoons E + E \\ K_D &= [E]^2/[EE] \end{aligned} \tag{6}$$

where [EE] is the concentration of dimeric enzyme and K_D is the enzyme dimerization constant. It will be assumed that the dimeric form of the enzyme binds inhibitor with a binding constant much larger than that for the monomeric species. For the purpose of analysis, it will be assumed that only monomeric enzyme binds inhibitor molecules. Evidence (17) suggests that the active site of α-chymotrypsin is involved in the dimerization interaction which makes this a reasonable assumption. Proflavin apparently binds only to the monomeric form of α-chymotrypsin at pH 6.2 (19).

The enzyme now exists in three possible forms, and $[E_o]$ is given by eq 7.

$$[E_o] = [E] + [EI] + 2[EE] \tag{7}$$

This expression yields an equation for [E] which can be substituted into eq 4 to give eq 8.

$$K_I = \frac{([E_o] - [EI] - 2[EE])([I_o] - [EI]}{[EI]} \tag{8}$$

If this same expression for [E] is substituted into eq 6, the following equation involving [EE], $[E_o]$, K_D, and [EI] is obtained.

$$K_D = \frac{([E_o] - [EI] - 2[EE])^2}{[EE]}$$

This relationship can be rearranged to yield an expression for [EE].

$$[EE] = \frac{K_D/4 + [E_o] - [EI] - (K_D^2/16 + \frac{1}{2}\{[E_o] - [EI]\})^{\frac{1}{2}}}{2} \qquad (9)$$

If this formula for [EE] is substituted into eq 8, the following cubic equation in [EI] is obtained.

$$[EI]^3 - [EI]^2(K_I + 2[I_o] + [E_o] - 2K_I^2/K_D) + [EI](2[E_o][I_o]^2 + K_I[I_o]) - [E_o][I_o]^2 = 0 \qquad (10)$$

If K_D and K_I are known or assumed, this expression may be solved by Newton's Method to yield [EI]. In practice, this procedure tends to converge on physically meaningless roots so another approach is needed for determining [EI].

If eq 8 is solved for [EI], the following expression results.

$$[EI] = \frac{[E_o] + [I_o] + K_I - 2[EE] - (\{[E_o] + [I_o] + K_I - 2[EE]\}^2 - 4[I_o]\{[E_o] - 2[EE]\})^{\frac{1}{2}}}{2} \qquad (11)$$

Eqs 9 and 11 form a simultaneous pair which can be solved iteratively until self-consistent values for [EI] and [EE] are obtained. Thus, even with the complication of dimerization, [EI]

can be obtained from the experimentally measureable quantities $[E_o]$ and $[I_o]$ if values for K_I and K_D are known.

In theory, it should be possible to assume values for K_I and K_D and calculate [EI] from eq 11. A best fit (according to eq 3) between [EI] values so calculated and δ_{obs} values could yield K_I, K_D, and Δ. In practice, this is too many variables to extract from one type of experiment. Therefore, in addition to doing the normal experiment in which $[I_o]$ is varied at constant $[E_o]$ (this is the type of experiment which is done to determine K_I in the simple case discussed earlier), it seemed desirable to do the complementary experiment in which $[E_o]$ was varied at constant $[I_o]$. Such an experiment should be most useful for evaluating K_D since dimerization would be expected to greatly perturb enzyme concentration-dependent results.

In order to analyze this second type of experiment, the quantity K_{Iapp}, which is the apparent enzyme-inhibitor dissociation constant, is defined by eq 12a. This is the value of K_I which would be measured if the effect of dimerization is ignored. K_{Iapp} is defined analogously to K_I except that the [E] factor in the mass action expression is taken to be the sum of all forms of enzyme not complexed with inhibitor.

$$K_{Iapp} = \frac{([E] + 2[EE])[I]}{[EI]} \qquad (12a)$$

$$K_{Iapp} = \frac{[E][I]}{[EI]} + \frac{2[EE][I]}{[EI]} \qquad (12b)$$

$$K_{Iapp} = K_I + \frac{2[EE][I]}{[EI]} \qquad (12c)$$

Substitution for [EE] from eq 6 yields eq 13.

$$K_{Iapp} = K_I + 2K_I[E]/K_D$$

$$K_{Iapp} = K_I(1 + 2[E]/K_D)$$

$$K_{Iapp} = K_I + \frac{2K_I^2[EI]}{K_D[I]} \qquad (13)$$

From eq 13 it can be seen that a plot of K_{Iapp} versus $[EI]/[I]$ should yield a straight line with an intercept of K_I and a slope of $2K_I^2/K_D$ from which K_D can be obtained. Thus, the problem requires that K_{Iapp} and $[EI]/[I]$ be determined in order to determine K_I and K_D. Solving eq 3 for [EI] yields the following expression.

$$[EI] = \frac{\delta[I_o]}{\Delta} \qquad (14)$$

If Δ is known and δ_{obs} is experimentally measured at some inhibitor concentration $[I_o]$, [EI] can be determined. Eq 12a for K_{Iapp} can be rewritten as

$$K_{Iapp} = \frac{([E_o] - [EI])([I_o] - [EI])}{[EI]} . \qquad (12d)$$

Thus, from the experimental values $[E_o]$ and $[I_o]$ and the value of $[EI]$ calculated from eq 14, it is possible to calculate K_{Iapp} for a given set of concentrations. A set of such points (preferably taken at constant $[I_o]$ and varying $[E_o]$ since K_D is being determined) can be used in a plot of K_{Iapp} versus $[EI]/[I] = [EI]/([I_o] - [EI])$ to yield K_I and K_D (eq 13).

Thus, by using eqs 9 and 11, it is possible to analyze experiments in which $[I_o]$ is varied at constant $[E_o]$ to yield best values of K_I if K_D is known. From K_I, Δ can be determined. With this Δ, eqs 13 and 14 can be used to determine K_D (and also K_I as a check) from experiments in which $[E_o]$ is varied at constant $[I_o]$. By analyzing complementary sets of experiments in this manner it should be possible to extract K_I, K_D, and Δ.

In practice this procedure works very well for analysis of the binding of inhibitor molecules to α-chymotrypsin. From very preliminary experiments, it was determined that Δ (for the trifluoroacetyl group of N-trifluoroacetyl-labeled amino acids) was on the order of -100 Hz (the minus sign indicates a downfield shift from solution environment). With data from enzyme concentration-dependent experiments, the value of K_D corresponding to values of Δ from -50 Hz to -250 Hz (a range which surely included the proper value) was determined from a least squares

fit of the experimental data to eq 13. It turned out that the value of K_D determined varied over a relatively small range (3×10^{-5} M to 2×10^{-4} M at pH 5.0).

With this range of values, experiments in which $[I_o]$ was varied at constant $[E_o]$ were analyzed according to eqs 9 and 11. This analysis yielded values of Δ which varied very little with varying K_D (64-68 Hz over the K_D range listed above). The K_I value which gave the best fit in such an analysis was, however, very sensitive to K_D. Fortunately, the value of Δ had been severely limited. With this narrow range of Δ's, the process was repeated to determine a new range of K_D values. It happened that over the range 64-68 Hz for Δ, K_D varied between 4.8×10^{-5} to 5.2×10^{-5} M (pH 5.0). Thus, fairly narrow limits had been placed on K_D. From these K_D values, a narrow range of K_I values was determined from eqs 9 and 11. This process can be repeated until the range of values lies within the probable experimental errors. For this work, it turned out that the analysis converged rapidly, probably as a result of the fact that two semi-independent types of experiments were done and analyzed cooperatively.

Extension of the Analysis to Include a Second Inhibitor Molecule

As mentioned before, it is also desirable to examine the effect of a second inhibitor on the analysis of binding curve data.

Such an analysis can be used to determine information for an inhibitor molecule which itself is not suitable for monitoring by nmr because of small concentration dependent chemical shifts. Both dimerization and a second inhibitor molecule will be considered since this is necessary for analysis with α-chymotrypsin. The analysis without dimerization is similar (and simpler) and can be used for general enzyme systems.

Basically this perturbation introduces a new species EI' which is the complex between the enzyme and the second inhibitor molecule I'. The material balance equations for E_o, I_o, and I_o' are

$$[E_o] = [E] + 2[EE] + [EI] + [EI'] \tag{15}$$

$$[I_o] = [I] + [EI] \tag{16}$$

$$[I_o'] = [I'] + [EI'] . \tag{17}$$

The dissociation constant, K_I', for the second inhibitor is defined by eq 18.

$$K_I' = \frac{[E][I']}{[EI']} \tag{18}$$

Substitution for [E], [I], and [I'] from eqs 15-17 into eqs 4, 6, and 18 yields the following set of simultaneous equations.

$$[EI] = \frac{[E_o] + [I_o] + K_I - [EI'] - 2[EE] - (\{[E_o] + [I_o] + K_I - [EI'] - 2[EE]\}^2 - 4[I_o]\{[E_o] - [EI'] - 2[EE]\})^{\frac{1}{2}}}{2} \quad (19)$$

$$[EI'] = \frac{[E_o] + [I_o'] + K_I' - [EI] - 2[EE] - (\{[E_o] + [I_o'] + K_I' - [EI] - 2[EE]\}^2 - 4[I_o']\{[E_o] - [EI] - 2[EE]\})^{\frac{1}{2}}}{2} \quad (20)$$

$$[EE] = \frac{[E_o] + K_D/4 - [EI] - [EI'] - (K_D\{([E_o] - [EI] - [EI'])/2 + K_D^2/16\})^{\frac{1}{2}}}{2} \quad (21)$$

Since the second inhibitor molecule cannot be monitored by nmr techniques, we observe a chemical shift, δ, for the first inhibitor which is given by

$$\delta = ([EI]/[I_o])\Delta \quad (22)$$

where [EI] is diminished due to competition for enzyme sites by the second inhibitor (it is, of course, assumed that the second inhibitor is competitive with the first). The introduction of a new equilibrium makes extraction of all the desired parameters very

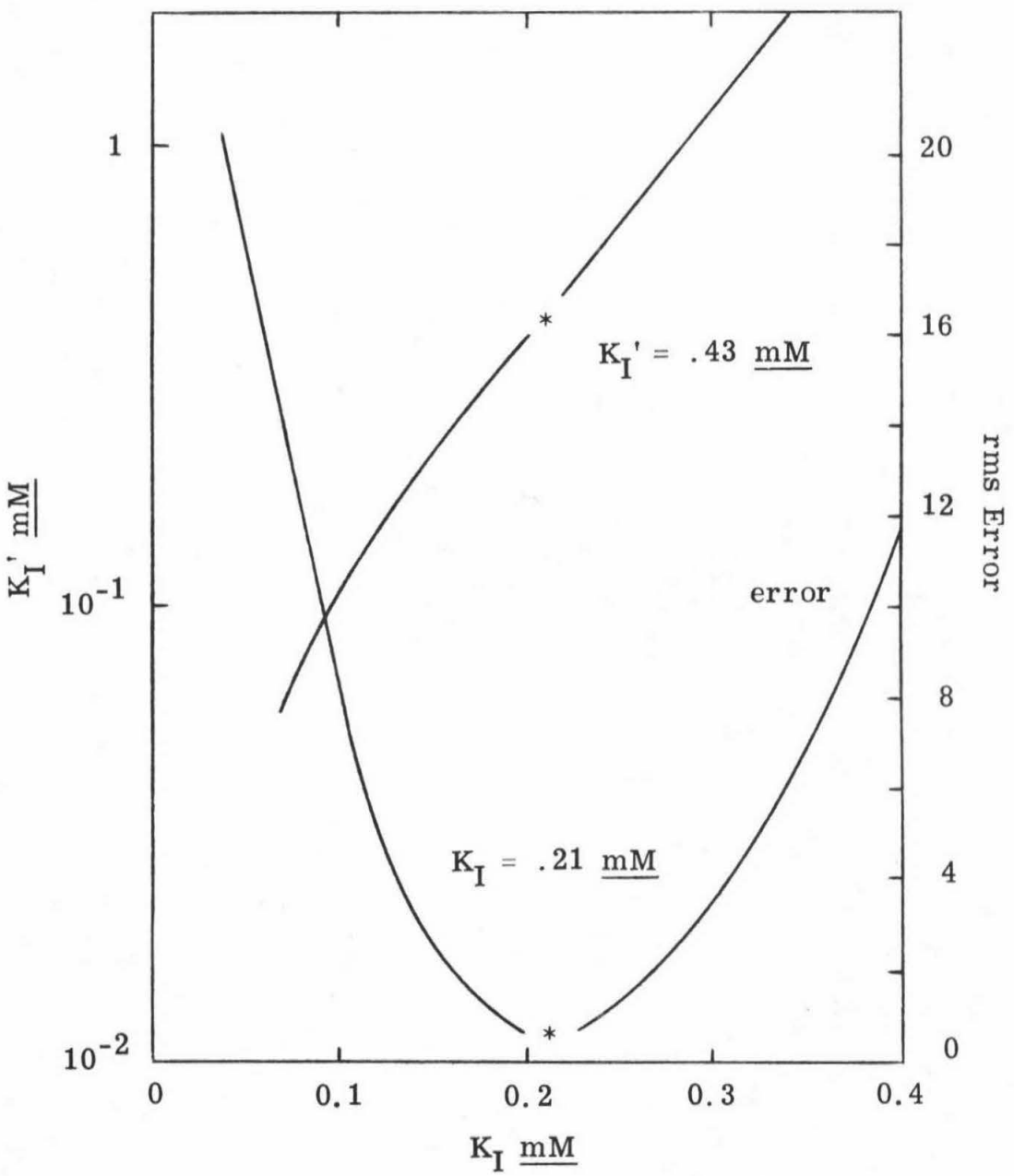
difficult even if both types of concentration dependent experiments previously described are done. For this reason, it was desirable to measure certain of the parameters in experiments which were not complicated by the presence of a second inhibitor. Therefore, experiments were done, as previously described, in which only dimerization needed to be considered. From these experiments (N-trifluoroacetyl-D-p-fluorophenylalanine used as the inhibitor) values for K_D at the necessary pH values were determined. These values were then used in the analysis of a second inhibitor. In this work the second inhibitor was N-TFA-L-tryptophan which cannot be studied directly by nmr due to a small concentration dependence of δ. By monitoring the effect of the L-isomer on N-TFA-D-tryptophan (an inhibitor which can be monitored by nmr), it is possible to obtain information about N-TFA-L-tryptophan. Therefore, separate experiments were done on the D-isomer alone to produce values of Δ and K_I for this inhibitor. Both of these values could then be used in the analysis for the L-isomer to determine K_I'. It was decided, however, to use only Δ from such experiments and to determine both K_I and K_I' from experiments involving both isomers. Although this was not necessary, it served as a check to insure that the system was well understood. With these known values of K_D and Δ, experimental data for a system containing both inhibitors can be analyzed according to eqs 19, 20, and 21.

First a value is assumed for K_I and for K_I'. Iteration between eqs 19-21 yields self-consistent numbers for [EI], [EI'], and [EE]. From this [EI] and the previously determined value for Δ, it is possible to calculate δ from eq 2. These calculated δ's can be compared to those experimentally observed, and root-mean-square errors can be calculated. These numbers are a measure of how well the assumed values fit the experiment. K_I' is then varied over an appropriate range until the best K_I' is determined for the originally assumed K_I. This process is repeated for an appropriate range of K_I's, and that combination of K_I and K_I' which gives the best fit is taken as the correct pair. Figure 2 shows a plot of K_I versus error and a plot of K_I' versus K_I. The error is seen to show a sharp minimum which indicates that a reliable K_I can be obtained. From the steepness of the K_I' plot, it can be seen that K_I' is very sensitive to the value of K_I determined by the analysis. It is therefore necessary to determine K_I accurately if a reliable K_I' is to be calculated.

Summary

A mathematical method has been developed for considering all the equilibria which are thought to occur in a solution of α-chymotrypsin containing one or two inhibitor molecules. With slight modifications, this method can be applied to other enzyme systems for determination of binding constants and enzyme-induced chemical shifts.

FIGURE 2. Plot of K_I' and rms Error Versus K_I at pH 5.5



RESULTS

Studies with N-trifluoroacetyl-D, L-p-fluorophenylalanine

A solution containing N-trifluoroacetyl-D, L-p-fluorophenylalanine and α-chymotrypsin exhibits two fluorine nmr peaks for both the trifluoroacetyl and aromatic fluorines. The aromatic fluorine appears as a singlet for each isomer (the AA'BB'X multiplet usually seen for the aromatic fluorine is collapsed by proton noise decoupling). For both types of fluorines, one peak is fairly sharp and exhibits about the same chemical shift as the inhibitor molecule without enzyme. The other peak is broadened and appears downfield of the position without enzyme. Preliminary concentration studies revealed that the upfield peak exhibited little change in chemical shift and linewidth as the ratio of inhibitor concentration to enzyme concentration was changed. The downfield peak, however, was observed to broaden and shift further downfield as the ratio of inhibitor concentration to enzyme concentration decreased. Experiments in which the pure L-isomer was observed at varying concentration ratios indicated that it was the D-isomer which exhibited the varying shift and linewidth.

If the L-isomer is added to an α-chymotrypsin solution containing the D, L-racemate, the chemical shift difference between the two resonances decreases very slightly as a result of the

D-isomer moving upfield. By variation of the amount of L-isomer added over a range, it is possible to determine an approximate binding constant for this isomer. From such experiments, it was found that at all pH values studied the enzyme-inhibitor dissociation constant for the L-isomer exceeded that for the D-isomer by a factor of ten or more (3×10^{-3} M versus 3×10^{-4} M at pH 6.0). The L-isomer therefore does not effectively compete for binding sites with the D-isomer. The fluorine resonance of the L-isomer was therefore used as an internal reference from which to measure chemical shifts of the D-isomer. This made it unnecessary to correct for diamagnetic susceptibility changes as a result of the addition of the enzyme to the solution.

As mentioned, it was desirable to measure not only K_I and Δ for N-TFA-D-p-fluorophenylalanine, but also to determine K_D, the enzyme dimerization constant, to aid in the analysis of the data for this and other inhibitors. Therefore, data were collected from experiments in which either the concentration of the inhibitor or the enzyme was varied while the other concentration was kept constant. No attempt to determine quantitative values for the L-isomer was made since its dissociation constant is too large to be accurately determined by this method.

For experiments in which the enzyme concentration was varied, $[I_o]$ (the concentration of the D-isomer) was held constant at ~ 1.5 mM and $[E_o]$ was varied between 1.9 mM and 5×10^{-2}

mM. Eight experimental points were taken at pH 5.0, 6.0, and 7.0. The active enzyme concentration was taken as 80% of the total protein concentration since an active site titration indicates that only 80 ± 2% of commercial α-chymotrypsin is active (29). At pH 5.0 and 6.0, 0.1 M sodium citrate buffers were used to prepare samples. At pH 7.0, 0.1 M tris buffer was used. As will be shown later, α-chymotrypsin behaves identically in both buffer systems.

For experiments in which $[I_o]$ was to be varied, the enzyme concentration was held constant at ~ 1.9 mM (corrected for inactive protein). The D-inhibitor concentration was varied between 0.5 and 20 mM. Since the inhibitor was added as the D, L-racemate, each sample contained an equal concentration of L-isomer from which chemical shifts were measured. Eight points were taken at each half pH unit between 5.0 and 8.0. Experiments were run in 0.1 M sodium citrate buffer at pH 5.0 to 7.0 and in 0.1 M tris buffer from pH 7.0 to 8.0. The experiments at pH 7.0 were done in both buffers and indicated no dependence on the buffer system used.

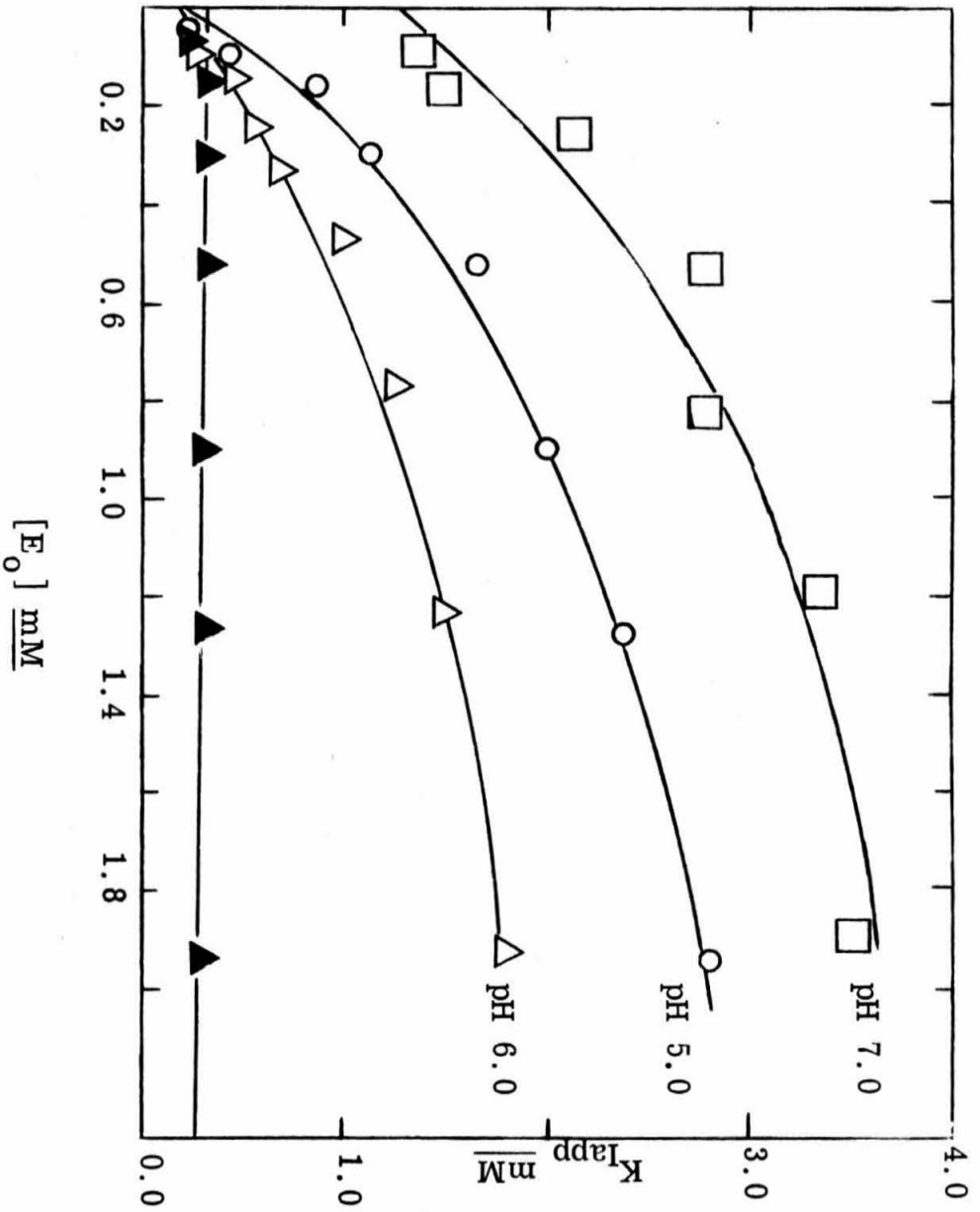
N-TFA-D-p-fluorophenylalanine has two fluorines which can be monitored by nmr. In the experiments using varying $[I_o]$, both were monitored. Because of the greater line broadening (probably due mostly to increased exchange broadening (28)) and lower sensitivity (the three fluorines of the trifluoroacetyl group are

equivalent) of the aromatic fluorine, it was possible to observe the trifluoroacetyl fluorines at much lower concentrations than could be used for the aromatic fluorines. Therefore, both types of fluorines were observed only at the four highest inhibitor concentrations. In the experiments using a constant inhibitor concentration, only the trifluoroacetyl fluorines were observed. The experimentally observed values of δ_{obs} at various values of $[E_o]$ and $[I_o]$ were analyzed according to eqs 3, 9, 11, and 13 to determine K_I, K_D, and Δ for the N-TFA-D-p-fluorophenylalanine-α-chymotrypsin system.

In Figure 3 is shown a plot of K_{Iapp} (determined for Δ = -65 Hz from eqs 12d and 14) versus enzyme concentration. This clearly shows the dramatic effect which enzyme oligomerization has on the analysis. At pH 5.0, K_{Iapp} varies by an order of magnitude (0.3 to 3.0 mM) as the enzyme concentration ranges between 0.05 and 1.9 mM. Extrapolation to infinitely dilute enzyme concentration corrects for dimerization and should yield the proper value for K_I. It is seen that the data at pH 5.0 and 6.0 extrapolate to the same limiting value of K_{Iapp} and indicate that K_I is equal at these two pH's. The higher limiting value obtained at pH 7.0 is a result of decreased apparent binding at the higher pH.

The value of K_D at these pH's can be determined by a least squares fit of K_{Iapp} versus [EI]/[I] (eq 13). The slope of such a plot will be equal to $2K_I^2/K_D$. If this is done, the dimerization

FIGURE 3. Dependence of K_{Iapp} on $[E_o]$ Neglecting Enzyme Self-association at pH 7.0 (□), 5.0 (⊙), and 6.0 (△). If Self-association is included, K_I does not depend on $[E_o]$ (△ are for pH 5.0 and $K_D = 5 \times 10^{-5}$ M).



constants given in Table I at pH 5.0, 6.0, and 7.0 are obtained. Also listed are the values of K_I obtained from the intercept of a plot of K_{Iapp} versus [EI]/[I]. As previously discussed, only the observed shifts of the trifluoroacetyl fluorines were used in this analysis because of the difficulty in obtaining aromatic fluorine spectra. Note that the values of K_D are rather small (indicating strong self-association) and increase rapidly at higher pH. It is obviously risky to ignore dimerization constants of this value. If dimerization is included in the determination of K_{Iapp}, the value obtained is essentially independent of enzyme concentration as is shown by the darkened triangles in Figure 3 at pH 5.0. This line corresponds to $K_D = 5 \times 10^{-5}$ M indicating a considerable amount of dimerization. Note that at pH 5.0 and 6.0 the corrected and uncorrected data go to the same limiting value at low enzyme concentration as would be expected. If the value of K_D determined in this manner is used to calculate the amount of monomeric protein present, the results agree well with the value which would be obtained using the previously reported values of $K_D = 9.6 \times 10^{-4}$ M and K_T (for trimerization: $E_3 \rightleftharpoons EE + E$) $= 2.9 \times 10^{-4}$ M at pH 6.2 (19).

As a further evidence of the importance of dimerization on α-chymotrypsin equilibria, K_I values have been determined with and without correction for dimerization. Data gathered at constant enzyme concentration (~ 1.9 mM) were analyzed according to eqs

TABLE I. Enzyme-Inhibitor and Enzyme-Dimer Dissociation Constants from Analysis of Type (ii) Experiments With α-Chymotrypsin and N-TFA-D-p-fluorophenylalanine[a]

pH	K_I	K_D
5.0	3.6×10^{-4} M	5×10^{-5} M
6.0	3.2×10^{-4} M	9×10^{-5} M
7.0	1.2×10^{-4} M	4×10^{-4} M

[a]The inhibitor concentration was ~ 1.5 mM and the enzyme concentration was varied from 0.05 to 1.94 mM.

3 and 5 as if there were no dimerization. The values obtained are listed in Table II and plotted in Figure 4. Note that the low pH values for K_{Iapp} are in the millimolar range and that there is an apparent minimum around pH 6.5.

If the same experimental data is analyzed with the inclusion of dimerization, quite different results are obtained. The corrected values for K_I are also given Table II and plotted in Figure 5. The binding constants are now about an order of magnitude smaller, and there is no minimum. At low pH the value of K_I is essentially constant. The minimum observed before was the result of the competing effect of decreasing dimerization (leading to more free enzyme and greater apparent binding) and decreasing binding at high pH.

Note that there is a slight difference between the corrected values of K_I given in Table II and those listed in Table I. The values listed in Table I represent the intercept of a plot of K_{Iapp} versus $[EI]/[I]$. Those given in Table II represent refined values obtained by using the K_D values determined in the enzyme concentration dependent experiments to analyze the data obtained at constant $[E_o]$. The values in Table II represent an iterative refinement and are the most accurate obtainable by this method. The rather small differences between these values indicate how rapidly this procedure converged.

TABLE II. Enzyme-Inhibitor Dissociation Constants for α-Chymotrypsin and N-TFA-D-p-fluorophenylalanine[a]

pH	Buffer	K_{Iapp}[b] mM	K_I[c] mM	pK_I
5.0	0.1 M citrate	2.94	0.35	3.46
5.5	0.1 M citrate	2.19	0.31	3.50
6.0	0.1 M citrate	0.93	0.33	3.49
6.5	0.1 M citrate	1.64	0.43	3.37
7.0	0.1 M citrate	3.03	0.91	3.04
7.0	0.1 M tris	3.21	0.96	3.02
7.5	0.1 M tris	7.69	2.79	2.55
8.0	0.1 M tris	18.0	7.08	2.15

[a]The enzyme concentration was 1.94 mM and the inhibitor concentration varied between 0.5 and 20 mM.

[b]Calculated assuming all active enzyme is available for binding.

[c]Calculated assuming only enzyme monomer binds inhibitors.

FIGURE 4. Variation in K_{Iapp} (dimerization neglected) as a Function of pH

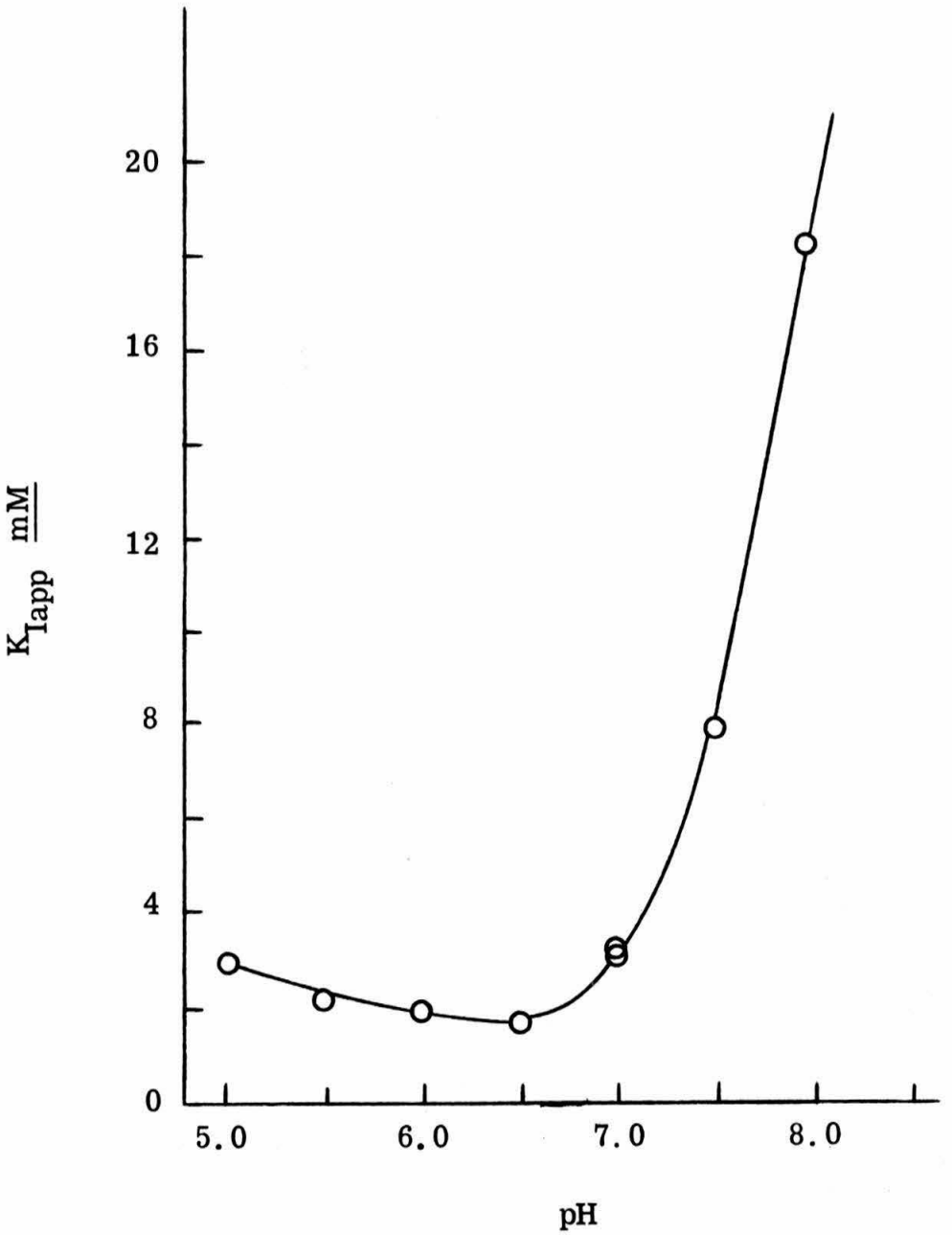
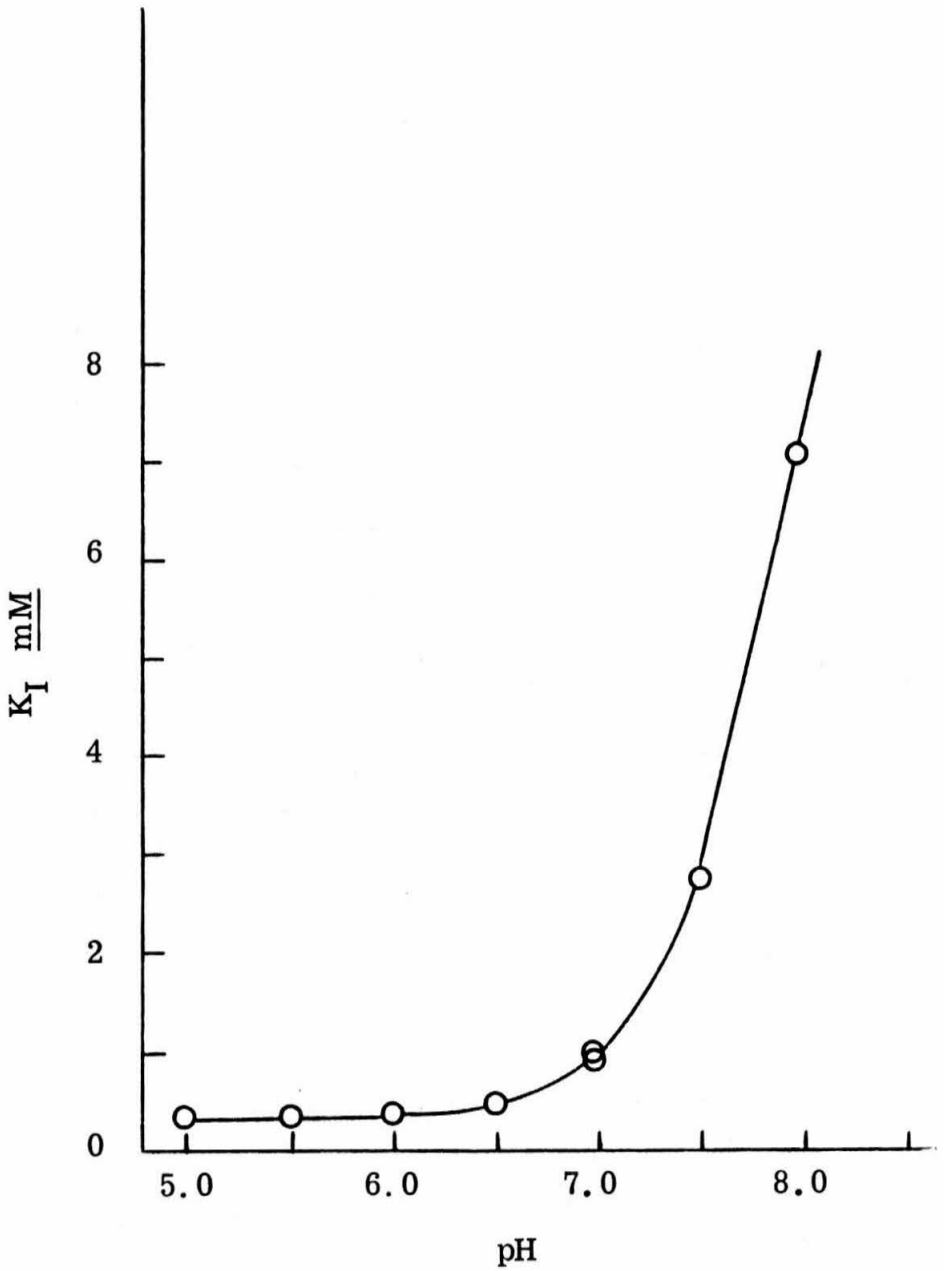


FIGURE 5. Variation of K_I (dimerization included) as a Function of pH



At all pH's the value of Δ obtained for the acetyl fluorines of N-TFA-D-p-fluorophenylalanine was -65 Hz (-.69 ppm). That is, these fluorines resonate 65 Hz downfield of their solution frequency when bound to the enzyme. If the data gathered for the aromatic fluorines is analyzed with the values listed in Table II, a value of Δ = -115 (±15) Hz (-1.22 ppm) is obtained at each pH (again, as in the case of the N-TFA group of the D-isomer, a fairly large downfield shift). The K_I values in Table II were used to analyze the aromatic data since it was felt that the few aromatic spectra observed were not adequate for determining reliable binding constants. If such an analysis is attempted, K_I values in reasonable agreement (within a factor of 2) with the correct values are obtained, but the least squares errors are larger than those calculated when analyzing the trifluoroacetyl data. The agreement is sufficiently good to indicate that both ends of the inhibitor molecule interact with the same binding constant.

These results were obtained using the L-isomer of N-TFA-p-fluorophenylalanine as an internal reference. As previously discussed, due to weak binding and strong inhibition from the D-isomer, the L-isomer does not exhibit a concentration dependent chemical shift in a solution containing the D, L-racemate. If the L-isomer is observed alone, measurable shifts are seen, and some analysis is possible. Therefore, the concentration dependent shift of the L-isomer was observed as a function of

inhibitor concentration at pH 6.0. With the approximate binding constant of 3 mM determined by inhibition of the D-isomer, these shifts were analyzed to yield values of $\Delta < 5$ Hz (downfield) for the trifluoroacetyl fluorines and $\Delta \cong -120$ Hz (± 20 Hz) for the aromatic fluorines. The value obtained for the trifluoroacetyl fluorines differs greatly from the D-isomer value. Within experimental error, however, the aromatic fluorines exhibit identical shifts when bound to the enzyme.

Studies with N-trifluoroacetyl-D, L-tryptophan

A solution of N-TFA-D, L-tryptophan and α-chymotrypsin exhibits a fluorine nmr spectrum which is similar to that seen with N-TFA-D, L-p-fluorophenylalanine. The L-isomer appears as a fairly sharp peak at about the same frequency observed without enzyme. The D-isomer is shifted downfield and broadens as a function of enzyme to inhibitor concentration. If, however, the L-isomer is added to a solution of the D, L-racemate, the chemical shift difference between the two isomers decreases markedly. Thus, even though the L-isomer exhibits very little shift, it effectively competes for binding sites. This makes it undesirable to use the L-isomer as an internal standard from which to measure chemical shifts. The strong binding of N-TFA-L-tryptophan should allow the binding constant of this inhibitor to be measured by nmr techniques. Since the L-isomer

exhibits such a small chemical shift, it is not a good nmr probe. Therefore, experiments were done in which the effect of the L-isomer on the D-isomer shift was measured. These experiments were analyzed by the second inhibitor technique previously described to yield K_I' values for the L-isomer.

The analysis of experiments done by varying the L-isomer concentration are most easily analyzed if K_I and Δ are known for the D-isomer. In order to determine these values, binding curves were run at each half pH unit between 5.0 and 8.0. The enzyme concentration was held constant at 1.92 mM (corrected for inactive protein), and the inhibitor concentration was varied between 0.8 mM and 22 mM. The complementary experiments in which $[E_o]$ is varied at constant inhibitor concentration was not done since K_D was already known from the N-TFA-p-fluorophenylalanine results.

Since the L-isomer could not be used as an internal reference from which to measure chemical shifts, it was necessary to find another standard. N-TFA-glycine was chosen and was added to each sample at a concentration of $\sim$ 2 mM. In order to ensure that N-TFA-glycine did not also inhibit the binding of N-TFA-D-tryptophan to α-chymotrypsin, several nmr samples were prepared in which the N-TFA-glycine concentration was varied between 1 and 80 mM while the N-TFA-D-tryptophan and α-chymotrypsin concentrations were held constant at 2.3 and 1.92 mM, respectively. For each of these samples the chemical shift difference between

the glycine and tryptophan resonances was measured. Only at very high concentrations of glycine was there any noticeable difference. This allowed the determination of a lower limit of 6 mM for the dissociation constant of the N-TFA-glycine-α-chymotrypsin complex at pH 6.0. Since this value exceeds the value for N-TFA-D-tryptophan by a factor of forty, at a concentration of 2 mM there should be little competition for binding sites by the internal standard.

Experiments were run with N-TFA-D-tryptophan in the same buffers and at the same concentrations as with N-TFA-p-fluorophenylalanine. The results were analyzed according to eqs 9 and 11 using the values of K_D previously determined. The results obtained for K_I are listed in Table III and plotted in Figure 6. If dimerization is not considered, the K_I values obtained are about an order of magnitude larger, and there is a pronounced minimum at pH 6.5. Correction for dimerization has essentially the same effect as was found for N-TFA-D-p-fluorophenylalanine results.

The value of Δ for the trifluoroacetyl fluorines of N-TFA-D-tryptophan was found to be -70 Hz (0.74 ppm) at each pH. This value is very similar to that obtained for the trifluoroacetyl fluorines of N-TFA-D-p-fluorophenylalanine and indicates a similar environment for the two inhibitors when bound to the enzyme.

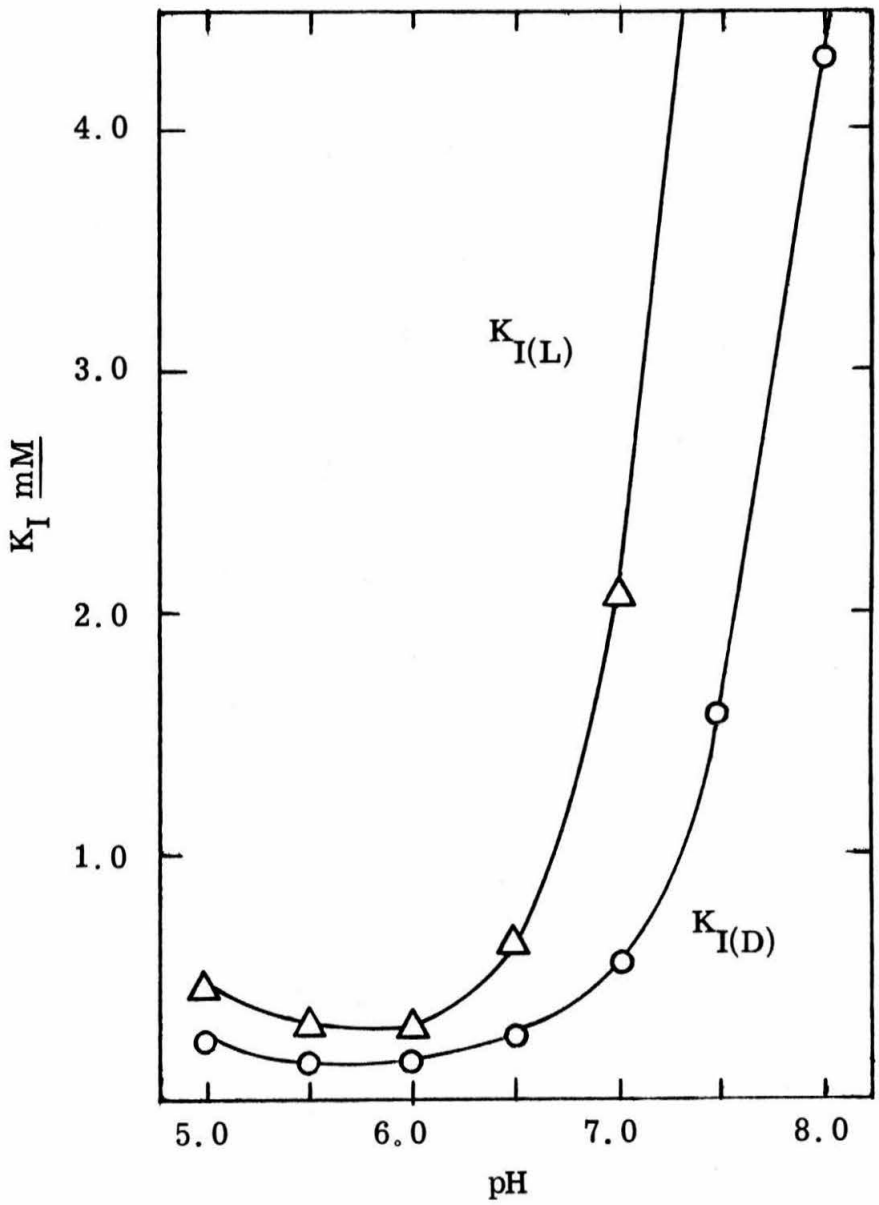
TABLE III. The Enzyme-Inhibitor Dissociation Constants, K_I and K_I', for α-Chymotrypsin and N-TFA-D-(and L-)tryptophan[a]

pH	Buffer	K_I mM	K_I' mM
5.0	0.1 M citrate	0.26	0.46
5.5	0.1 M citrate	0.18	0.26
6.0	0.1 M citrate	0.16	0.26
6.5	0.1 M citrate	0.30	0.61
7.0	0.1 M tris	0.59	2.12
7.5	0.1 M tris	1.60	[b]
8.0	0.1 M tris	4.24	[b]

[a]The initial enzyme concentration was 1.92 mM and the inhibitor concentration varied between 0.7 mM and 22 mM.

[b]Too large for measurement by our methods.

FIGURE 6. Dependence of Binding Constants $K_{I(L)}$ (Δ) and $K_{I(D)}$ (⊙) on pH.



With these values of Δ and K_I for the D-isomer, it is possible to determine K_I' for the L-isomer of N-TFA-tryptophan by competition experiments. Data were collected at each half pH unit between 5.0 and 8.0 in the buffers previously used. Equal amounts of the D- and L-isomers were added to each nmr sample. The enzyme concentration was held constant at ~ 1.9 mM, and the total inhibitor concentration was varied from 1 to 20 mM. The shift between the D- and L-isomers was measured and analyzed according to eqs 19-21 with K_D, K_I, and Δ values previously determined. Values of K_I' obtained by this method are listed in Table III and plotted in Figure 6. If the iterative procedure is allowed to choose both K_I and K_I' (from known values of K_D and Δ), similar values to those listed in Table III are obtained. Since the K_I values obtained for the D-isomer alone are probably more reliable (due to the simpler analysis) than those obtained in the D, L experiments, the values listed in Table III are the best obtainable by this method. From Figure II, it has already been seen that the value of K_I' is very sensitive to the value of K_I used in the analysis. It is therefore important to use the best K_I value possible. Note that at pH 7.5 and 8.0 K_I' for the L-isomer is too large to measure by this method.

From these values of K_I', it is possible to estimate Δ for the L-isomer by going to very low concentrations of the pure L-isomer. If this is done, some shift is seen, and a limiting

value of -5 Hz can be assigned to the trifluoroacetyl group of the L-isomer. Thus, the trifluoroacetyl fluorines of the L-isomer show a much smaller chemical shift than the D-isomer when bound to the enzyme even though both isomers bind about equally well.

DISCUSSION

The binding constants and chemical shift data obtained in the present work agree qualitatively with most previously reported nmr studies on the binding of inhibitor molecules to α-chymotrypsin; however, the quantitative agreement is poor (2, 9, 10, 13). Most of the discrepancy is due to the small chemical shifts (often less than 2 Hz) observed in other work and improper analysis of the data (for example, neglect of enzyme self-association). This results in binding constants which are generally an order of magnitude larger than those reported here. Analysis of the data obtained in the present work without consideration of oligomerization gives essentially the same numbers reported previously. Since a much larger range of concentrations has been used here than previously, a wide range of chemical shifts has been experimentally observed making the absolute magnitudes of the K_I and Δ values obtained here more reliable than those reported elsewhere.

Spotswood et al. (2) reported a K_I of $6 \pm 2 \times 10^{-3}$ M and a Δ of -83 ± 5 Hz for the aromatic fluorine of N-acetyl-D-p-fluorophenylalanine. These experiments were carried out by observing the D-L splitting of the undecoupled aromatic fluorine spectrum. Due to the overlap of the multiplets, accurate chemical shift data were difficult to obtain. The large downfield shift of the

aromatic fluorine is in qualitative agreement with the present work and indicates a similar behavior between N-acetyl- and N-trifluoroacetyl-amino acid derivatives. A more rigorous analysis of the data reported would undoubtedly produce very similar results in the two systems.

Sykes (10) has reported values of $K_I = 4.9 \times 10^{-2}$ M and $\Delta = +41$ Hz for the trifluoroacetyl fluorines of N-TFA-D-phenylalanine at pH 7.5. The binding constant is reasonable considering the high pH at which the experiments were done. The upfield shift reported is hard to understand. The present work indicates that the trifluoroacetyl fluorines of D-amino acid derivatives should show a downfield shift, and it is not reasonable that the trifluoroacetyl group of N-TFA-D-phenylalanine should be in an environment which is so different from that experienced by tryptophan and p-fluorophenylalanine derivatives. The upfield shift observed may be the result of improper choice of the origin from which to make measurements. Sykes' experiments were carried out at varying enzyme concentrations, but no systematic correction for changes in the bulk diamagnetic susceptibility produced by the addition of enzyme was made. Since the diamagnetic shift is as large as 3 Hz (at $[E_o] = 2$ mM) and Sykes observed shifts which were two cycles or less, it is doubtful that his data are at all reliable.

A downfield shift for the trifluoroacetyl fluorines of N-TFA-D-phenylalanine has been reported (9). This work reports Δ = -164 Hz for the D-isomer and Δ = -110 Hz for the L-isomer. The present work indicates that the L-isomer of N-TFA-amino acids should not show an appreciable shift (this has in fact been observed by R. B. Moon for N-TFA-L-phenylalanine), and the large Δ value reported is surprising. The data analysis used in obtaining this value did not allow for the -3 Hz diamagnetic susceptibility change caused by the addition of enzyme to the system. Probably, most of the shift observed for the L-isomer can be accounted for by this phenomenon. A more realistic value of Δ for the L-isomer is about -5 Hz. If the value of Δ reported for the D-isomer is corrected by that for the L-isomer, a value of Δ = -54 Hz is obtained. This number is about what would be expected from the results of the present work with D-isomers of other amino acids.

Ashton and Capon (13) reported a pH dependence of the K_I and Δ for N-TFA-D-tryptophan. These workers observed an increase in K_I at high pH just as was observed in the results reported here. They also reported a value of Δ which varied erratically from -112 to -255 Hz with pH and indicated no explainable correlation of Δ with any ionization. These workers made all measurements from external trifluoroacetate and did not correct for diamagnetic susceptibility differences between the

reference and sample solutions. This correction amounts to almost 25% of the largest shift observed (~ -13 Hz) and undoubtedly produces considerable error in the calculated value of Δ. The highly variable value of Δ is a result of K_I values which are not quantitatively correct due to the neglect of oligomerization in the data analysis. These workers only made measurements at four pH's and therefore could not define the pK value associated with the increase in K_I they observed.

The present work has cleared up some of the inconsistencies reported previously and presents a systematic approach to the nature of inhibitor-α-chymotrypsin equilibria. It is felt that the results presented here have fully characterized the interaction of the protein with anionic inhibitor molecules.

Mode of Binding

The values of Δ obtained for the inhibitors used in this study have a bearing on the mode of binding of these molecules to α-chymotrypsin and are particularly useful in discussing a comparison of the binding of D- and L-isomers. From the results obtained with N-TFA-D-tryptophan and N-TFA-D-p-fluorophenylalanine, it is seen that the trifluoroacetyl fluorines of these inhibitor molecules experience a rather large and similar shift of 65-70 Hz downfield when bound to α-chymotrypsin. This indicates that the trifluoroacetyl groups of both compounds interact with the enzyme

and are in very similar environments. For the L-isomers, the results are quite different, and Δ values of less than 5 Hz downfield are calculated indicating that the trifluoroacetyl groups of L-isomers are little perturbed on interaction of L-amino acid derivatives with the enzyme. The N-TFA group of these substances experiences an environment very similar to that of solution. In the case of N-TFA-p-fluorophenylalanine, the aromatic fluorines of both isomers experience the same downfield shift of about 115 Hz. Thus, the aromatic rings of both D- and L-isomers are in a similar environment in the enzyme.

The active site region of α-chymotrypsin has been mapped by Niemann and his co-workers (30-32) by observing the effect which groups of various sizes and polarities have on the catalysis rates of various chymotrypsin substrates. From this work, it was proposed that there are three important loci at the active site which determine the mode and strength of binding of substrate and inhibitor molecules to α-chymotrypsin. Cohen and co-workers (33, 34) using cyclized α-tetrasubstituted substrates which had locked geometries arrived at similar conclusions although a fourth site was included. The crystal structure of α-chymotrypsin (24) has given a molecular basis for this description and has assigned specific amino acids to each of the loci previously indicated.

The most important locus in binding is the "hydrophobic pocket" or tosyl hole (24a) seen in the crystal structure. This

nonpolar region is responsible for the specificity of α-chymotrypsin toward amino acids with aromatic or large hydrophobic side chains. The interaction of the amino acid with this region is probably principally responsible for the binding of inhibitors to α-chymotrypsin. Crystallized derivatives of α-chymotrypsin have indicated that the aromatic moiety of covalently bound tosyl (24a) and indoleacrylolyl (24d) groups as well as the side chains of bound N-formyl-L-tryptophan and N-formyl-L-phenylalanine (24c) reside in this locus. The residues involved in shaping the hydrophobic pocket have been identified (24c) as Ser 190, Cys 191, Met 192, Try 215, and Gly 216. The aromatic side chain is positioned parallel to the plane of the peptide bond between 190-191 and 191-192 on one side and 215-216 on the other side. The narrowness of the pocket fairly well restricts the orientation of the aromatic ring with respect to the pocket.

A second locus of the active site is the hydrolytic area which contains the residues responsible for catalysis, His 57 and Ser 195. The carboxyl group of bound N-formyl-L-tryptophan is found in this region (24c), and presumably the ester or amide bond to be cleaved would be directed to this site. At pH's below the pK of His 57, this region will have a net positive charge. After ionization, the region will be somewhat negatively charged due to the presence of ionized Asp 102 which is hydrogen bonded to His 57 (24b). This change in charge as His 57 is deprotonated

probably causes the decrease in binding affinity of negatively charged inhibitors at high pH observed in this work.

The third important region of the active site has been shown to be essentially contiguous with the "hydrophobic pocket" and is near the γ-methylene of Met 192 (24c). The α-proton of N-formyl-L-tryptophan has been seen to be in van der Waals contact with this region of the enzyme. Niemann (30) observed from kinetic studies that replacement of the α-proton of amino acid substrates with any group larger than methyl led to decreased affinity of α-chymotrypsin for the substrate. A similar conclusion was reached by Cohen (34) using cyclized substrates. The close association of the α-proton of N-formyl-L-tryptophan with the γ-methylene of Met 192 indicates that a larger group would probably interact unfavorably if crowded into this region (24c). Strong binding of a molecule with α-chymotrypsin probably requires that no large group be situated in this region of the active site.

Cohen (34) extended Niemann's theory to include a fourth locus in which the acylamido group of L-substrates was bound. This area was thought to be near the surface of the enzyme and to form a hydrogen bond with some group of the enzyme. Crystal studies (24c) have indicated that this hypothesis is probably correct. The formyl group of N-formyl-L-tryptophan lies close to the surface of the enzyme near the backbone carbonyl of Ser 214. It was postulated that in solution the amido proton of specific

substrates probably form a hydrogen bond to this carbonyl oxygen. These interactions properly align the substrate for cleavage in the hydrolytic locus. The nmr data obtained here supports this theory for the binding of substrates and inhibitors to α-chymotrypsin and confirms that the mode of binding seen in the crystal is like that observed in solution.

Because of the specificity of α-chymotrypsin for aromatic side chains, binding of both D- and L-isomers undoubtedly places the aromatic side chain of the amino acid into the hydrophobic pocket. This should put the aromatic fluorine of both isomers of N-TFA-p-fluorophenylalanine in a very similar environment, and the identical chemical shift seen for both isomers indicates that this is indeed the case for these inhibitors. Presumably a similar situation exists for N-TFA-D, L-tryptophan.

From what has been said about the site near the γ-methylene group of Met 192, it is reasonable that, for the inhibitors studied in this work, the α-proton is probably bound in this region since neither the carboxyl nor trifluoroacylamido group could be accomodated without strongly unfavorable steric interactions which should lead to much larger K_I values than are observed. It is therefore reasonable that the aromatic side chain and α-proton of both isomers of the inhibitors studied here reside in essentially the same locus of the enzyme active site. This requires that the other two groups attached to the asymmetric carbon of these amino acid derivatives

exchange places on the enzyme on going from one antipode to the other.

Since ester and amide derivatives of L-amino acids are hydrolyzed so much more rapidly than the D-isomers, it seems reasonable that the carboxyl group of the L-amino acids used in this study should lie in the hydrolytic locus near His 57 and Ser 195. This agrees with what is seen with N-formyl-L-tryptophan in the crystal structure (24c). The trifluoroacetyl group must therefore lie near the enzyme surface in the vicinity of the carbonyl oxygen of Ser 214. For the D-isomer inhibitors, the opposite would be expected with the trifluoroacylamido group lying near the hydrolytic locus and the carboxyl group lying near the surface.

This hypothesis is consistent with the chemical shift data obtained in this work. The trifluoroacetyl group of the L-isomers experience very little shift when bound to the enzyme ($\sim$ 5 Hz). They therefore experience a very solution-like environment which would be anticipated as the fluorines would be near the enzyme surface and not necessarily interact with any enzymic groups. This possibly rules against the hydrogen bond between the amide proton of the inhibitor and the carbonyl oxygen of Ser 214 proposed from crystal studies (24c). It is not possible to say whether such an interaction (which is four bonds removed from the observed fluorines) would have a larger effect on the fluorine chemical shift than is observed.

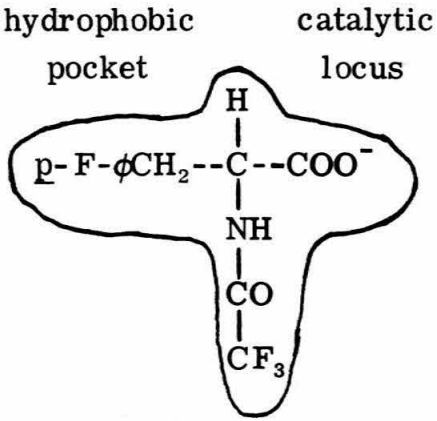
The trifluoroacetyl fluorines of the D-isomers exhibit much larger shifts (65-70 Hz) as would be expected if they are indeed near His 57 and Ser 195 as proposed. The large value of Δ found for these compounds indicates that they may be interacting strongly and similarly with the enzyme as would be the case if they follow the generalized binding hypothesis discussed above. The nearness of the fluorines to the catalytic residues would certainly have an appreciable effect on their chemical shifts. The reversal of the carboxyl and trifluoroacylamido groups quite adequately accounts for the trifluoroacetyl chemical shifts calculated in this work. The proposed mode of binding of the two isomers is diagrammed in Figure 7.

Interpretation of Chemical Shifts

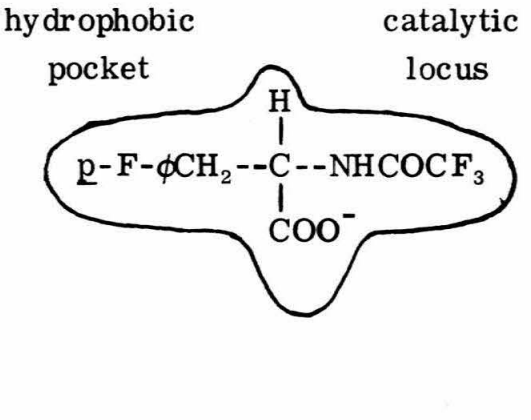
Since nmr studies of this type give chemical shift data which are dependent on the environment which an individual nucleus experiences when bound to a protein, it is in principle possible to obtain detailed information about the exact nature of that part of the enzyme with which the nucleus interacts. In practice this turns out to be very difficult due to the uncertainties involved in interpreting fluorine chemical shifts.

Because fluorine contains occupied p-orbitals, the primary influence on fluorine chemical shifts is the paramagnetic term in Ramsey's equation (35). Such shifts are dependent on the extent

FIGURE 7. Proposed Stereochemistry of Binding of D- and L-Inhibitors to α-Chymotrypsin



Binding of N-TFA-L-p-fluorophenylalanine



Binding of N-TFA-D-p-fluorophenylalanine

the p-electron density of a given fluorine deviates from spherical symmetry. Large deviations produce large downfield shifts. Since deviations from spherical symmetry can be caused by many factors, it is not possible to correlate fluorine chemical shifts strictly with electron density as is usually done for protons.

Theories have been developed (36, 37) for correlating fluorine chemical shifts with changes in electron density and distribution as a result of changes in ionic or hybrid character of a fluorine system. Since no covalent interaction occurs between α-chymotrypsin and the inhibitors used in this work, it is unlikely that changes of this type are involved in the shifts observed

More likely, the shifts seen here represent a change in gross environment seen by the inhibitor molecule. The effects of solvent induced perturbations such as diamagnetic susceptibility, van der Waals interactions, and permanent and induced electric fields on fluorine chemical shifts have been investigated by Emsley and Philips (38) and Evans (39). Correlations between chemical shift and solvent induced shifts is very complex even for simple systems.

The very small chemical shift seen for the trifluoroacetyl fluorines of the L-isomers used in this study are probably indicative of little or no interaction with the enzyme upon binding as discussed previously. Compensating effects of strong interactions could coincidently produce a small net shift, but this seems unlikely. That these fluorines still see a solution-like

environment seems a more reasonable explanation.

The larger shifts seen by the trifluoroacetyl fluorines of the D-isomers are difficult to interpret unambiguously. These fluorines are in the vicinity of a serine hydroxyl group and a protonated histidine residue when bound to the enzyme. The positive charge could lead to decreased electron symmetry on the fluorines (and hence a downfield shift) through electrostatic interactions or by specific hydrogen bonding of the positively charged locus to the carbonyl oxygen or amide proton of the trifluoroacylamido group of the inhibitor molecule. Ring current effects from the imidazolium ion of protonated His 57 or van der Waals interactions may also lead to a downfield shift of the magnitude seen (38). Electric fields which can polarize the electrons in fluorine bonds can also be responsible for large shifts. The trifluoroacetyl fluorines of D-isomers are located between the positive charge of His 57 and the deprotonated carboxyl group of the inhibitor molecule and are therefore in an electric field which could easily lead to the shifts seen. As discussed in Part II, inhibitors in which the carboxyl group has been neutralized (with an amide or semicarbazide group) give much smaller shifts when bound to the enzyme indicating that the electric field effect may be responsible for the shift seen with the anionic inhibitors used in this work.

The large downfield chemical shift observed for the aromatic fluorine of N-TFA-D, L-p-fluorophenylalanine is likewise hard to unambiguously attribute to any one interaction. Quite possibly, this shift is the result of van der Waals interactions with the enzyme which would be expected if the aromatic side chain of the inhibitor molecule fits tightly into the specificity pocket. It is also possible that a specific hydrogen bond is formed with the hydroxyl proton of Ser 189 which is buried deeply in the hydrophobic pocket (24a). The electric field created by the dipole between the ammonium group of Ile 16 and the carboxyl group of Asp 194 could also account for the shift of the aromatic fluorine.

It seems quite likely that the shifts of inhibitor molecules when bound to α-chymotrypsin are the net result of many factors and that unambiguous assignment of a specific interaction to an observed shift is presently impossible.

pH Dependence of Binding Constants

Figures 5 and 6 show a sharp increase in the enzyme-inhibitor dissociation constant above pH 6.5 for N-TFA-D, L-tryptophan and N-TFA-D-p-fluorophenylalanine. Above pH 8.0, the binding is so poor that it is not possible to obtain sufficiently accurate chemical data to give reliable values of K_I. A similar behavior was observed for N-acetyl-D-tryptophan by Johnson and Knowles (26) using an equilibrium dialysis study. Their values of

K_I are about an order of magnitude too large as a result of neglecting enzyme oligomerization, but the pH dependence they observed compares well with work reported here.

The data on the dependence of K_I on pH can be conveniently analyzed by the method of Dixon and Webb (40) which plots pK_I versus pH. Such a plot theoretically consists of straight line segments which connect at the pK values of ionizations on the free enzyme, free inhibitor, or enzyme-inhibitor complex. If an ionization results in a decrease in slope then the free inhibitor or enzyme is ionizing. An increase in slope indicates an ionization on the enzyme-inhibitor complex.

Figures 8 and 9 show such plots of pK_I versus pH for the D-isomers of N-TFA-p-fluorophenylalanine and N-TFA-D-tryptophan, respectively. These figures show that the binding of both inhibitors depends strongly on an ionization of the free enzyme with a pK_a of 6.6 (since the inhibitors used exhibit no ionization around 6.6 the free inhibitor can be ruled out as the source of the observed ionization). This ionization causes a sharp decrease in binding. The binding constant after ionization is not measurable by nmr methods because it is too large and adequate chemical shifts are not observed. If plots such as those shown in Figures 8 and 9 are made for K_I values obtained by neglecting dimerization, then a pK_a value of 6.9 is indicated and illustrates again the importance of dimerization on the interpretation of binding data

FIGURE 8. pK_I for N-TFA-D-p-fluorophenylalanine Versus pH. The intersection of the two linear segments indicates a pK_a for the free enzyme of 6.6.

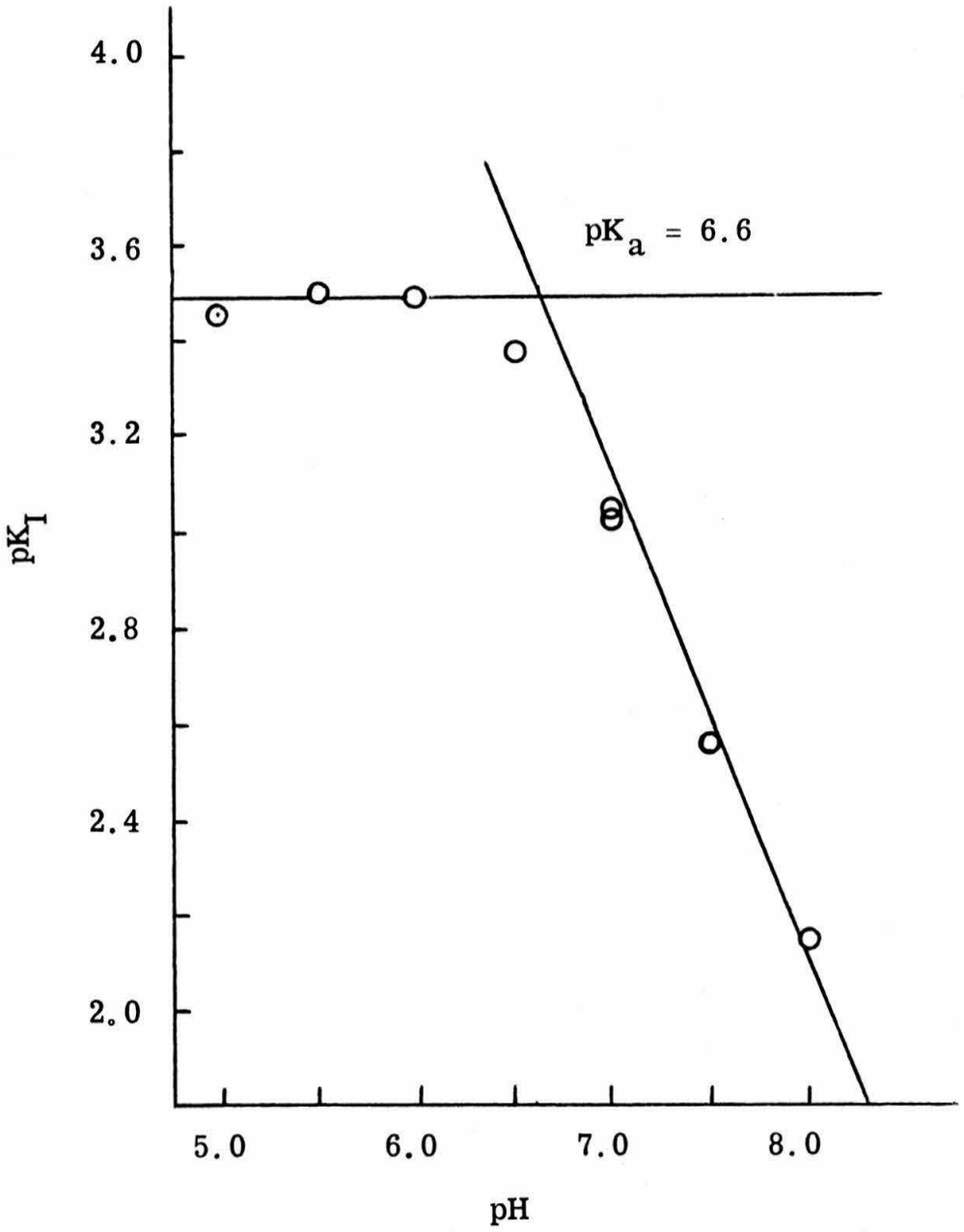
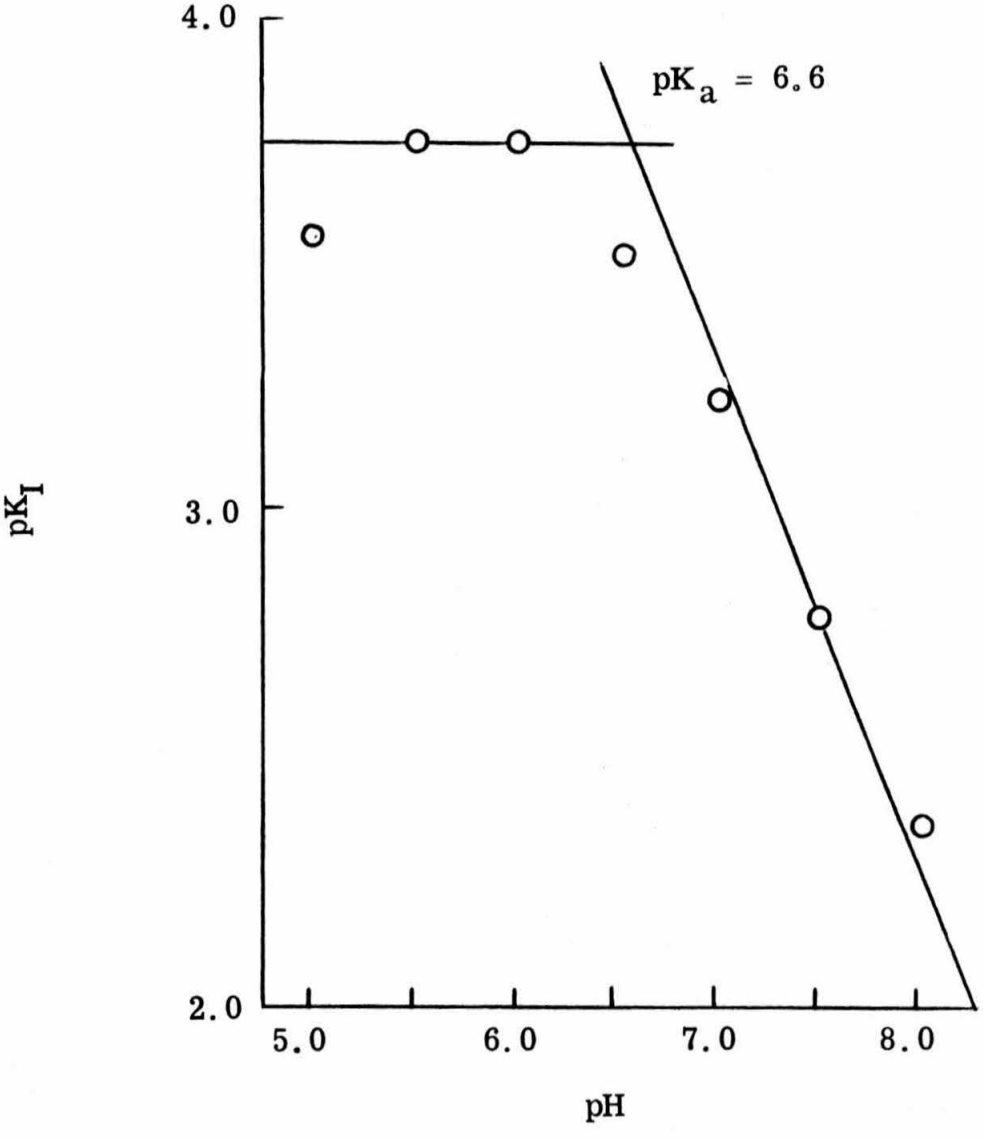


FIGURE 9. Plot of pK_I for N-TFA-D-tryptophan Versus pH. The intersection of the two linear segments indicates a pK_a of 6.6 for the free enzyme.



obtained with α-chymotrypsin. The decrease in observed binding is a result of decreasing amounts of the strongly binding form of the protein at higher pH.

This large increase in K_I explains why the Δ values obtained in this work are pH independent over the range studied. Binding to the form of the enzyme produced by the ionization with pK_A = 6.6 is so poor that it does not contribute to the observed chemical shifts. All the shifts observed are the result of binding to the low pH form of the enzyme.

The group with pK_A = 6.6 which causes the large increase in K_I is most probably His 57. Kinetic studies have previously implicated a group with a pK between 6.5 and 7.0 in the catalytic mechanism of α-chymotrypsin (41). Reaction of His 57 with chloromethylketones (42) and methyl p-nitrobenzenesulfonate (43) modify this residue and cause a drastic if not complete reduction in the ability of the enzyme to hydrolyze ester and amide bonds. Most importantly, crystallographic studies (24a) have placed His 57 at the active site of α-chymotrypsin.

Ionization of His 57 should leave the catalytic locus with a net negative charge due to the presence of the carboxylate anion of Asp 102. This negative charge would cause strong electrostatic repulsion with the anionic inhibitors used in this study and would account for the decrease in binding observed after the deprotonation of His 57. Figure 6 demonstrates that the L-isomer of

N-TFA-tryptophan seems to show an increase in K_I at a lower pH than the D-isomer. Analysis of this data also indicates a pK_A of 6.6 on the free enzyme. The apparent earlier break indicates that the K_I for interaction of the L-isomer with the deprotonated form of the enzyme is even larger than that for the D-isomer. This is reasonable when the mode of binding of the two isomers previously discussed is considered. In this scheme, the carboxyl group of the L-isomer is placed very near to His 57 and would be expected to interact more unfavorably after ionization.

That the observed effect is indeed an unfavorable electrostatic effect is indicated by the fact that a neutral inhibitor, N-acetyl-D-tryptophan amide, does not show the binding decrease observed for anionic inhibitors (26). Although there is another residue (His 40) near the active site of α-chymotrypsin which might ionize around pH 6.6, it does not seem likely that this residue could cause the large electrostatic interaction observed since it is probably at least 7.0 Å away from the bound inhibitor molecule (24d) and does not produce a negatively charged region on the enzyme upon ionization.

Figure 6 reveals that the dissociation constants for both isomers of N-TFA-tryptophan show a slight decrease between pH 5.0 and 5.5. A similar, but larger, effect was seen when dimerization was neglected in the analysis. The data in Figure 6

have been corrected for dimerization, and the pH dependence is puzzling since it was not observed with N-TFA-p-fluorophenylalanine. The effect is rather small (~ .1 mM for the D-isomer and .3 mM for the L-isomer) and may simply mean that insufficient dimerization has been included. This possibly also represents a real conformational change (which was not observed with N-TFA-p-fluorophenylalanine) leading to decreased binding at lower pH, a possibility which has been previously discussed (44).

Enzyme Oligomerization

Although the phenomenon of enzyme oligomerization is not yet fully understood mechanistically, it has been quantitatively accounted for in this work to provide values of K_I which are independent of enzyme concentration in the range studied. Thus, the effect of self-association has been effectively eliminated by this treatment even though the values obtained for dimerization constants must be considered as effective numbers (perhaps representing several self-association equilibria). The dimerization constants calculated in this work could possibly also have corrected for any perturbation caused by the twenty per cent inactive protein found in commercial samples of α-chymotrypsin. If this material interacts with the native protein and inhibits the binding of inhibitor molecules, its effect should be dependent on its concentration which will vary linearly with $[E_o]$. The effect of the

inactive protein would probably be to decrease the amount of monomeric protein available for binding inhibitor molecules which is analogous to the effect of self-association and has therefore been partially corrected for with the effective dimerization constants calculated in this work.

The pH dependence of K_D found here is in agreement with what has been previously reported from sedimentation experiments (20). The amount of enzyme self-association decreases sharply above pH 5.0. Since oligomerization is highly dependent on temperature and ionic strength (21), the effective dimerization constants determined here are correct only for the experimental conditions used.

EXPERIMENTAL

N-Trifluoroacetyl-D, L-p-fluorophenylalanine

D, L-p-Fluorophenylalanine (10g, 0.036 mole) was dissolved in anhydrous trifluoroacetic acid (60 ml). The solution was cooled to -5° in an ice/salt bath, and trifluoroacetic anhydride (18 ml) was added dropwise with stirring over a 10 minute period. After two hours at 0°, trifluoroacetic acid and anhydride were removed by distillation under vacuum (~ 1 Torr) at 25°. The solid residue was dissolved in ether, and the solution was filtered and then dried over anhydrous sodium sulfate. After reduction of the solution to $\frac{1}{3}$ the original volume, the product crystallized. Recrystallization from ether-n-hexane yielded 5.2 g (50%) of white crystalline solid. Mp 143-144°. Anal. Calcd for $C_{11}H_9O_3NF_4$: C, 47.32; H, 3.25; N, 5.02. Found: C, 47.17; H, 3.02; N, 4.63.

D, L-p-Fluorophenylalanine Methyl Ester Hydrochloride

To a solution of thionyl chloride (10 ml) in absolute methanol (45 ml) in a Dry Ice/acetone bath was added D, L-p-fluorophenylalanine (10.0 g). The mixture was refluxed for 2 hours, and solvent and excess reagent were removed on a rotary evaporator (20-30°, 20 Torr). The residue was dissolved in a minimum

amount of absolute methanol, and the solution was filtered. Addition of anhydrous ether caused precipitation of white crystals which were collected by filtration and dried over sodium hydroxide. Yield 13.1 g (96%).

N-Trifluoroacetyl-D, L-p-fluorophenylalanine Methyl Ester

D, L-p-Fluorophenylalanine methyl ester hydrochloride (13.1 g) was placed in a separatory funnel with saturated sodium bicarbonate solution (400 ml) cooled with ice, and ethyl acetate (400 ml) was added. The mixture was shaken vigorously, and the phases were separated. The aqueous phase was extracted a second time with ethyl acetate (200 ml). The two ethyl acetate fractions were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness on a rotary evaporator leaving a gummy residue. Crystallization was not attempted. The gummy residue was dissolved in anhydrous ethyl acetate (300 ml), and fresh trifluoroacetic anhydride (10 ml) was added dropwise with stirring. The solution was allowed to stand at room temperature for 30 minutes with occasional stirring. Additional trifluoroacetic anhydride (8 ml) was then added with stirring, and the solution was allowed to stand for another 30 minutes with occasional stirring. The ethyl acetate solution was extracted with 10% aqueous sodium bicarbonate (300 ml), dried over anhydrous sodium sulfate, and filtered, and the ethyl acetate was evaporated. The

resulting gummy residue was dissolved in a minimum amount of ethyl ether, and this solution was added to five volumes of n-hexane. The solution was evaporated on a rotary evaporator to give a white powder. Yield 12.2 g (76%). Mp 59-61°.

N-Trifluoroacetyl-L-p-fluorophenylalanine

N-Trifluoroacetyl-D, L-p-fluorophenylalanine methyl ester (12.2 g) was dissolved in methanol (80 ml). Aliquots (10 ml) of this solution were added to a solution of α-chymotrypsin (250 mg) in water (750 ml). The pH was maintained at 7.5 by addition of 0.203 N NaOH, and all the ester was added over a period of one hour. The pH remained essentially constant after 98.5 ml of base had been added (corresponding to hydrolysis of 96% of the original L-isomer). The aqueous solution was extracted twice with ethyl acetate (400 ml) and lyophilized to dryness. The residue was dissolved in a minimum amount of water and precipitated by addition of concentrated hydrochloric acid to pH 2. The white solid was filtered with suction and washed on the filter with cold 0.01 N HCl. Then it was dissolved in a minimum amount of methanol, filtered, and precipitated by slow addition of 0.01 N HCl. The fine, needle-shaped crystals were filtered, washed with cold 0.01 N HCl, and dried in a vacuum desiccator with NaOH pellets overnight. Yield 2.08 g. Mp 133-134°. Anal. Calcd for $C_{11}H_9O_3NF_4$: C, 47.32; H, 3.25; N, 5.02. Found:

C, 47.14; H, 3.05; N, 4.74.

p-(N, N, N-Trimethylammonium)cinnamic Acid Iodide p-Nitrophenyl Ester

The titrant was prepared by R. B. Moon using the method of Knowles and Preston (29) with several modifications suggested by Parker (45).

N-Trifluoroacetyl-D-tryptophan (46)

Dry D-tryptophan (2.04 g, 0.01 mole) was dissolved in anhydrous trifluoroacetic acid (11.2 ml) with stirring. Anhydrous ethyl ether (13 ml) was added to the solution, and the reaction mixture was cooled to 0^{o} in an ice bath. Trifluoroacetic anhydride (2 ml) was added dropwise over a period of ten minutes. After about five minutes a precipitate formed. The mixture was allowed to stand another fifteen minutes and then filtered. The filtrate was washed with water and dried thoroughly. The crude product was recrystallized twice from toluene to yield 1.9 g (63%) of white needles. Mp 162-163^{o} (Lit. mp 163-164^{o} (47)).

N-Trifluoroacetyl-L- and N-Trifluoroacetyl-D, L-tryptophan were prepared in the above manner. Mp D 162-163^{o}; mp D, L 154-155^{o}.

N-Trifluoroacetylglycine (48)

Dry glycine (0.75 g, 0.10 mole) was dissolved in anhydrous trifluoroacetic acid (60 ml), and the solution was cooled to -10° in an ice/salt bath. Trifluoroacetic anhydride (17.6 ml) was then added dropwise with stirring. After all the anhydride had been added, the bath was removed, and the mixture was stirred for an additional 30 minutes at room temperature. The mixture was then stripped to dryness on a rotary evaporator. The residue was dissolved in ethyl ether (200 ml), filtered, and concentrated to half valume on a rotary evaporator. Hot toluene (150 ml) was added to the solution, and the remaining ether was removed at room temperature. The crystalline product was collected by suction filtration and recrystallized once from toluene. Mp 116-116.5° (Lit. mp 116° (46)).

α-Chymotrypsin

Three times recrystallized, salt-free α-chymotrypsin was obtained from Sigma Chemical Company and stored in a freezer when not in use. Its activity, as determined below, was ~ 80%.

Enzymatic Activity

The activity of the α-chymotrypsin was determined by the method of Knowles and Preston (29) using, as titrant,

p-(N, N, N-trimethylammonium)cinnamic acid iodide p-nitrophenyl ester. Absorbances were measured on a Cary 14 Spectrophotometer at 400 nm, and the final pH was measured on a Radiometer pH meter. Calculations assumed $pK_a = 7.04$ for p-nitrophenol and $\epsilon = 18,320$ for p-nitrophenolate anion. The enzymatic activity was determined before samples were placed in the probe and after observation. The activities agreed within experimental error and were consistently 80 ± 2%.

Nmr Samples

Samples for nmr measurements were prepared in 0.1 M citrate buffer for pH 5.0 to 7.0 and 0.1 M tris buffer for pH 7.0-8.0. Appropriate amounts of α-chymotrypsin and inhibitor were accurately weighed into a 5 ml graduated centrifuge tube. Buffer (2 ml) was added, and the mixture was stirred and centrifuged to dissolve solid. Appropriate volumes of buffer at various pH's were then added to give a final volume of 4 ml and a pH within 0.05 of the reported values. After a final centrifugation to remove any undissolved protein (only minimal amounts of material do not dissolve), the solutions were filtered into 12 mm nmr tubes. The tubes were equilibrated in a constant temperature bath at 32° (the probe temperature) for about eight minutes before being transferred to the nmr probe. The pH of representative samples was checked after observation of the nmr spectrum. It

never changed significantly.

Determination of Spectra

Spectra were recorded on a Varian HA-100-15 (or, in a few cases of control experiments, XL-100-15) spectrometer. Frequency sweep was provided by a Fabri-tek 1062 time averaging computer driving a very stable voltage-controlled oscillator. The number of scans accumulated varied between 2 and 32, depending on the concentration of inhibitor. The spectrometer (HA-100-15) was field-frequency locked on a small capillary of hexafluoroacetone held concentrically by a Teflon vortex plug inside the 12 mm tubes.

REFERENCES

1. E. W. Thomas, Biochem. Biophys. Res. Commun., 24, 611 (1966).
2. T. M. Spotswood, J. M. Evans, and J. H. Richards, J. Amer. Chem. Soc., 89, 5052 (1967).
3. F. W. Dahlquist and M. A. Raftery, Biochemistry, 7, 3269 (1968).
4. J. C. K. Roberts and O. Jardetzky, Advan. Protein Chem., 24, 447 (1970).
5. D. H. Meadows, J. L. Markly, J. S. Cohen, and O. Jardetzky, Proc. Natl. Acad. Sci. U. S., 58, 1307 (1967).
6. M. A. Raftery, F. W. Dahlquist, S. I. Chan, and S. M. Parsons, J. Biol. Chem., 243, 4175 (1968).
7. P. G. Schmidt, G. R. Stark, and J. D. Baldeschwieler, J. Biol. Chem., 244, 1860 (1969).
8. D. H. Meadows, G. C. K. Roberts, and O. Jardetzky, J. Mol. Biol., 44, 491 (1969).
9. E. Zeffren and R. E. Reavill, Biochem. Biophys. Res. Commun., 33, 727 (1968).
10. B. D. Sykes, J. Amer. Chem. Soc., 91, 949 (1969).
11. J. T. Gerig and J. D. Reinheimer, J. Amer. Chem. Soc., 92, 3146 (1970).

12. J. T. Gerig and R. A. Rimerman, Biochem. Biophys. Res. Commun., 40, 1149 (1970).

13. H. Ashton and B. Capon, J. Chem. Soc. D, 513 (1971).

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

15. B. H. Bittner and J. T. Gerig, J. Amer. Chem. Soc., 92, 5001 (1970).

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

17. F. J. Kezdy and M. L. Bender, Biochemistry, 4, 104 (1965).

18. S. N. Timasheff, Arch. Biochem. Biophys., 132, 165 (1969).

19. L. D. Faller and R. E. LaFond, Biochemistry, 10, 1033 (1971).

20. K. C. Aune and S. N. Timasheff, Biochemistry, 10, 1069 (1971).

21. K. C. Aune, L. C. Goldsmith, and S. N. Timasheff, Biochemistry, 10, 1617 (1971).

22. J. A. Pople, W. J. Schneider, and K. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Company, New York, 1959, p. 317.

23. Ibid., p. 307.

24. (a) B. W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, Nature, 214, 652 (1967).

(b) P. B. Sigler, D. M. Blow, B. W. Matthews, and R. Henderson, J. Mol. Biol., 35, 143 (1968).

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

(d) D. R. Henderson, J. Mol. Biol., 54, 341 (1970).

25. (a) B. Zeiner and M. L. Bender, J. Amer. Chem. Soc., 86, 3669 (1964).

(b) M. L. Bender, G. E. Clement, F. J. Kezdy, and H. D. A. Meck, J. Amer. Chem. Soc., 86, 3680 (1965) and references therein.

26. C. H. Johnson and J. R. Knowles, Biochem. J., 101, 56 (1966).

27. J. A. Pople et al., pp. 221-223.

28. S. H. Smallcombe, B. Ault, and J. H. Richards, J. Amer. Chem. Soc., 94, 4585 (1972).

29. J. R. Knowles and J. M. Preston, Biochem. Biophys. Acta, 151, 290 (1968).

30. J. Hein and C. Niemann, J. Amer. Chem. Soc., 84, 4495 (1962).

31. R. J. Foster, H. J. Shine, and C. Niemann, J. Amer. Chem. Soc., 77, 2378 (1955).

32. R. J. Foster and C. Niemann, J. Amer. Chem. Soc., 77, 3370 (1955).

33. S. G. Cohen, A. Milovanovic, R. M. Schultz, and S. Y. Weinstein, J. Biol. Chem., 244, 2664 (1969).

34. S. G. Cohen and L. W. Lo, J. Biol. Chem., 245, 5718 (1970).

35. J. A. Pople et al., p. 168.

36. A. Saika and C. P. Slichter, J. Chem. Phys., 22, 26 (1954).

37. M. Karplus and T. P. Das, J. Chem. Phys., 34, 1683 (1962).

38. J. W. Emsley and L. Phillips, Mol. Phys., 11, 437 (1966).

39. F. D. Evans, J. Chem. Soc., 877 (1960).

40. M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, 1964, p. 166.

41. B. Zeiner and M. L. Bender, J. Amer. Chem. Soc., 86, 3669 (1964) and references therein.

42. K. G. Stevenson and L. B. Smillie, J. Mol. Biol., 12, 937 (1965).

43. Y. Nakagawa and M. L. Bender. Biochemistry, 9, 259 (1970).

44. A. R. Fersht, presented at Cold Spring Harbor Symposium on Protein Structure and Function, June 1971.

45. L. Parker, personal communication, 1970.

46. F. Weygand and R. Geiger, Chem. Ber., 89, 647 (1956).

47. E. E. Schallenberg and M. Calvin, J. Amer. Chem. Soc., 77, 2779 (1955).

48. F. Weygand and E. Csender, Angew. Chem., 64, 136 (1952).

PART II

Nuclear Magnetic Resonance Studies of the Interaction of N-TFA-D-tryptophan Semicarbazide with α-Chymotrypsin

INTRODUCTION

Part I of this thesis reported results on the binding of anionic inhibitor molecules to α-chymotrypsin. Since these inhibitors are negatively charged in the pH range studied, they exhibited a sharp decrease in binding affinity above pH 6.5 due to the ionization of His 57 and made impossible a determination of Δ values for the complex between these inhibitor molecules and α-chymotrypsin with a neutral His 57. Since this ionization is thought to be responsible for converting α-chymotrypsin to its active form (1-3), knowledge of its effect on the environment of a bound inhibitor is desirable. Part II of this thesis reports results for the inhibitor N-TFA-D-tryptophan semicarbazide. Since this molecule is neutral in the pH range of interest, it would not be expected to exhibit a drastic decrease in binding at pH 6.6 (4, 5). It should therefore be possible to measure Δ for the trifluoroacetyl fluorines of this inhibitor molecule when bound to α-chymotrypsin with both a protonated and deprotonated His 57 residue. Thus, the effect of the ionization of His 57 on the environment of a bound inhibitor molecule can be determined. In addition, it should be possible to observe the decrease in binding between pH 8.0 and 9.0 which has been attributed (5) to the ionization of Ile 16 that results in a collapse of the specificity pocket.

That neutral inhibitors bind well to α-chymotrypsin after the ionization of His 57 has been demonstrated with equilibrium dialysis studies (4) on N-acetyl-D-tryptophan amide and by stop-flow kinetic studies (5) on furylacryloyltryptophan amide. Some nmr binding studies on N-TFA-D-tryptophan amide are reported here; however, due to the low solubility of this inhibitor, it was impossible to observe the necessary concentration range to obtain reliable numbers. A recent kinetic study (7) with α-chymotrypsin reported the synthesis of the semicarbazide derivative of N-formyl-L-phenylalanine. This material was quite soluble in water and uncharged in the pH range of interest. N-TFA-D-tryptophan semicarbazide was therefore synthesized and used for binding studies in this work. Since the L-isomer of this compound is a substrate for α-chymotrypsin, it cannot be used in binding studies.

MATHEMATICAL ANALYSIS

In Part I of this thesis it was never necessary to consider the analysis of nmr data obtained under conditions in which two forms of the enzyme were contributing to the observed chemical shifts. Binding of anionic inhibitors to the form of α-chymotrypsin with a deprotonated His 57 is so poor that only the protonated form of the enzyme contributed to the chemical shift of the inhibitor molecules. With neutral inhibitor molecules, however, it is probable that both forms of the enzyme will bind and affect the chemical shift of the inhibitor molecule. The analysis of nmr data must therefore incorporate shifts from two enzyme-inhibitor complexes.

The following equations describe the case in which the enzyme exists in two forms related by a single ionization.

$$E_1I \rightleftharpoons E_1 + I \tag{1}$$

$$K_{I_1} = \frac{[E_1][I]}{[E_1I]} \tag{2}$$

$$E_2I \rightleftharpoons E_2 + I \tag{3}$$

$$K_{I_2} = \frac{[E_2][I]}{[E_2I]} \tag{4}$$

$$E_1 \rightleftharpoons E_2 + H \tag{5}$$

$$K_a = \frac{[E_2][H]}{[E_1]} \tag{6}$$

$$E_1I \rightleftharpoons E_2I + H \tag{7}$$

$$K_a' = \frac{[E_2I][H]}{[E_1]} \tag{8}$$

E_1 and E_2 are the forms of the enzyme, and E_1I and E_2I are the inhibitor complexes with the two forms of the enzyme. K_a and K_a' are the ionization constants for the free enzyme and the enzyme-inhibitor complex, respectively.

In general, each form of the enzyme will have its own value of K_I and Δ. Since the observed chemical shift of an inhibitor molecule in fast exchange with a mixture of the two forms of the enzyme is a population weighted average (8) of the shift in solution and on the two forms of the enzyme, the observed chemical shift, δ, is given by

$$\delta = \frac{[I]}{[I_o]}\Delta_{soln} + \frac{[E_1I]}{[I_o]}\Delta_1 + \frac{[E_2I]}{[I_o]}\Delta_2 \tag{9}$$

where $[I]$ is the free inhibitor concentration, $[I_o]$ is the total inhibitor concentration, and Δ_{soln}, Δ_1, and Δ_2 are the chemical shifts of the observed nucleus in its three possible environments.

If measurements are made relative to the shift in solution, $\Delta_{soln} = 0$ and eq 9 reduces to

$$\delta = \frac{[E_1I]}{[I_o]}\Delta_1 + \frac{[E_2I]}{[I_o]}\Delta_2 . \tag{10}$$

If eqs 2 and 4 are solved for $[E_1I]$ and $[E_2I]$ and these values are substituted into eq 10, the following relationship is obtained.

$$\delta = \frac{1}{[I_o]}\left[\frac{[E_1][I]}{K_{I_1}}\Delta_1 + \frac{[E_2][I]}{K_{I_2}}\Delta_2\right] \tag{11}$$

Since the relative amounts of free enzyme in the two forms is controlled by one ionization, the following expressions can be written.

$$[E_1] = f_1[E] \tag{12}$$

$$[E_2] = f_2[E] = (1 - f_1)[E] \tag{13}$$

$[E]$ is the total concentration of free enzyme, and f_1 and f_2 are the fractions of the free enzyme in each form. f_1 and f_2 are determined by K_a and are given by eqs 14 and 15.

$$f_1 = \frac{[H]}{[H] + K_a} \tag{14}$$

$$f_2 = \frac{K_a}{[H] + K_a} \tag{15}$$

[H] is the concentration of $H^{\oplus}$ ion. Substitution for $[E_1]$ and $[E_2]$ into eq 11 yields

$$\delta = \frac{[E][I]}{[I_o]}\left[\frac{f_1}{K_{I_1}}\Delta_1 + \frac{f_2}{K_{I_2}}\Delta_2\right]. \qquad (16)$$

It is convenient to define the quantity [EI] which is given by eq 17 and is equal to the total concentration of enzyme-inhibitor

$$[EI] = [E_1I] + [E_2I] \qquad (17)$$

complex in all ionization states. The dissociation constant associated with this quantity is defined by eq 18 and is an effective

$$K_I = \frac{([E_1] + [E_2])[I]}{[E_1I] + [E_2I]} = \frac{(f_1 + f_2)[E][I]}{(f_1' + f_2')[EI]} = \frac{[E][I]}{[EI]} \qquad (18)$$

constant for all enzyme forms present. f_1' and f_2' are the fractions of enzyme-inhibitor complex in each ionization state and are given by eqs 19 and 20 where K_a' is the ionization constant in the enzyme-

$$f_1' = \frac{[H]}{[H] + K_a'} \qquad (19)$$

$$f_2' = \frac{K_a'}{[H] + K_a'} \qquad (20)$$

inhibitor complex. If eq 18 is solved for the product [E][I] and this expression is substituted into eq 16, the following formula for

δ results:

$$\delta = \frac{[EI]}{[I_o]}\left[\frac{K_I}{K_{I_1}} f_1\Delta_1 + \frac{K_I}{K_{I_2}} f_2\Delta_2\right] \qquad (21)$$

With the facts that $f_2 = 1 - f_1$ and $K_{I_2} = K_{I_1}K_a/K_a'$, it is possible to rearrange this expression to yield the following equation:

$$\delta = \frac{[EI]}{[I_o]} \frac{K_I f_1}{K_{I_1} f_1'} (f_1'\Delta_1 + f_2'\Delta_2) \qquad (22)$$

Recalling eq 2 for K_{I_1} and using the previously defined fractions f_1 and f_1', one can write the following expression:

$$K_{I_1} = \frac{[E_1][I]}{[E_1I]} = \frac{f_1[E][I]}{f_1'[EI]} = \frac{f_1}{f_1'} K_I \qquad (23)$$

Therefore, the second factor on the right hand side of eq 22 is equal to one, and the expression for δ can be written as follows:

$$\delta = \frac{[EI]}{[I_o]} (f_1'\Delta_1 + f_2'\Delta_2) \qquad (24)$$

The K_I value defined here is just the pH dependent effective K_I used in the mathematical analysis of Dixon and Webb (9). Since the inhibitor molecule does not ionize in the pH range of interest, the factor f_I (which is the reciprocal of the fraction of inhibitor molecule in a certain ionization state) used in the Dixon and Webb derivation

is one here and need not be included. Note also that the fractions defined here are the reciprocals used by Dixon and Webb.

From eq 24, it is seen that even when two enzyme forms are being considered the observed value of δ should be a linear function of the total enzyme-inhibitor complex concentration and that the slope of such a plot is a function of Δ values for the two forms of the complex. Eq 24 is analogous to the expression

$$\delta = \frac{[EI]}{[I_o]} \Delta$$

used in Part I.

If nmr data obtained in binding studies are analyzed according to eq 24, the effective numbers obtained for K_I and Δ are meaningful. The K_I value obtained is a Dixon and Webb type K_I. A plot of pK_I versus pH should therefore define straight line segments which intersect to define pK_a's of the various species in solution. The effective value of Δ obtained as a function of pH should follow a simple titration curve defined by the pK_a of the enzyme-inhibitor complex and limiting values equal to Δ in the two ionized forms of the complex. That Δ titrates with the pK_a of the complex is important in discussions that follow.

Thus, chemical shift data can be analyzed by the method developed in Part I to yield effective K_I and Δ values for the two forms of the enzyme which should have a pH dependence related to

the pK_a of the ionizing group in the free enzyme and in the enzyme-inhibitor complex.

RESULTS

Nmr samples containing N-TFA-D-tryptophan semicarbazide and α-chymotrypsin were prepared at each half pH unit between 5.0 and 9.0 (except 5.5). In general, eight samples were prepared at each pH. In all cases, the enzyme concentration was held constant at ~ 1.9 mM (corrected for inactive protein). The concentration of inhibitor was varied from about 0.05 M to 0.3 mM. Below pH 7.0, 0.1 M citrate buffer was used to prepare samples. At pH 7.0 and above, 0.1 M tris buffer was used. Samples were prepared in volumetric centrifuge tubes and transferred to 12 mm nmr tubes to be run. It was not possible to carry out experiments at pH values above 9.0 due to base catalyzed hydrolysis of the trifluoroacetyl group.

Since all chemical shifts were measured relative to internal water, no correction for bulk diamagnetic susceptibility changes due to the presence of protein was necessary. It was, however, observed that a plot of δ versus $[EI]/[I_o]$ did not pass through the origin, but instead intersected the y-axis at about 3.6 Hz instead of at zero. This intercept was constant with pH and may partly result from the different geometry and polarity of the trifluoroacetyl group relative to bulk water. Because of these differences, water may not experience exactly the same enzyme induced solvent effects as do the observed fluorines (10). Since the shifts measured in Part I

were made relative to an internal fluorine-containing group of geometry similar to the fluorine moiety being observed, this effect should have been absent and was not in fact observed.

This non-zero intercept may also reflect a nonspecific, concentration independent interaction of the inhibitor molecule with the surface of the enzyme which produces a shift above that caused by interaction with the active site. This type of shift was not observed with the anionic inhibitors studied in Part I perhaps because the negative charge prevented close association with the enzyme except at the active site.

Unfortunately, the range of chemical shifts observed at varying concentrations of inhibitor was rather small. At pH 5.0, the total range observed was only 2 Hz. At other pH values a range of from 6 to 10 Hz was seen. Although these numbers are measurable and the shifts always increased as expected at lower inhibitor concentrations, the values are not large enough to define K_I and Δ as well as the results reported in Part I. For this reason, the quantitative results obtained are probably not as reliable as the results obtained with N-TFA-D-tryptophan and N-TFA-D-p-fluorophenylalanine.

Since all of the α-chymotrypsin-small molecule systems examined so far exhibit rather small chemical shifts above pH 7.5 (either because of poor binding or small Δ values), it has not been feasible to measure effective dimerization constants by the nmr

method discussed in Part I. It was therefore necessary to use literature values for these constants. Although the absolute values determined in Part I did not agree with all the literature values, there is general agreement on the pH dependence of α-chymotrypsin oligomerization. From the pH dependence reported by Egan (11) and Timasheff (12), approximate values of K_D were calculated at higher pH values and used in the analysis of data obtained here. Since enzyme oligomerization is greatly reduced at high pH, it is felt that these numbers are accurate enough to remove the effect of enzyme self-association from the results obtained for K_I and Δ. The K_D values used in this work are listed in Table I.

The chemical shift data were analyzed according to eqs 9 and 11 of Part I to yield the best values of K_I and Δ at each pH. At pH 5.0, values of 0.15 mM and 3.9 Hz were obtained. At higher pH values, the results were scattered and no precise pK_a value for the change in K_I or Δ was obviously defined. It was apparent, however, that both K_I and Δ were increasing with a pK_a between 6.0 and 7.0. K_I increased steadily above pH 5.0 to a value of about 1.2 mM at pH 8.0. Over the same pH region, Δ increased to an average plateau value of 30 Hz downfield above pH 7.5.

K_I continued to increase above pH 8.0 to a value of $\sim$ 40 mM at pH 9.0 as would be expected if the ionization of Ile 16 was affecting binding (6). Attempts to fully characterize this effect by going to pH values above 9.0 failed due to the hydrolysis of the

TABLE I. Effective Dimerization Constants for α-Chymotrypsin

pH	K_D M
5.0	5.0×10^{-5}
6.0	9.0×10^{-5}
6.5	2.0×10^{-4}
7.0	4.0×10^{-4}
7.5	7.0×10^{-4}
8.0	1.0×10^{-3}
8.5	2.0×10^{-3}
9.0	5.0×10^{-3}

trifluoroacetyl group from the inhibitor molecule at the higher pH values.

Attempts were also made to carry out experiments on N-TFA-D-tryptophan amide which is also a neutral inhibitor. Due to the low solubility and small observed chemical shift of this molecule, it was not feasible to obtain analyzable results directly. Using the analysis for a second inhibitor molecule reported in Part I, however, it was possible to do competition experiments in which the effect of N-TFA-D-tryptophan amide on the chemical shift of N-TFA-D-tryptophan was measured. This was done at pH 6.0 and 7.75. K_I values of 0.3 and 0.4 mM, respectively, were determined. Thus, the decrease in binding affinity observed with this inhibitor is much smaller than that observed with the semicarbazide derivative. Although reliable values of Δ could not be obtained, the trend toward small chemical shifts observed with N-TFA-D-tryptophan semicarbazide was also evident with the amide inhibitor.

A pK_a value for N-formyl-L-phenylalanine semicarbazide of 10.03 has been reported (7) with the hydrazine group titrating as an acid. N-TFA-D-tryptophan semicarbazide was titrated in 0.1 M sodium phosphate buffer by observing the pH dependence of the fluorine and carbon-13 resonances. The inhibitor exhibited a pK greater than 9.5. An accurate value could not be obtained due to substrate hydrolysis at high pH. No ionization which would affect the molecule in the pH 5.0 to 9.0 range was observed.

DISCUSSION

From the results reported in Part I and from other studies (1-3) with α-chymotrypsin, an ionization with a pK_a of 6.6 has been identified which could affect the binding and chemical shift of an inhibitor molecule. Presumably this is the ionization of His 57. This ionization might therefore be responsible for the changes observed here. Since the ionization caused K_I to change from 0.15 mM to 1.2 mM, it can be seen from eq 21 that if His 57 is responsible for the decrease in binding the pK_a of His 57 in the enzyme-inhibitor complex must be around 7.5. The effective Δ value obtained from the analysis of the nmr data should therefore have increased from $\sim$ 4.0 Hz to $\sim$ 30.0 Hz with a pK_a of about 7.5. It is easily shown that there should have been little change in the Δ found at pH 6.0 and 6.5 relative to pH 5.0. It was found, however, that Δ had increased to 16.5 Hz at pH 6.0 and to 19.5 Hz at pH 6.5 indicating that Δ was titrating with a pK_a considerably lower than 7.5.

Inspection of the experimentally observed chemical shifts reveals a similar descrepancy. At pH 6.0 and 6.5, experimental shifts for N-TFA-D-tryptophan semicarbazide of 4.6 and 7.2 Hz, respectively, were found. Since both of these numbers exceed the expected value of Δ, it is not possible that an ionization of 7.5 is

responsible for the titration of Δ. Thus, the assumption of one pK_a of 6.6 in the free enzyme which causes a decrease in binding will not reasonably explain the experimental data.

Since the results obtained from the analysis of the N-TFA-D-tryptophan semicarbazide were too scattered to yield reliable values for K_I and Δ, the following model is proposed to explain the results. Because of the stereochemistry of the binding of D-isomer inhibitors to α-chymotrypsin proposed in Part I, it is reasonable that the ionization of His 57 should have a rather strong effect on the observed chemical shift of an N-TFA group on the inhibitor. From crystallographic studies (1, 2, 6) on α-chymotrypsin, it has been postulated that conversion of the hydrolytic locus to its active form upon ionization of His 57 can be accomplished with little change in the conformation of the active site with only the formation of one hydrogen bond being required. It is thus not necessary that this ionization strongly affect the binding of an uncharged inhibitor molecule to the enzyme. For these reasons, it is proposed that the change in Δ is caused by the ionization of His 57 and that this ionization does not affect binding of the inhibitor molecule. The pK_a of His 57 is therefore 6.6 in both the free enzyme and the enzyme-inhibitor complex, and Δ would be expected to titrate with a pK_a of 6.6. This would explain why the values of Δ obtained at pH 6.0 and 6.5 are considerably higher than the value obtained at pH 5.0. If it is assumed that Δ titrates between 3.9 Hz and

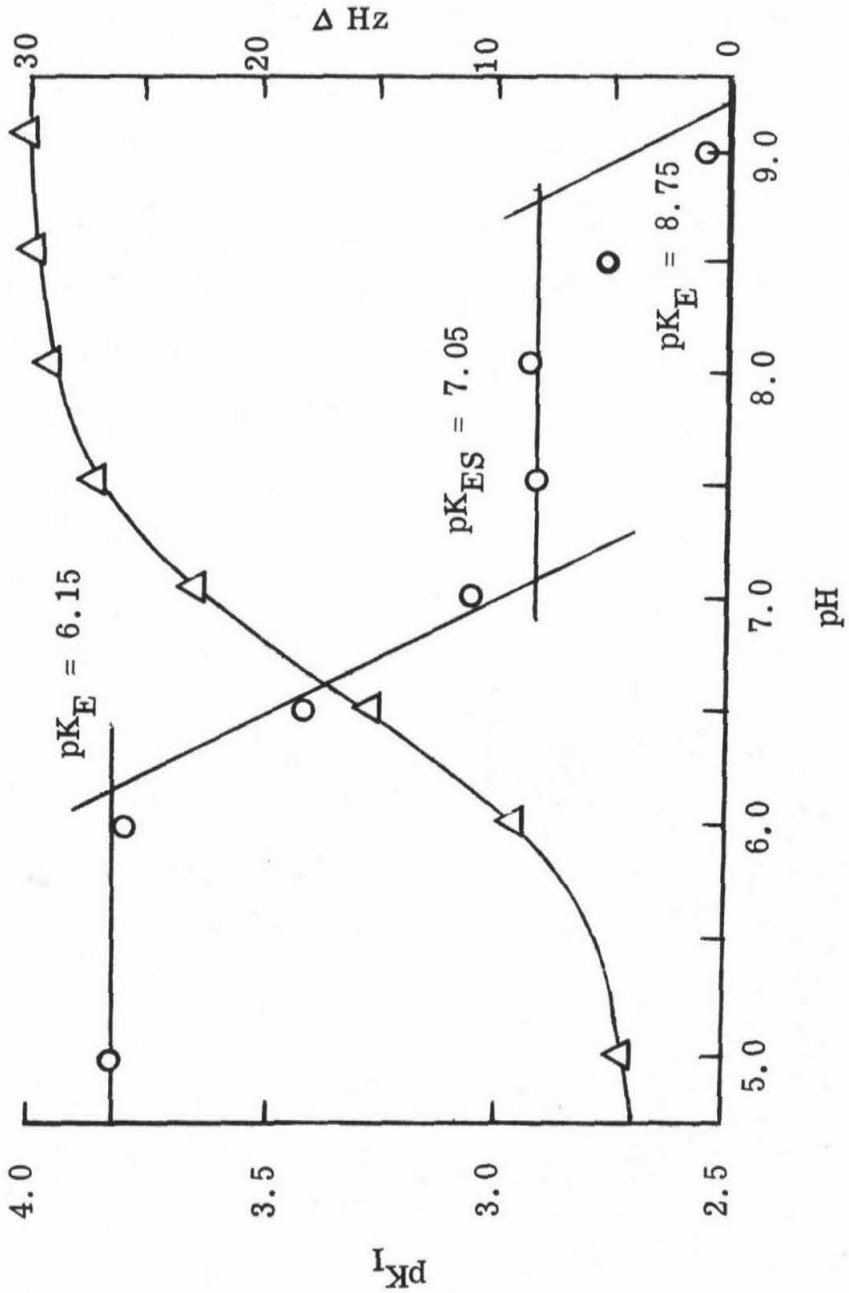
30 Hz with a pK_a of 6.6, it is possible to calculate what value of Δ should be obtained at each pH value.

When the experimentally observed chemical shifts are computer analyzed using a best fit procedure, the output contains what value of K_I provides the best fit at a certain value of Δ. Although the combination which gives the smallest average error is nominally the best combination, with the small chemical shifts found in this work the minimum error is not well defined, and it is therefore not possible to precisely define K_I and Δ. If, however, one of the values is known, the other can be determined quite accurately. Therefore, the Δ values calculated above at each pH can be used to generate pH dependent K_I values which are very reliable if the Δ values used are correct. From the calculated pH values of Δ based on a pK_a of 6.6, it is possible to determine K_I at each pH. Table II lists K_I and Δ values determined in this manner. Since this effective K_I is that used in the Dixon and Webb analysis (9), a plot of pK_I versus pH should yield the pK of the second ionizing group in the free enzyme and the enzyme-inhibitor complex. Figure 1 shows a plot of pK_I versus pH for the numbers calculated in this work. The solid line is composed of straight line segments of integral slope with intercepts corresponding to pK_a values of 6.15, 7.05, and 8.7. The experimental points fit this line quite well. Under this model it can be seen that there is an ionization with a pK_a of 6.15 in the free enzyme which is perturbed to a pK_a of 7.05

TABLE II. Calculated Values of Δ and K_I Assuming a pK_a of 6.6 for His 57 and Δ Values of 3.3 Hz and 30.0 Hz for the Enzyme-Inhibitor Complex with a Protonated and Deprotonated His 57

pH	Δ Hz	K_I M
5.0	3.95	1.51×10^{-4}
6.0	8.66	1.63×10^{-4}
6.5	15.12	3.97×10^{-4}
7.0	22.39	8.91×10^{-4}
7.5	27.01	12.57×10^{-4}
8.0	28.98	11.93×10^{-4}
8.5	29.67	17.24×10^{-4}
9.0	29.89	30.05×10^{-4}

FIGURE 1. Plot of pK_I (⊙) and Δ (Δ) Versus pH for N-TFA-D-tryptophan Semicarbazide



in the enzyme-inhibitor complex. Also, another ionization of pK_a ~ 8.7 on the free enzyme is evident.

Since there are only two residues in α-chymotrypsin which might be expected to titrate in the 6.0 to 7.0 pH range, it seems reasonable to assign the ionization affecting binding to His 40. Although this residue has never been observed in binding or kinetic studies, there is some information to indicate that its pK is in this range. From studies (5) on fluorescence quenching of tryptophans in α-chymotrypsin, Hess observed that a residue with a pK_a of 6.0 was affecting such quenching. Chemical modification of His 57 with L(-1-tosylamido-2-phenyl)ethylchloromethyl ketone did not change the apparent pK_a of the group although this modification is reported to lower the pK_a of His 57 by 0.8 pH units (13). Since His 40 is closely associated with a tryptophan residue in the tertiary structure of the enzyme, the pK_a of 6.0 was assigned to the imidazole ring of His 40. Using the technique of competitive labeling, Kaplan (14) has determined a pK_a of 6.7 for His 40. The present results do not prove either of these assignments, but it seems apparent that His 40 does ionize in the proposed pH range.

Crystallographic studies with α-chymotrypsin have shown that His 40 is near the active site of α-chymotrypsin coming within 7.0 Å of a covalently bound tosyl group (3). The position of this residue near the cleft which runs above and below the active site might well enable His 40 to affect the binding affinity of inhibitor molecules.

A comparison of the crystal structure of α-chymotrypsin at pH 3.9 with that at pH 6.7 has indicated that His 40 shows some movement in this range (15) perhaps as a result of ionization.

The binding constant of 0.15 mM found with N-TFA-D-tryptophan semicarbazide is similar to the value determined for N-TFA-D-tryptophan and N-TFA-D-p-fluorophenylalanine in Part I. After correction for dimerization, this is also about the value obtained from equilibrium dialysis studies (4) with N-acetyl-D-tryptophan amide and from stop-flow kinetic studies (5) on furyl-acryloyltryptophan amide. The semicarbazide inhibitor thus binds with an affinity similar to other neutral molecules at low pH.

The eight-fold increase in K_I observed between pH 5.0 and 7.5 was not expected since an effect this large is not seen with other neutral inhibitor molecules. Knowles (4) reported that within his experimental error, K_I for N-acetyl-D-tryptophan amide was pH-independent between pH 5.0 and 8.0. A similar pH-independent K_I has been reported for furylacryloyltryptophan amide (5). Results in the present work have shown that K_I for N-TFA-D-tryptophan amide changes from 0.2 mM at pH 6.0 to 0.3 mM at pH 7.75. The results for the anionic inhibitors reported in Part I also showed no decrease in binding affinity which could not be explained with one ionization at pH 6.6. These results tend to indicate that the effect on binding here attributed to His 40 is a result of the rather large size of the semicarbazide group relative to a carboxylate anion or a

simple amide group.

A recent crystallographic study (16) aimed at determining whether or not α-chymotrypsin interacts with amino acid residues removed from the bond to be hydrolyzed has shown that Ser 214, Trp 215, and Gly 216 are involved in hydrogen bonding with the polypeptide chain extending from the amino end of a covalently bound molecule. It thus seems possible that for the D-isomer used here some interaction may occur with the semicarbazide group attached to the carboxyl end of the molecule. Such interaction would be less likely with a carboxylate anion or simple amide derivative. A conformational change induced by His 40 ionizing might produce an unfavorable binding interaction with the semicarbazide group leading to decreased binding above pH 6.0.

Because the semicarbazide derivative is still binding fairly strongly at pH 8.0, it is possible to observe the sharp decrease in binding expected above pH 8.5. Kinetic studies (17) with α-chymotrypsin have shown that there is an ionization in the pH 8.5-9.0 range which leads to almost complete loss of binding of large molecules. This ionization has been attributed to Ile 16 and results in a disruption of the Ile 16-Asp 194 salt bridge thought to stabilize the specificity pocket of the enzyme (6). In Figure 1, a reasonable pK_a of 8.7 has been assigned to this group although the experimental points do not really define the number very closely. Unfortunately, it is not possible to do experiments at higher pH due to hydrolysis of the N-TFA group from the inhibitor molecules.

The small value for Δ of -3.9 Hz obtained at pH 5.0 indicates that the enzyme does not strongly affect the environment of the trifluoroacetyl fluorines relative to their solution environment. There is no reason to expect that this indicates a significantly different placement of the trifluoroacetyl fluorines of the inhibitor molecule than that proposed in Part I for N-TFA-D-tryptophan. A similar small shift was observed with N-TFA-D-tryptophan amide in this work. This small shift perhaps indicates that the larger shift of -70 Hz found for N-TFA-D-tryptophan was a result of the placement of the trifluoroacetyl fluorines in the electric field between the positively charged His 57 and negatively charged carboxylate group of the inhibitor molecule (10). Since the negative charge is absent in the semicarbazide and amide inhibitors, this effect would be expected to be substantially reduced.

The change in chemical shift experienced by the trifluoroacetyl group of N-TFA-D-tryptophan semicarbazide is proposed to result from the ionization of His 57. A downfield shift of about 26 Hz is observed. This shift could result from a change in the charge field which the inhibitor molecule sees. The strong repulsion of anionic inhibitor molecules upon ionization of His 57 indicates that deprotonation results in a substantial change in the ionic character of the active site. The chemical shift of the bound inhibitor molecule reflects this change. Also, a repositioning of the inhibitor molecule or the active site residues after ionization might result in a closer

association of the trifluoroacetyl fluorines with some enzymic group and a corresponding downfield shift from van der Waals forces (10). Such an interaction might, however, be expected to result in a change in binding with the ionization of His 57 contrary to what is proposed here.

The assumption that the ionization at 6.6 affects only Δ and the other ionization affects only K_I is perhaps not quite right. Varying the effect of each ionization on K_I and Δ might produce a fit as good as that shown in Figure 1. Such an analysis seems futile at this point due to the uncertainty of the numbers involved. The important point to be made is that the results obtained with the semicarbazide inhibitor require two ionizations on the enzyme.

EXPERIMENTAL

Synthesis of N-TFA-D-tryptophan Semicarbazide

N-TFA-D-tryptophan (10 g, 0.033 mole) prepared by the method of Schallenberg and Calvin (18) and 2.48 g (0.033 mole) of semicarbazide were dissolved in 99 ml of dry acetonitrile. 7.52 g (0.0366 mole) of dicyclohexylcarbodiimide was added, and the mixture was stirred for five hours at room temperature. After the addition of 0.5 ml of glacial acetic acid, the mixture was filtered. The filtrate was evaporated to dryness to yield a yellowish gum which was thoroughly extracted with hot water ($\sim 60^{\circ}$). The slightly yellowish water solution was extracted twice with $\frac{1}{3}$ volume of ether and lyophilized to yield a white powder. Recrystallization from n-hexane-ethanol gave a white crystalline product. Yield 5.2 g. Mp 140-141°. Anal. Calcd for $C_{14}H_{14}F_3N_5O_3$: C, 47.05; H, 3.96; N, 19.60. Found: C, 46.90; H, 3.88; N, 19.41.

Synthesis of N-TFA-D-tryptophan Amide

D-Tryptophan Methyl Ester Hydrochloride. D-tryptophan (10 g) was suspended in 150 ml of dry methanol. Dry hydrogen chloride gas was bubbled through the suspension until all solid had dissolved. The solution was cooled to 0° and saturated with hydrogen chloride.

After the solution was left overnight at -2°, a white crystalline product formed and was collected by filtration. The volume of the filtrate was reduced to about 30 ml and cooled to yield a second crop of crystals. The product was combined and dried in vacuo over sodium hydroxide to yield 7.0 g of white crystalline product.

D-Tryptophan Methyl Ester. The above material was placed in a separatory funnel with 50 ml of ethyl ether. Solid sodium carbonate was added, and the mixture was shaken until no more carbon dioxide was released. The ether layer was collected, dried over sodium sulfate, and evaporated to dryness on a rotary evaporator to yield a yellowish gum which could not be crystallized.

D-Tryptophan Amide. The gum prepared above was placed into 50 ml of methanol saturated with ammonia at 0°. This solution was allowed to stand for $3\frac{1}{2}$ days. The solvent was removed on a rotary evaporator to yield a syrup. Infrared analysis indicated that the ester had been completely converted to the amide.

N-TFA-D-tryptophan Amide. The above syrup was dissolved in 400 ml of anhydrous ethyl acetate. 4.8 ml of trifluoroacetic anhydride was added, and the mixture was allowed to stand at room temperature for $\frac{1}{2}$ hour with occasional stirring. An additional 4.0 ml of anhydride was added, and the solution was allowed to stand for another $\frac{1}{2}$ hour. The ethyl acetate solution was extracted three times with 70 ml of 10% aqueous sodium bicarbonate and dried over calcium sulfate. Evaporation in vacuo yielded a yellowish

syrup which was dissolved in ether and again evaporated to dryness. This process was repeated until a white crystalline product formed. This material was recrystallized twice from water-methanol to give 3.5 g of white needles. Mp 161-161.5°; lit. mp 162° (19).

Enzyme Preparations

Calbiochem three times-recrystallized α-chymotrypsin was used for all experiments. The enzyme was kept desiccated and frozen when not in use. An active site titration indicated an activity of 80% based on a molecular weight of 25,000.

Nmr Samples

Nmr samples were prepared as described in Part I. All solutions were temperature equilibrated at probe temperature before final spectra were taken. A Varian XL-100 spectrometer operating at 94.6 MHz in the Fourier transform mode was used to record all spectra. A 30 to 40 μsec pulse width and an acquisition time of 0.5 to 1.0 second were used. From 5 to 500 transients were time averaged before Fourier transformation. The nmr was locked on internal water making corrections for diamagnetic susceptibility changes unnecessary. The chemical shift of the fluorine peaks was determined using the marking routine supplied with the 16K FT program.

REFERENCES

1. G. Schoellman and E. Shaw, Biochem. Biophys. Res. Commun., 7, 36 (1962)
2. B. W Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, Nature, 214, 652 (1967).
3. R. Henderson, J. Mol. Biol., 54, 341 (1970).
4. C. H. Johnson and J. R. Knowles, Biochem. J., 101, 56 (1966).
5. G. P. Hess, J. McConn, E. Kim, and G. McConkey, Phil. Trans. Roy. Soc. Lond. B, 257, 89 (1970).
6. P. B. Sigler, D. M. Blow, B. W. Matthews, and R. Henderson, J. Mol. Biol., 35, 143 (1968).
7. A. R. Fersht and Y. Requena, J. Amer. Chem. Soc., 93, 7079 (1971).
8. J. A. Pople, W. J. Schneider, and K. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Company, New York, 1959, pp. 221-223.
9. M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, 1964, p. 136.
10. J. W. Emsley and L. Phillips, Mol. Phys., 11, 437 (1966).
11. R. Egan, H. O. Michel, R. Schleuter, and B. J Jandorf, Arch. Biochem. Biophys., 66, 366 (1957).

12. S. N. Timasheff, Arch. Biochem. Biophys., 132, 165 (1969).

13. D. M. Glick, Biochemistry, 7, 3391 (1968).

14. H. Kaplan, unpublished results, 1972.

15. R. L. Vandlen and A. Tulinsky, Fed. Proc. Abstr., 30, 1129 (1971).

16. D. M. Segal, J. C. Powers, G. H. Cohen, D. R. Davies, and P. E. Wilcox, Biochemistry, 10, 3728 (1971).

17. M. L. Bender and F. J. Kezdy, Ann. Rev. Biochem., 34, 49 (1965).

18. E. E. Schallenberg and M. Calvin, J. Amer. Chem. Soc., 77, 2779 (1955).

19. H. T. Huang and C. Niemann, J. Amer. Chem. Soc., 74, 101 (1952).

PART III

^{13}C-Nuclear Magnetic Resonance Studies of Methylated α-Chymotrypsin

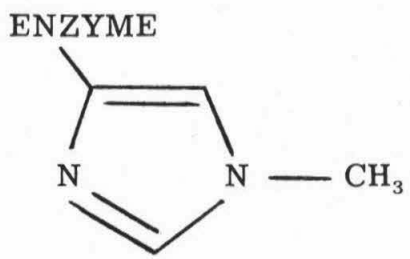
INTRODUCTION

Chemical modification studies on enzymes have proved very useful for identifying the enzymic residues involved in the catalytic mechanism of the enzyme (1, 2). Amino acid analysis of the modified protein allows determination of exactly which group or groups on the enzyme have reacted. The effect of the chemical label on the ability of the enzyme to catalyze certain reactions or bind substrate molecules can be correlated to the role which the modified group plays in catalysis. Although any label must produce some perturbations, careful examination of the effect of the modification can yield valuable information about the native enzyme.

The proteolytic enzyme α-chymotrypsin has been the subject of numerous chemical modification studies (3-5). Chemical labels have implicated the residues Ile 16, His 57, Met 192, and Ser 195 as being important in the observed catalytic properties of this enzyme. His 57, which is thought to be involved in the hydrolysis of amide and ester bonds, has been modified with several reagents including phenoxymethylchloromethyl ketone (6) and 2-phenyl-1, 4-dibromoacetoin (7). These enzyme derivatives exhibited essentially complete loss of catalytic activity which indicated that His 57 was involved in catalysis. Each of these chemical labels is an

α-chymotrypsin substrate analog containing an aromatic group (8). Since His 57 is located near the "hydrophobic pocket" seen in crystal studies (9) as the binding site for aromatic side chains, it is probable that the aromatic moiety of the covalently bound modifying label resides in the hydrophobic pocket and prevents access to the catalytic groups. This would render the enzyme inactive even if the modification of His 57 itself did not reduce the ability of the enzyme to catalyze hydrolytic reactions. Modification studies using such labels do not, therefore, unambiguously implicate His 57 as an important residue. Only the nearness of this group to the active site can be established in this manner.

A more convincing modification study has been reported (8). This involved the reaction of α-chymotrypsin with methyl p-nitrobenzenesulfonate. This reagent contains an aromatic group which probably directs the reactive sulfonate ester to the active site region where the p-nitrobenzenesulfonyl group can easily be displaced from the methyl group by a nucleophile. Sequencing of the product from this reaction revealed that one of the two histidine residues of the enzyme, His 57, had been methylated at the $N^{\xi 2}$ position to yield 3-methyl His 57 (1). The pH-rate profile for



1

this reaction is similar to that for most α-chymotrypsin catalyzed reactions. A bell-shaped curve defined by pK values of ~ 7.0 and ~ 9.5 describes the reaction. Presumably, the pK at 7.0 is deprotonation of His 57 rendering the imidazole nitrogen more nucleophilic. The higher pK has been assigned to the breakup of the Ile 16-Asp 194 salt bridge causing collapse of the specificity pocket.

Methylated α-chymotrypsin shows greatly reduced catalytic activity toward substrates (8). Since the small methyl group should not have sufficient steric effect to prevent substrate molecules from binding at the active site, it was felt that this particular modification clearly implicated His 57 as an important catalytic residue.

In a further study (10) of this modified form of α-chymotrypsin, modification of His 57 with a methyl group did not totally abolish the activity of the enzyme toward the hydrolysis of ester linkages. A rate reduction of from 5000 to 200,000 fold relative to the native enzyme was observed, but for other His 57 modifications a rate reduction of at least 100 times greater was found. These kinetic studies were carried out using the proflavin displacement method which rules out the possibility that the residual activity comes from a small impurity of native α-chymotrypsin. The modified enzyme exhibited binding constants for proflavin and N-acetyl-L-tyrosine ethyl ester (measured as K_M) which were

identical to those of the native enzyme. The modified protein also formed an acyl enzyme with indolylacryloylimidazole indicating that the active site was essentially intact.

It is difficult to understand how methylated α-chymotrypsin could exhibit any residual activity if the native conformation is maintained after the modification (10). The accepted mechanism of α-chymotrypsin hydrolysis (11, 12) involves transfer of the hydroxyl proton of Ser 195 to the $N^{\xi 2}$-nitrogen of His 57 concerted with attack of the serine oxygen at the carbonyl carbon of the substrate. Methylation of the nitrogen of His 57 should leave the oxygen of Ser 195 with about the same reactivity as a normal hydroxyl. Even if the proximity of the substrate to the serine as a result of binding is considered, such a group should not attack ester linkages at pH 7.0.

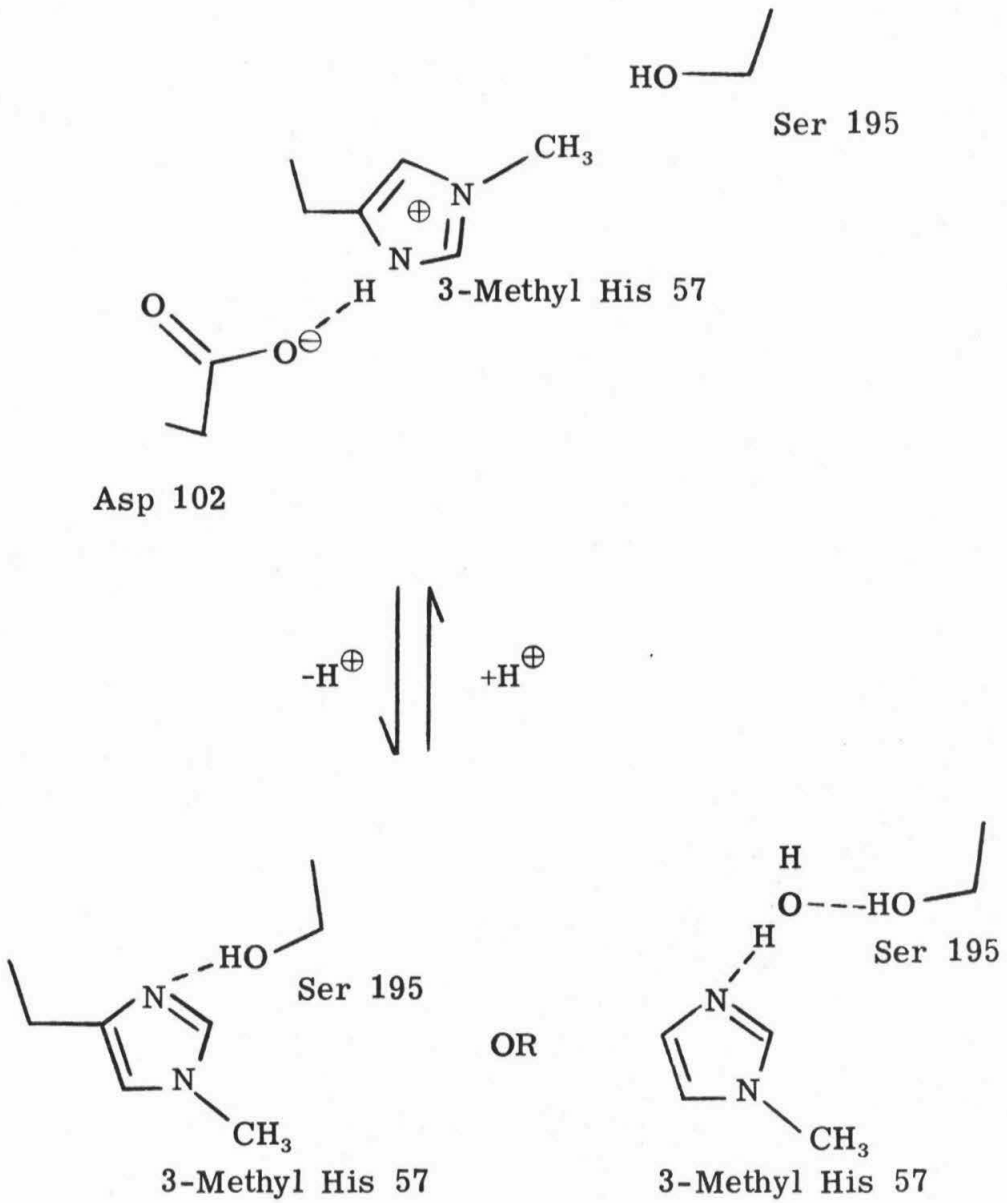
It was therefore proposed that the methylation of His 57 resulted in a conformational change involving 3-methyl His 57 (10). This change might involve flipping of the imidazole ring in such a manner as to direct the other ring nitrogen toward Ser 195. This nitrogen might then act as a general base to increase the nucleophilicity of Ser 195 making catalysis at lower pH possible. Model building studies indicated that such a conformational change would allow a poor hydrogen bond to be formed between Ser 195 and the imidazole nitrogen. Placement of a water molecule into this system produced a very good hydrogen bonded

network. Figure 1 diagrams the relevant conformations discussed.

The crystal structure of methylated α-chymotrypsin has been reported (13). The modified protein crystallized isomorphously with the native enzyme. Both proteins have essentially identical structures except for small changes in the active site region expected from the placement of a methyl group on His 57. Since the crystals used for this study were grown at pH 4.0, 3-methyl His 57 is undoubtedly protonated at the $N^{\delta 1}$ position. This should allow formation of a hydrogen bond between the methylimidazole ring and Asp 102 as in the native enzyme which is observed in the crystals. The modifying methyl group is directed toward Ser 195 which indicates that at low pH a conformation similar to that of the native protein is maintained. As discussed previously, this conformation is unlikely to be active even at high pH since the formation of the charge transfer network is precluded by the presence of the methyl group. This led the crystallographers to conclude that the active conformation found in solution is different from that seen in the crystalline phase. Model building studies allowing for free movement of 3-methyl His 57 indicated that it was possible to achieve a configuration which might explain the pH-rate profile observed in kinetic studies.

These studies on methylated α-chymotrypsin suggested that a carbon-13 nuclear magnetic resonance (nmr) study of the protein might prove interesting. Part III of this thesis reports a partial

FIGURE 1. Relevant Conformational Changes Associated with Ionization of 3-Methyl His 57



study of this system. Selective enrichment of the modifying methyl group with carbon-13 has allowed assignment of this resonance. Since the resonance comes in a fairly open region of the spectrum (as would be expected since there are no other methyl groups attached to a nitrogen in the molecule), it is possible to observe its position as a function of pH and to observe the ionization of the methylimidazole ring of the modified protein. Because of the low catalytic activity of the enzyme, it is stable for the rather long times required to obtain good ^{13}C-nmr spectra. These preliminary experiments have indicated that methylated α-chymotrypsin is well suited for ^{13}C-studies related to the native enzyme.

RESULTS

Figures 2 and 3 show the upfield region of the carbon-13 nmr spectrum of two samples of methylated α-chymotrypsin which were prepared identically except for the use of ^{13}C-enriched methyl p-nitrobenzenesulfonate in the preparation of the material used to obtain the spectrum in Figure 3. The spectra are quite similar except for the large peak at -26.5 ppm (relative to external tetramethylsilane) which can be assigned to the resonance of the modifying methyl group. Careful examination of the spectra indicates a few other differences (14), but these are mainly the result of slightly different phasing for the two spectra and the difference in the amount of power put into a Fourier transform RF pulse on different days (these spectra were taken two weeks apart).

The methyl resonance was monitored as a function of pH from pH 3.5 to 9.7. Figure 4 shows a plot of chemical shift versus pH for this resonance. The solid line is theoretical for a pK_a of 6.75 and limiting values of 1011.0 and 1042.5 Hz. The values plotted in Figure 4 are listed in Table I. This peak shifts downfield about 31 Hz with a pK_a of 6.75. Only one break is observed in this pH range indicating that only one ionization appreciably affects the environment which the methyl resonance experiences. The points in this plot represent spectra taken on

FIGURE 2. ^{13}C-Spectrum of 3-Methyl His 57 α-Chymotrypsin

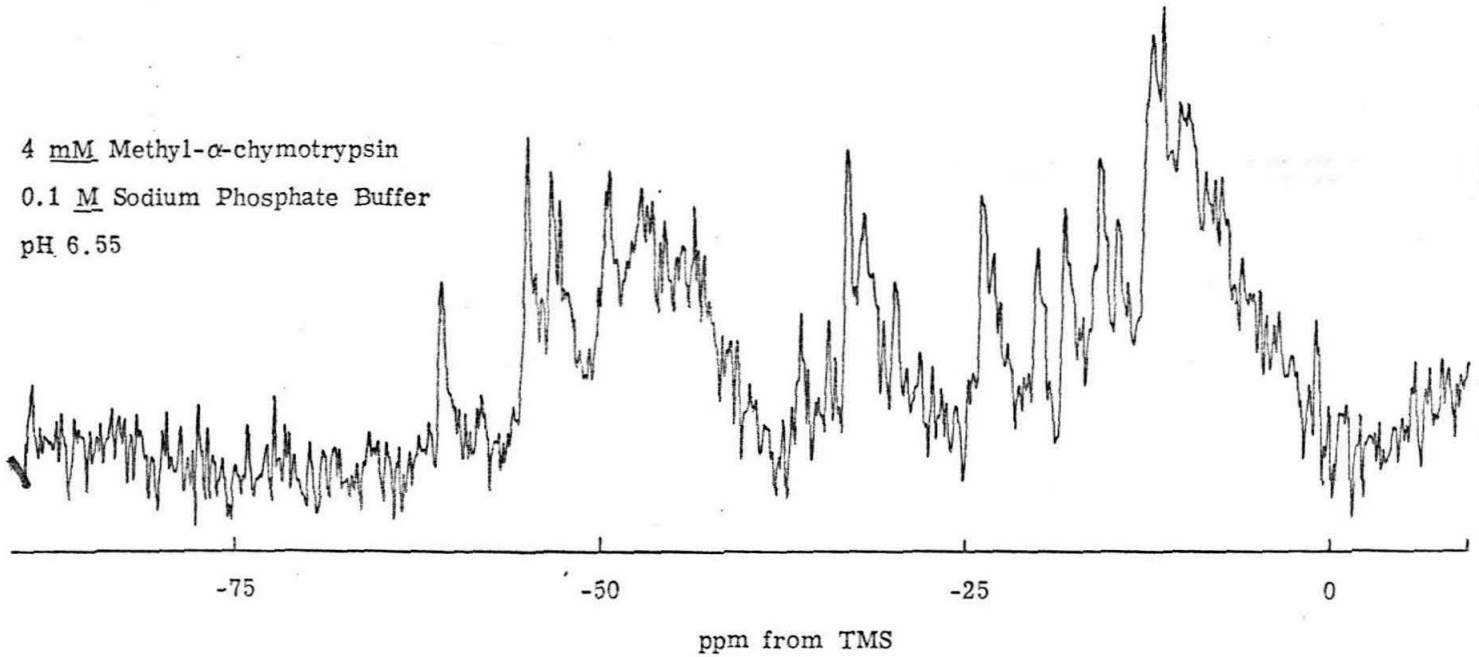


FIGURE 3. ^{13}C-Spectrum of ^{13}C-Enriched 3-Methyl His 57 α-Chymotrypsin

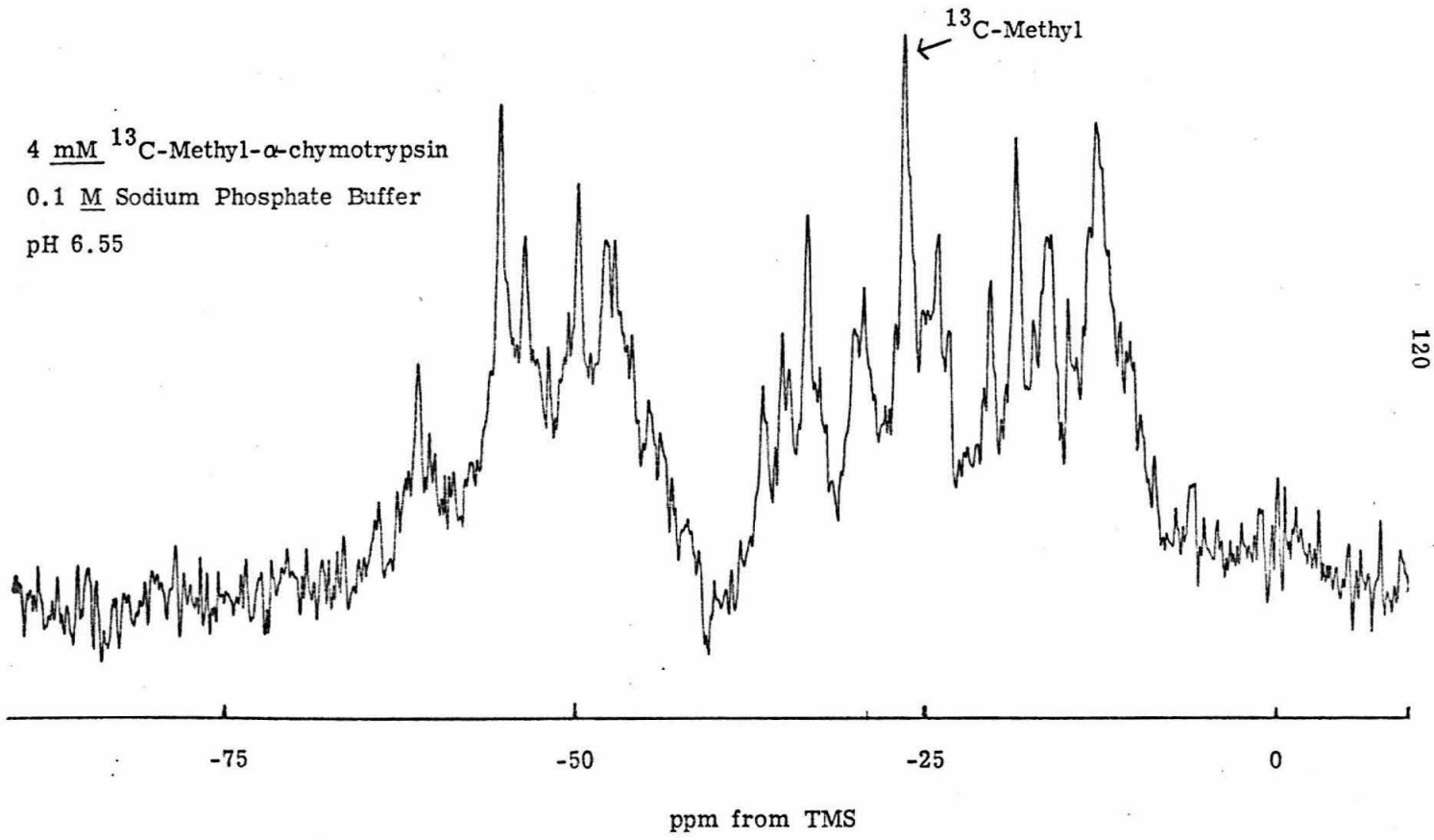


FIGURE 4. Chemical Shift of the Enriched Methyl Resonance of Methylated α-Chymotrypsin

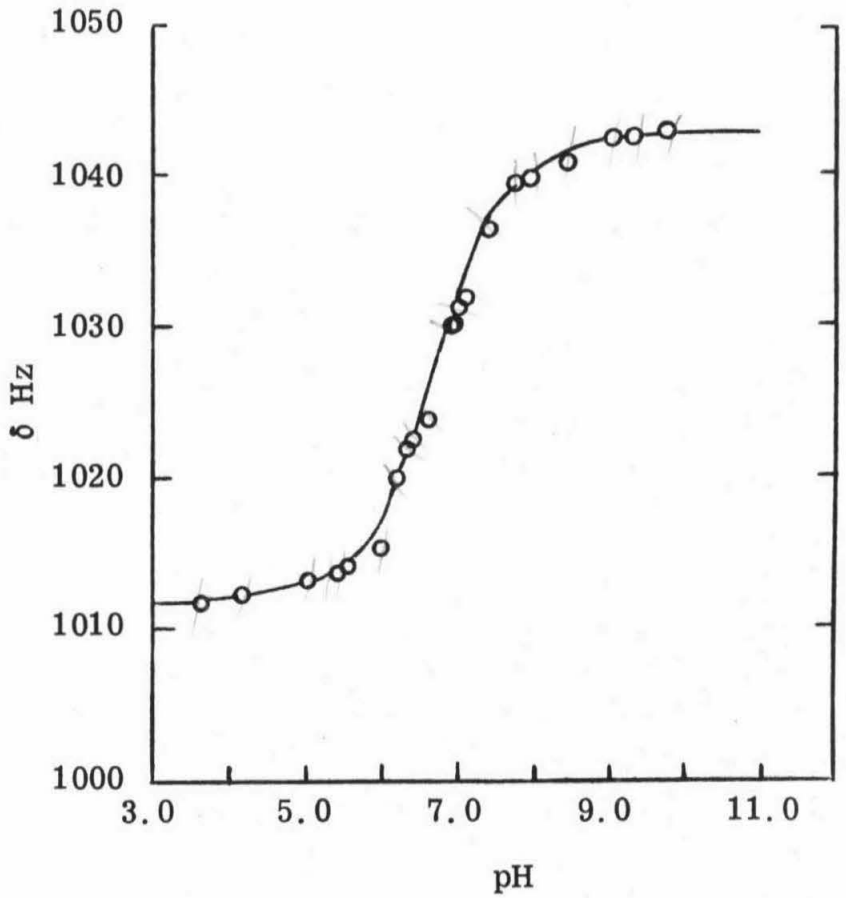


TABLE I. Chemical Shift of Enriched Methyl Group as a Function of pH

pH	δ Hz
3.68	1012.7
4.24	1012.2
5.05	1013.2
5.46	1013.7
5.60	1014.2
6.00	1015.3
6.22	1020.1
6.33	1022.1
6.34	1022.7
6.57	1023.8
6.91	1030.1
6.98	1030.4
7.00	1031.3
7.04	1031.8
7.42	1036.6
7.73	1039.0
7.95	1039.5
8.43	1042.6
9.00	1042.6
9.25	1042.2
9.70	1043.3

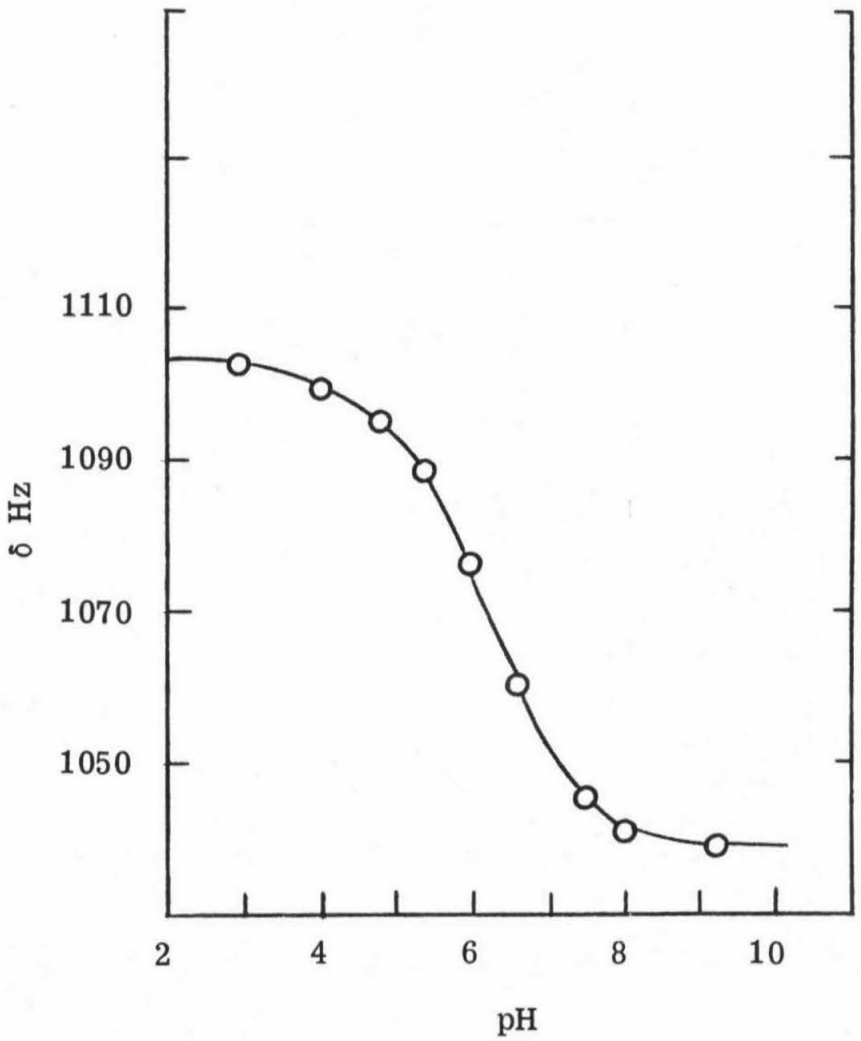
three different days. The titration was shown to be fully reversible.

In order to determine the effect of the ionization of 3-methyl His 57 on the chemical shift of the methyl resonance, the ^{13}C-nmr signal from the molecule 3-methylhistidine was observed as a function of pH. The values obtained are listed in Table II and plotted in Figure 5. Ionization of the imidazole ring is accompanied by an upfield shift of about 60 Hz with a pK_a of ~ 6.2. The points in Figure 5 do not define a perfect, one-ionization titration curve because of the perturbations produced by the charges on the carboxylate and amino groups. A similar pH effect has been observed for the protons on the imidazole ring of histidine (15). These charge effects do not seriously perturb the direction and magnitude of chemical shifts caused by ionization of the imidazole ring in histidine (15). Since the imidazole of histidine has a pK_a of 6.0 (16), the effect of methylation is to raise the pK_a slightly.

TABLE II. Chemical Shift as a Function of pH for the Methyl Resonance of 3-Methylhistidine

pH	δ Hz
3.00	1102.9
4.02	1100.0
4.82	1096.2
5.40	1089.6
5.99	1077.1
6.62	1061.3
7.52	1046.3
8.08	1042.4
9.20	1040.1

FIGURE 5. Chemical Shift of the Methyl Carbon of 3-Methylhistidine as a Function of pH



DISCUSSION

The pK_a of 6.75 affecting the chemical shift of 3-methyl His 57 must be the ionization of the methylimidazole group since no other break is seen in the pH range studied (3.5-10.0). The pH-rate profile exhibits a pK of ~ 7.0 (10), and, since His 57 is so intimately associated with the serine residue involved in catalysis, the ionization of 3-methyl His 57 is likely responsible for the increase in hydrolysis rate above pH 7. This histidine probably plays a role in the catalytic mechanism of the native enzyme, presumably that suggested from kinetic and crystallographic studies (11, 12).

Comparison of the titration curves shown in Figures 4 and 5 shows interesting features. Ionization of the methylimidazole ring of 3-methylhistidine is accompanied by an upfield shift of about 60 Hz. Such a shift would be expected from simple application of Ramsey's equation (17). Deprotonation of the ring increases the electron density on the observed carbon and thereby increases the symmetry of the p-orbitals of this carbon resulting in an upfield shift. In the enzyme, however, this same methyl group shifts downfield by 30 Hz upon ionization. This could well mean that the conformational change needed to put the modified enzyme into what could be an active configuration does indeed occur upon ionization.

The low pH form of the enzyme in solution probably has an active site arrangement much like that seen in the crystal structure (13). At these pH's, the methyl imidazole is protonated, and its most stable conformation is undoubtedly that with a hydrogen bond formed to the deprotonated Asp 102 carboxyl. This is the inactive conformation seen in the crystals. Ionization of this ring involves removal of a proton from the nitrogen directed toward Asp 102. The hydrogen bond is therefore broken. In the native enzyme, ionization removes a proton from the $N^{\xi 3}$-nitrogen (which is methylated in the modified enzyme) but leaves the Asp 102-His 57 hydrogen bond intact. Subsequent hydrogen bonding to Ser 195 in the native enzyme completes the very stable charge transfer network with little movement of the imidazole ring (9). For the modified enzyme, however, another stabilizing hydrogen bond can form only if the methylimidazole ring moves allowing formation of a hydrogen bond directly or indirectly (via a water molecule) to the hydroxyl proton of Ser 195. It may be that this is the most stable configuration for the modified enzyme and is therefore the high pH form observed in solution. This might explain why the modified enzyme retains some activity in the same pH range exhibited by the native protein.

Such a reorganization of the active site would also explain the unexpected direction of movement of the ^{13}C-resonance of the modifying methyl group. Removal of the proton from the

methylimidazole should produce an upfield shift based on theory and what is seen for 3-methylhistidine. The shift would be expected to be somewhat less than 60 Hz since formation of a hydrogen bond to Asp 102 has presumably partly removed the proton before ionization occurs. If, however, ionization is accompanied by a fast (on the nmr time scale) reorganization of the hydrolytic locus, then a competing effect such as a change in van der Waals forces or the charge distribution of neighboring atoms (18) would influence the ^{13}C resonance. The sum of the two competing effects could be a net downfield shift of the magnitude seen. The fact that a downfield shift is observed seems to require that something other than deprotonation occur when the methylimidazole moiety titrates. As discussed before, this could lead to a partially active enzyme.

That the observed methyl group experiences only one pK in the pH range observed is interesting. Presumably His 40 also titrates in this region, but it evidently has little effect on the conformation of the hydrolytic locus unless it also has a pK_a of about 6.75 and thus titrates with His 57. Protonation of Asp 102 at very low pH should probably influence the chemical shift of the resonance since this would result in the breakup of the hydrogen bond to Asp 102. No effect at low pH is seen down to pH 3.5; therefore, this group must protonate well below pH 3.0. At the other extreme, there does not seem to be an effect from

deprotonation of Ile 16 causing the hydrophobic pocket to collapse. This ionization comes between pH 8.5 and 9.0 and should have been observed if it significantly affects the environment of the observed methyl group.

The pK_a of 3-methylhistidine has been shown to be about 0.2 pK units higher than that of histidine. If the charges of the carboxylate and amino groups of histidine are blocked by incorporating histidine into a polypeptide chain (which does not introduce hydrogen bonding interactions with the imidazole ring), the pK_a is raised from 6.0 to 6.42 (18). Assuming a similar effect on methylhistidine, one would expect a pK_a of about 6.6 for the imidazole ring of 3-methyl His 57 if interactions with the protein are ignored. Methylation of His 57 on the enzyme causes the pK_a to increase by about 0.15 units compared to 0.2 units for methylation of histidine itself.

A pK_a of 6.75 for 3-methyl His 57 indicates that the enzyme is interacting with the imidazole in a manner which somewhat stabilizes the protonated form. This is probably a result of the hydrogen bonding interaction between Asp 102 and the methylimidazole ring. In fact, it would be expected that a greater increase in pK_a should be observed unless the form of the enzyme assumed after ionization is also stabilized perhaps through a favorable interaction with a water molecule or Ser 195 (10, 13). Crystallographic studies (13) have shown that the presence of the

modifying methyl group forces Ser 195 to move from its native location to one of two alternate positions. Movement of the methylimidazole group after ionization might relieve any unfavorable interactions produced by the movement of Ser 195. A pK_a of 6.6 for His 57 in the native enzyme indicates that the hydrogen bond to Asp 102 does not raise the pK_a much above the expected value of 6.42 (18) for a histidine residue in a polypeptide chain. The pK_a of 6.75 indicates that the active site region of the protein does not have sufficient hydrophobic character to drastically increase the pK_a for ionization of His 57 (which leads to a net negative charge compared with a net charge of zero before ionization). A similar argument would hold for His 57 in the native enzyme.

Due to the line width of the titrated methyl resonance ($\sim$ 20 Hz), there is some uncertainty in the chemical shift which should be assigned to a peak. In this work all shifts were measured using the marking routine supplied with the Varian 16K Fourier transform program, and the shifts therefore represent the point of maximum intensity of the peak. Since all measured peaks were symmetrical about the maximum, the uncertainty in the peak position should be considerably less than 10 Hz. Spectra taken on different days at the same pH agreed with one another to within 2 Hz.

The titration curve shown in Figure 4 appears to be a smooth curve defined by one pK_a of 6.75. However, since there

is some uncertainty in the position of a measured ^{13}C-resonance, it is possible that some other ionization (for example, His 40 or Ile 16) might have some small effect on the chemical shift of the methyl group. Although the smoothness of Figure 4 indicates that there is no large effect from these ionizations, the uncertainty of the measured chemical shifts might prevent observation of small changes.

EXPERIMENTAL

Synthesis of ^{13}C-methyl p-Nitrobenzenesulfonate

The following reactions were carried out in the dark. p-Nitrobenzenesulfonic acid (20.3 g, 0.1 mole) was dissolved in 450 ml of dry acetonitrile. To this solution was added silver oxide (11.6 g, 0.05 mole). The suspension was stirred for one hour at room temperature, filtered through Celite, and concentrated to a wet paste on a rotary evaporator. The paste was dried *in vacuo* (~ 1 Torr) at 65° (refluxing methanol). A grayish powder, silver p-nitrobenzenesulfonate, resulted. This material was stored desiccated and protected from light at all times.

The silver p-nitrobenzenesulfonate (6.3 g, 0.02 mole) was dissolved in 16.5 ml of dry acetonitrile. This mixture was cooled to 0°, and 1 g (0.007 mole) of 90% enriched ^{13}C-methyl iodide was added. The stirred mixture was allowed to slowly warm up to room temperature and stand for a total of 48 hours. A greenish precipitate of silver iodide formed and was removed by filtration. The addition of 30 ml of cold water to the filtrate produced a yellowish solid which was extracted three times with 20 ml of cold ethyl ether. After drying for 5 hours over sodium sulfate, the ether was removed on a rotary evaporator to yield a yellowish solid. Recrystallization from ligroin produced 1.2 g of a slightly

yellow crystalline solid. The yield was 82% of theoretical, based on methyl iodide. Mp 91-92°; lit. mp 91.5-92.5° (8).

Integration of the ^{13}C-satellites of the methyl proton resonance of this compound indicated ~ 90% enrichment of carbon-13 in the methyl group. This material is quite stable when dry, but as a precaution, it was kept desiccated and frozen.

Methylated α-Chymotrypsin

3-Methyl His 57-α-chymotrypsin was prepared essentially by the method of Nakagawa and Bender (8). Worthington three times-crystallized α-chymotrypsin was chromatographed on CM-Sephadex C-50 to yield protein which was essentially 100% active based on an active site titration.

Purified α-chymotrypsin (650 mg) was dissolved in 50 ml of 0.1 M sodium phosphate buffer to a final pH of 7.9. A ten-fold excess (39.8 mg) of ^{13}C-methyl p-nitrobenzenesulfonate was dissolved in 3 ml of acetonitrile and added dropwise to the stirred enzyme solution. The reaction was allowed to proceed for one hour at room temperature. An active site titration indicated a residual activity of about 2% at this point. Phenylmethanesulfonyl fluoride (13 mg) dissolved in 0.7 ml of propan-2-ol was added and allowed to react for 5 minutes to remove the last traces of activity. The reaction solution was quickly cooled in an ice bath, transferred to dialysis tubing, and dialyzed in the cold for 24

hours against four changes of distilled water (6 l. each). The dialyzed solution was then lyophilized to yield a white powder which exhibited no detectable activity in an active site titration.

Nmr Samples

Nmr samples were prepared in 0.1 M sodium phosphate buffer to the desired pH. The final protein concentration was 3.0 mM. The pH was checked before and after each spectrum was run and never varied by over 0.05 pH unit. Protein samples were transferred to 12 mm nmr tubes, and a 5 mm D_2O locking capillary held concentrically by two Teflon vortex plugs was placed into the nmr tube. Samples were temperature equilibrated in the nmr probe for at least 15 minutes before being run.

Nmr Spectra

Nmr spectra were taken on a Varian XL-100-15 nuclear magnetic resonance spectrometer operating in the Fourier transform mode. Data accumulation and Fourier transformations of the free induction decay were carried out by a Varian 620i computer interfaced to the spectrometer.

In general, protein spectra were taken at a 5000 Hz sweep width using a 60^{o} pulse and an acquisition time of 0.2 second. A sensitivity enhancement of 0.2 second was applied to each free

induction before carrying out the Fourier transformation. Proton noise decoupling was used in all cases. The spectra shown in Figures 2 and 3 represent about 325, 000 transients each.

Spectra of 3-methylhistidine (purchased from Sigma Chemical Company) were taken under similar conditions except that a 20^{o} pulse and a 1 second acquisition time were used.

All shifts were measured relative to external D_2O lock using the marking routine supplied with the Varian 16K Fourier transform program. Samples run on different days at the same pH were reproducible to at least 2 Hz. Experiments with dioxane indicated no correction for the external reference was necessary over the pH range studied.

REFERENCES

1. T. F. Spande, B. Witkop, Y. Degani, and A. Patchornick, Adv. Protein Chem., 24, 97 (1970).
2. G. R. Stark, Adv. Protein Chem., 24, 261 (1970).
3. E. F. Jansen, M. D. F. Nutting, and A. K. Balls, J. Biol. Chem., 179, 210 (1949).
4. W. J. Ray, Jr., and D. E. Koshland, Jr., Brookhaven Symp. Biol., 13, 135 (1960).
5. G. Schoellman and E. Shaw, Biochem. Biophys. Res. Commun., 7, 36 (1962).
6. K. J. Stevenson and L. B. Smillie, J. Mol. Biol., 12, 937 (1965).
7. H. J. Schramm, Biochem. Z., 342, 139 (1965).
8. Y. Nakagawa and M. L. Bender, Biochemistry, 9, 259 (1970).
9. B. W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, Nature, 214, 652 (1967).
10. R. Henderson, Biochem. J., 124, 13 (1971).
11. M. L. Bender and F. Kezdy, J. Amer. Chem. Soc., 86,
12. D. M. Blow, J. J. Birktoft, and B. S. Hartley, Nature, 221, 235 (1969).
13. G. S. Wright, G. P. Hess, and D. M. Blow, J. Mol. Biol., 63, 295 (1972).

14. There is a small peak at -29.2 ppm in Figure 3 which does not appear in Figure 2. This may possibly represent a second conformational form of the enzyme in slow exchange with the predominant form. In the spectra taken for titration purposes there was not sufficient signal to noise to allow a determination of the pH dependence of this peak.

15. D. H. Sachs, A. N. Schechter, and J. S. Cohen, J. Biol. Chem., 247, 6576 (1971).

16. E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Hafner Publishing Company, New York, 1943, p. 85.

17. J. A. Pople, W. J. Schneider, and K. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Company, New York, 1959, p. 168.

18. A. D. Buckingham, T. Schaefer, and W. G. Schneider, J. Chem. Phys., 32, 1227 (1972).

19. W. L. Koltum, J. Amer. Chem. Soc., 81, 295 (1959).

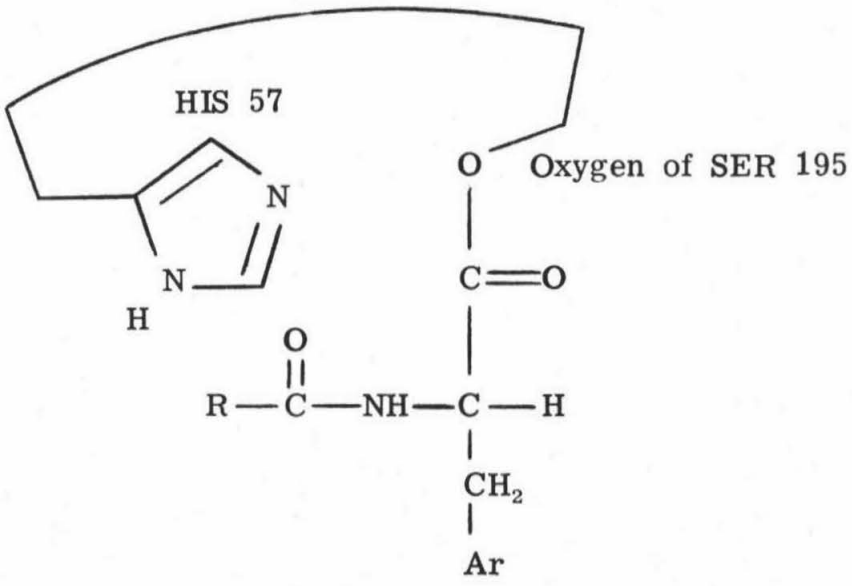
PART IV

Nuclear Magnetic Resonance Studies of Acylated Chymotrypsins

INTRODUCTION

The presently accepted mechanism (1, 2) for the proteolytic enzyme α-chymotrypsin involves the formation of an acyl enzyme with a covalent bond between the oxygen of Ser 195 and the carboxyl carbon of the acid group in the ester or amide substrate as shown in Figure 1. The formation of an acyl enzyme was originally proposed on the basis of results obtained from studies of the hydrolysis of p-nitrophenyl acetate (3-5) which is a nonspecific substrate for α-chymotrypsin. Subsequent steady state kinetic studies on specific ester substrates (6, 7) showed that formation of an acyl enzyme was probably a characteristic of all chymotryptic catalysis. More recent crystallographic studies (8) have definitely proven that a covalent bond exists between Ser 195 and the sulfonic acid group of the irreversible inhibitor toluenesulfonic acid. For amide substrates, it has been shown (9, 10) that the cleavage of the amide bond to yield a free amine and an acyl enzyme is rate limiting, and in this case a buildup of an appreciable amount of acyl enzyme would not be expected. For ester substrates, however, it is hydrolysis of the acyl enzyme to yield the native enzyme and a free acid which is rate determining (9, 10). With an ester substrate, it should be possible to have a steady state situation in which an appreciable amount of enzyme would be in the acyl form.

FIGURE 1. Structure of Acyl Enzyme Intermediate



Kinetic and spectroscopic evidence (11) has shown that this is indeed the case for the hydrolysis of N-acetyl-L-tryptophan p-nitrophenyl ester.

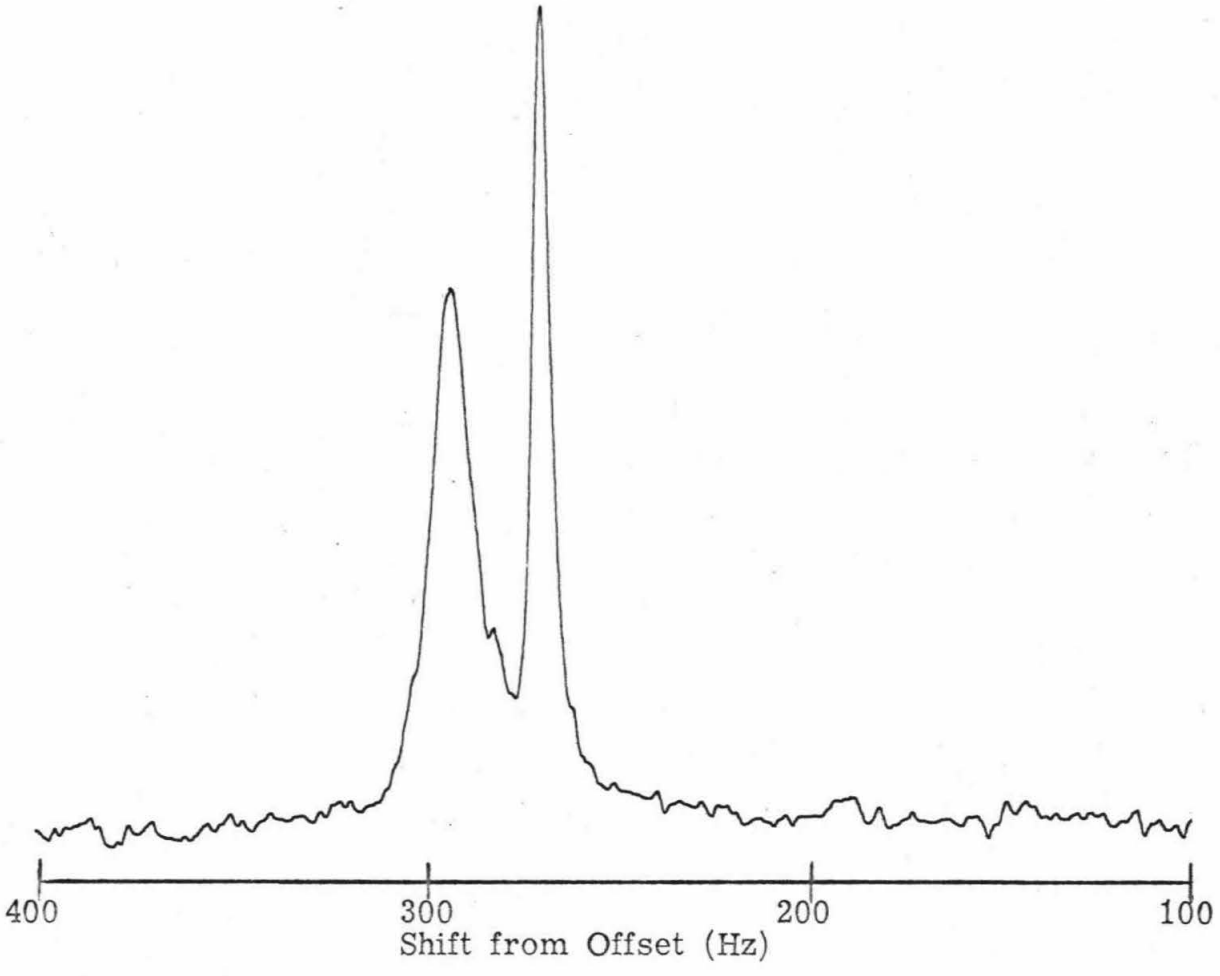
Part IV of this thesis reports preliminary fluorine nmr studies of α-chymotrypsin which has been acylated using fluorinated D-isomers of aromatic amino acids. Since acylation is rate limiting for these substrates, there is an appreciable buildup of enzyme in the acyl form, and nmr studies of the acylated enzyme are possible. Since D-isomers are not turned over to the free acid very rapidly (12), there is no rapid buildup of free acid to obscure observation of a relatively small amount of acyl enzyme as would occur with L-isomer substrates.

RESULTS

Figure 2 shows the fluorine nmr spectrum of a solution of N-TFA-D-p-fluorophenylalanine methyl ester (2.5 mM) and α-chymotrypsin (1.9 mM) in 0.1 M tris buffer at pH 7.5. The sharp peak at -262 Hz can be assigned to N-TFA-D-p-fluorophenylalanine methyl ester. The small shoulder at -274 Hz belongs to N-TFA-D-p-fluorophenylalanine, the enzymatic hydrolysis product. The broad peak ($\delta\nu_{\frac{1}{2}}$ = 10 Hz) at -285.2 Hz has been assigned to the acyl enzyme formed between N-TFA-D-p-fluorophenylalanine and α-chymotrypsin.

The intensity of the acyl peak increases as a function of time and reaches a near steady state concentration after about 1.5 hours. Integration of this peak indicates that about 60% of the enzyme in solution has been acylated. An active site titration of the enzyme showed a residual activity of ~40% as would be expected if the active site serine had been blocked by acylation. The formation of the acyl peak follows the expected pH dependence for α-chymotrypsin activity. The rate is slow below pH 5.5 and increases to a maximum at about pH 8.0. Disappearance of the methyl ester peak and formation of the acid peak shows a similar dependence during the initial reaction stages.

FIGURE 2. Fluorine Nmr Spectrum of a Solution of N-TFA-D-p-fluorophenylalanine Methyl Ester and α-Chymotrypsin at pH 7.5



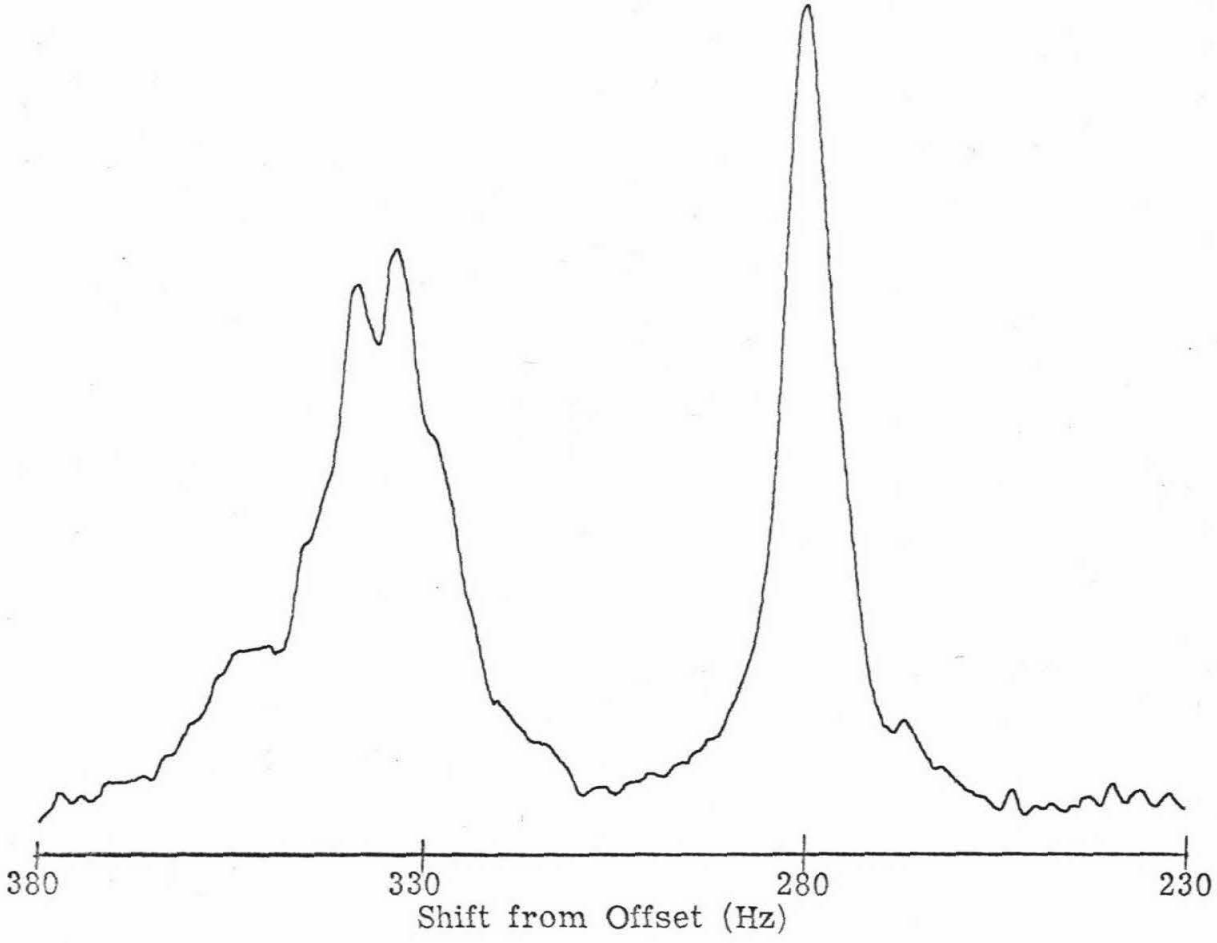
The nmr spectrum of an α-chymotrypsin solution with the methyl ester after 6 hours dialysis at 4° exhibits only one peak which corresponds to the resonance assigned to the acyl enzyme. The intensity of this peak slowly decreases, and the signal assigned to N-TFA-D-p-fluorophenylalanine slowly builds up as the acyl enzyme is hydrolyzed to yield the native enzyme and the free acid. That dialysis does not remove the acyl peak indicates that it does indeed represent a covalently bound intermediate.

If a solution of α-chymotrypsin is deactivated by addition of a three-fold excess of phenylmethanesulfonyl fluoride (13), there is no formation of either the acyl enzyme or free acid peak upon addition of the methyl ester to the solution. Since this reagent selectively reacts with the active site serine, the covalently bound intermediate must be at the active site of the enzyme, probably at Ser 195 (reaction with His 57 is not necessarily excluded since the size of the phenylmethanesulfonyl group would probably sterically block this residue even though it is not covalently bound to it).

Formation of a covalently bound peak has also been observed by nmr with N-TFA-D-p-fluorophenylalanine methyl ester and δ- and γ-chymotrypsin and with N-TFA-D-tryptophan methyl ester and α-chymotrypsin. Thus, it is a general phenomenon of chymotrypsin-D-substrate reactions.

Figure 3 shows the fluorine nmr spectrum of the aromatic region of a solution of N-TFA-D-p-fluorophenylalanine methyl ester

FIGURE 3. Aromatic Fluorine Nmr Spectrum of a Solution of N-TFA-D-p-fluorophenylalanine Methyl Ester and α-Chymotrypsin at pH 7.5



and α-chymotrypsin at pH 7.5. The peak at -280 Hz is from N-TFA-D-p-fluorophenylalanine. The rest of the spectrum is rather complex and poorly resolved due to broadening. The tallest signal can be assigned to N-TFA-D-p-fluorophenylalanine methyl ester. The rest of the spectrum represents the covalently bound acyl intermediate. It can be seen that the acyl enzyme appears as at least two poorly resolved peaks (-334 Hz and -350 Hz).

If the spectrum of the aromatic region is observed under conditions of gated decoupling (that is, with the proton noise decoupler on during acquisition of the free induction decay so a fully decoupled spectrum is obtained and off for a 2 second pulse delay between each Fourier transform pulse so no Overhauser enhancement of the fluorine signal is produced), the portion of the spectrum assigned to the covalently bound intermediate shows no detectable change in intensity. A similar experiment on the aromatic fluorine of N-TFA-D-p-fluorophenylalanine in buffer solution produced a 33% reduction of the fluorine signal . This reduction indicates that dipole-dipole relaxation is a dominant relaxational mechanism for this fluorine in a small molecule (14).

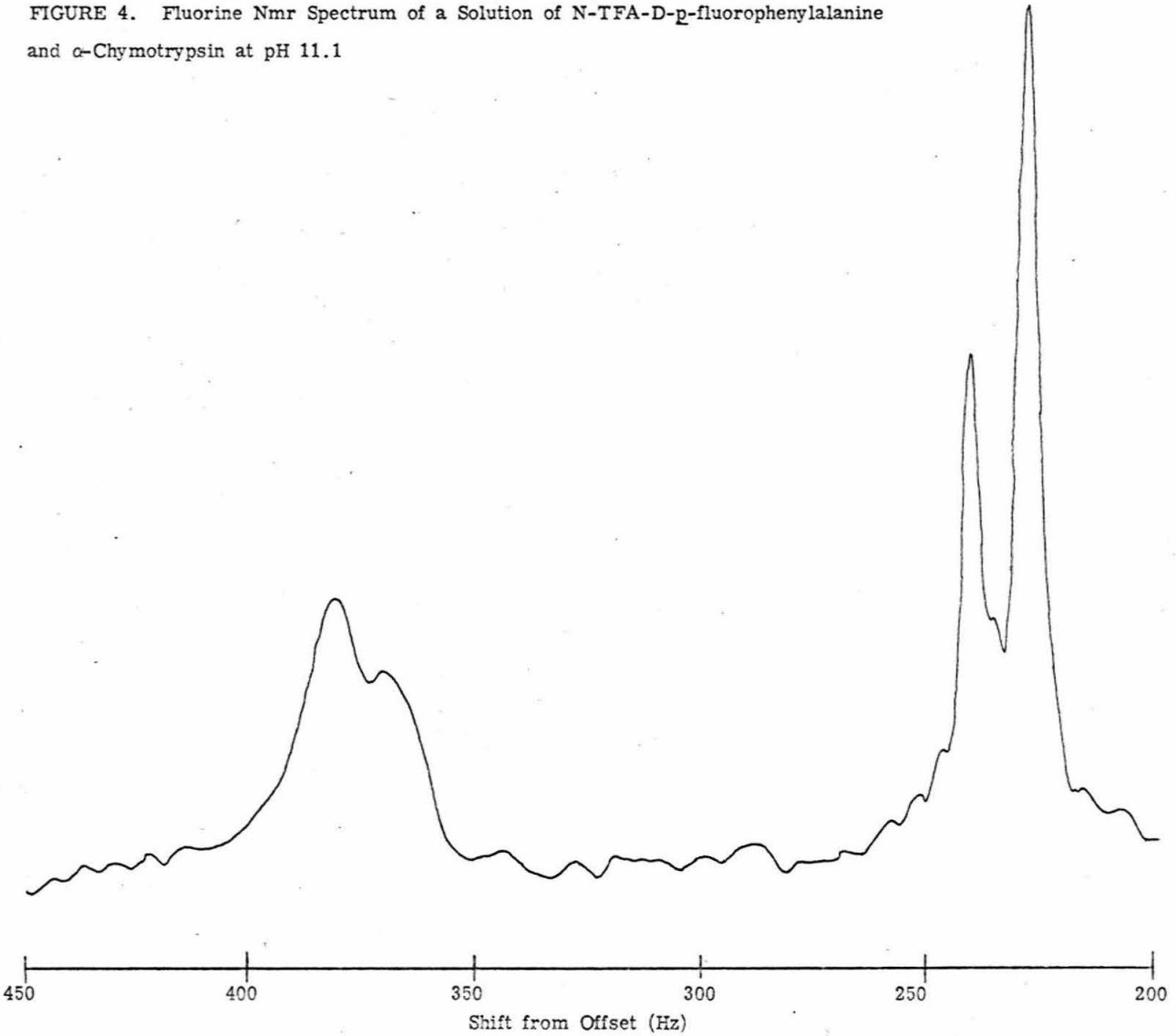
If the resonance assigned to the trifluoroacetyl fluorines of the acyl enzyme is observed as a function of pH, there is no appreciable change in chemical shift between pH 5.5 and 8.0. Because of the broadness of this peak and its nearness to the ester

and free acid resonances, it is doubtful that a shift of less than 3 Hz would be observed. Thus, the ionization of His 57 does not seem to significantly affect the chemical shift of the covalently bound intermediate. If the titration is carried to higher pH's, the acyl enzyme fluorines shift downfield about 158 Hz with an apparent pK_a of 10.8. A similar shift and pK_a is observed with the molecule N-TFA-D-p-fluorophenylalanine and results from titration of the activated amide proton of the N-trifluoroacetyl linkage.

The aromatic fluorine of the covalent intermediate moves upfield 50 Hz in the same pH range (pH 9-11.5). Again, this movement is observed with N-TFA-D-p-fluorophenylalanine methyl ester and results from titration of the amide proton. If the acyl enzyme is prepared using N-acetyl-p-fluorophenylalanine methyl ester, there is no significant movement of the fluorine resonance up to pH 13. The pK_a of the amide proton of this molecule is about 16 (15), and the proton would not titrate below pH 13. The total lack of movement indicates that ionizations on the enzyme have negligible effect on the position of the fluorine resonances of the covalently bound intermediate.

Interestingly, the trifluoroacetyl fluorines of the acyl enzyme exhibit more than one resonance while titrating. Figure 4 shows an nmr spectrum of the trifluoroacetyl peaks at pH 11.1. It can be seen that in addition to shifting downfield the peak has two

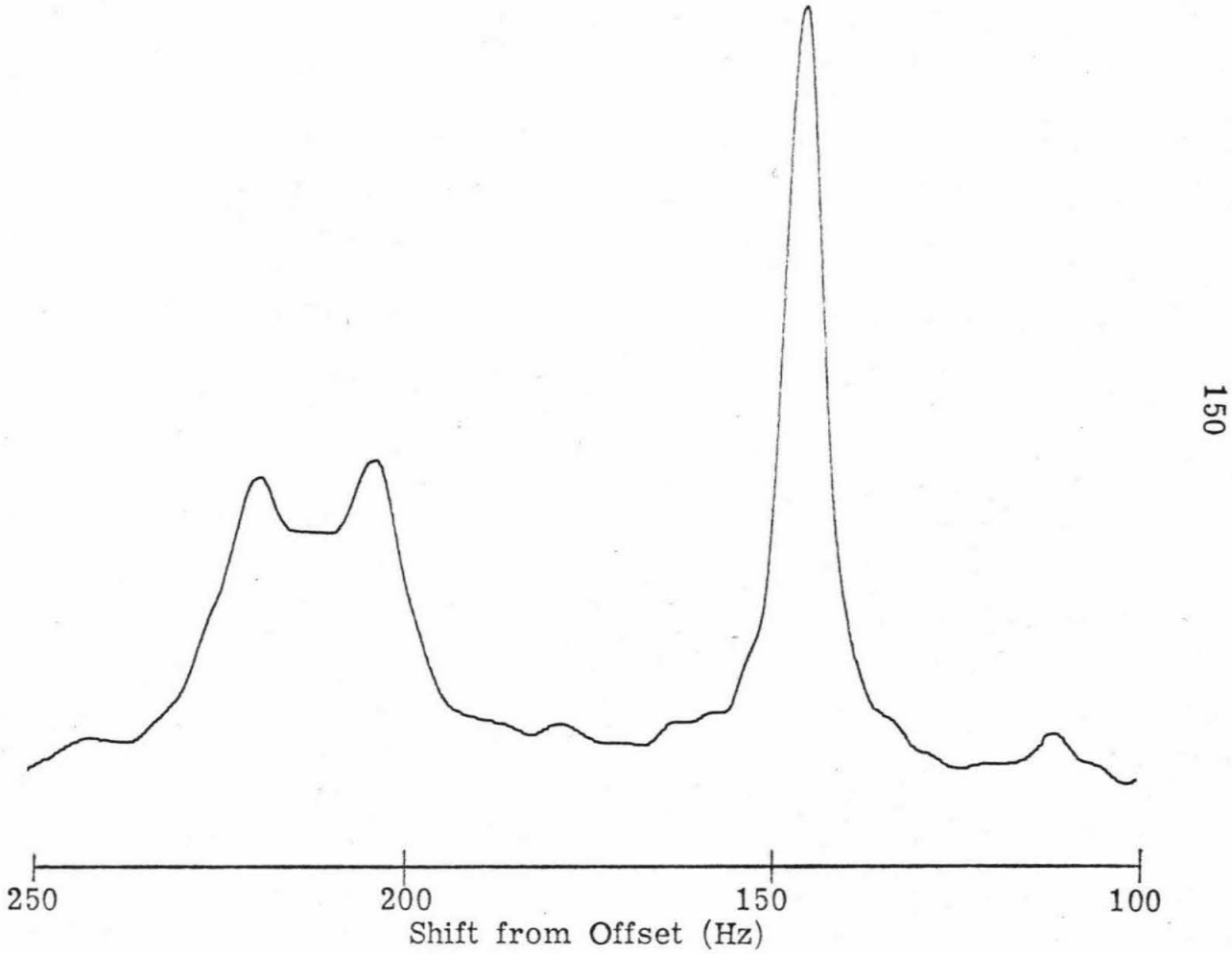
FIGURE 4. Fluorine Nmr Spectrum of a Solution of N-TFA-D-p-fluorophenylalanine and α-Chymotrypsin at pH 11.1



resolvable resonances. The two upfield peaks can be assigned to N-TFA-D-p-fluorophenylalanine and trifluoroacetate anion (from base hydrolysis of the N-trifluoroacetyl group). Figure 5 shows a spectrum of the aromatic region of the acyl enzyme formed with N-acetyl-p-fluorophenylalanine methyl ester. The aromatic resonances have not moved (since there is no ionization on the bound amino acid derivative), but the presence of two peaks is obvious. The upfield resonance of Figure 4 is from N-acetyl-D-p-fluorophenylalanine.

The addition of guanidine hydrochloride to the enzyme solution results in a sharpening of the peak assigned to the covalent intermediate. Even in 5 M guanidine hydrochloride, however, the covalent peak is broader than those assigned to small molecules in the solution. Thus, even in the presence of a large amount of denaturant, it is estimated that resolution of two covalently bound peaks separated by 3 Hz or less would be impossible.

FIGURE 5. Fluorine Nmr Spectrum of a Solution of N-acetyl-D-p-fluorophenylalanine and α-Chymotrypsin at pH 12.0



DISCUSSION

The experiments reported here strongly indicate that the nmr spectrum of an intermediate covalently bound at the active site of α-chymotrypsin has been observed. The covalent intermediate does not, however, seem to behave as would be expected for an acyl enzyme based on what is known about hydrolysis of D-amino acid acyl enzymes (12) and, by analogy, with L-amino acid acyl enzymes (7).

Although no accurate kinetic data has been obtained (work on this is presently in progress), the release of the free D-amino acid from the covalent complex and the hydrolysis of the complex is much slower than would be expected from previously reported kinetic results (12). Initially, the peak corresponding to the free acid builds up at an appreciable rate, but the rate seems to slow down as the acyl enzyme concentration builds up. Conversion of the acyl peak to free acid requires about five days which is much longer than would be predicted from the reported rate constants for deacylation of D-amino acid derivatives (12). A value of $0.015\ sec^{-1}$ for the deacylation of N-acetyl-D-phenylalanylchymotrypsin has been reported (12). This number is not at all consistent with the nmr results reported here.

The nmr spectra obtained for the aromatic fluorine of N-TFA-D-p-fluorophenylalanylchymotrypsin indicate that the aromatic ring of the acyl group is not in the hydrophobic pocket. The results reported in Part I of this thesis showed that placement of the ring in the pocket should perturb the chemical shift of the resonance by at least 100 Hz. A shift of no more than 20 Hz relative to the ester was observed (since the acyl enzyme involves an ester linkage, the chemical shift of the aromatic fluorine of the acyl enzyme should come close to that of the methyl ester if it is not perturbed by the enzyme). The gated decoupling experiments which were done on the aromatic fluorine of the acyl enzyme also indicate that the aromatic ring of the acyl enzyme does not reside in the hydrophobic pocket. Based on nmr (16) and fluorescence (17) studies, the correlation time of α-chymotrypsin has been reported to be about 3×10^{-8} seconds. It has been demonstrated (16) that a substituent on the para position of a covalently bound intermediate residing in the hydrophobic pocket assumes essentially the correlation time of the enzyme. From the theory of Solomon (14), it can be shown that if the para fluorine of the covalently bound intermediate had assumed the correlation time of the enzyme decoupling should cause a several-fold decrease in the intensity of the covalently bound peak (18) as the result of a negative Overhauser enhancement. The observed Overhauser enhancement of about 1 corresponds to a correlation time of about

2×10^{-9} seconds (18). Thus, the covalent intermediate has been significantly immobilized relative to a small molecule, but it still has rotational degrees of freedom relative to the enzyme.

It is possible that the dominant relaxation effect on the aromatic fluorine is something other than dipole-dipole relaxation with protons in which case the above conclusions based on Overhauser enhancements would be meaningless (14). This possibility is unlikely since similar experiments on the aromatic fluorine of N-TFA-D-p-fluorophenylalanine indicate a significant Overhauser effect.

The qualitative kinetic results indicate that the form of the acyl enzyme which has been observed by nmr is perhaps binding in a non-productive mode for efficient deacylation to yield free acid and the native enzyme. The nmr results on the aromatic fluorine indicate that this is the case since the aromatic side chain does not seem to reside in the "tosyl hole" and have proper alignment of the acyl bond for cleavage. A possible explanation is that somehow the aromatic ring of the acyl group comes out of the pocket because of the necessarily unfavorable interactions (for example, close contact of the trifluoroacetyl group with the γ-methylene of Met 192 (19)) associated with placing a D-isomer at the active site with its carboxyl group covalently bound to Ser 195 and its aromatic side chain in the specificity pocket. Removal of the aromatic ring to the surface of the enzyme would relieve this

strain and account for the small chemical shift and rotational freedom of the aromatic fluorine of the acyl group relative to the free ester. Once removed from the pocket, the ring might have difficulty reentering and producing proper alignment of the acyl bond for hydrolysis. This would explain the slow turnover of the acyl peak observed by nmr and apparent deactivation of the acylated enzyme.

If the above explanation is correct, then there must be at least two slowly exchanging conformations of the enzyme or the acyl group to account for the multiplicity of peaks observed. There is no precedent for several binding conformations of a covalently bound group, and it is impossible to describe the conformations on a molecular level at this time. Conformational equilibria in α-chymotrypsin which are affected by pH (20), temperature (21), and ionic strength (22) have been reported, but the effect of these equilibria on the bonding orientation of inhibitors and covalently bound species is not known. If two conformers do exist, the trifluoroacetyl fluorines of the covalently bound group evidently experience very similar environments at pH 7.5 since the acyl peaks are not resolved. The environment of the aromatic fluorines are somewhat different, and their nmr spectra show an obvious multiplicity at pH 7.5. Separation of the trifluoroacetyl fluorines upon ionization indicates that the pK_a of the trifluoroacetyl amide proton is somewhat different in the two forms or that ionization

is accompanied by a slightly different shift in the two conformers.

There are other explanations for the slow turnover of the acylated enzyme and the multiplicity of nmr resonances observed. It is conceivable that in addition to Ser 195 some acylation of the enzyme might occur at His 57. Reaction of His 57 with methyl p-nitrobenzenesulfonate (23) and the p-nitrophenylcarbonate monoester of Ser 195 (24) has been reported. This indicates the potential reactivity of this residue. Past attempts to detect acylimidazole intermediates with amino acid derivatives have been unsuccessful (25, 26), and it is generally felt that acylation of α-chymotrypsin at His 57 does not occur. The results obtained with phenylmethanesulfonyl fluoride indicate that whichever residue is being acylated must be at the active site. This would seem to exclude all residues except Ser 195 and His 57 as possible reaction points.

Another possible explanation is that the acyl enzyme observed by nmr may represent one or more states of enzyme denaturation as the result of tertiary structure disruption or peptide bond cleavage. This would explain the slow turnover of the acyl groups since the active site might be too disrupted to efficiently catalyze release of the acyl group. Since several states of denaturation can be imagined, this explanation could account for the multiplicity of nmr peaks. If this turns out to be the explanation, then the presence of the acyl group on the enzyme must somehow facilitate unfolding or cleavage of peptide bonds since, as reported in Part I,

α-chymotrypsin has not been observed to denature this rapidly in the presence of inhibitor molecules such as N-TFA-D-tryptophan.

In principle, a distinction between several conformations of native protein and several forms of a partially autolyzed enzyme could be made by observation of a fully denatured enzyme. In the former case, this should collapse the acyl peak into an obvious singlet. In the latter case, a singlet would not be expected although the chemical shift difference between an acyl group on denatured enzyme molecules differing by only a few covalent bonds might be very small. Addition of guanidine hydrochloride to the solution results in denaturation of the enzyme (27) and causes the resonance assigned to the acyl peak to sharpen somewhat, but it is still too broad to rule out two fluorine species separated by a small chemical shift difference.

Work is presently under way to quantitate the kinetic aspects of this phenomenon and to elucidate the cause of the multiplicity observed in the nmr spectra.

EXPERIMENTAL

Materials

N-TFA-D-p-fluorophenylalanine methyl ester and N-acetyl-D-p-fluorophenylalanine methyl ester were prepared by M. W. Hunkapiller.

α-Chymotrypsin was obtained from Calbiochem. δ- and γ-chymotrypsin were obtained from Sigma Chemical Company. All enzyme samples were stored frozen in a desiccator when not in use.

Preparation of Nmr Samples

Chymotrypsin (240 mg) was dissolved in 0.1 M tris or 0.1 M sodium phosphate buffer to the desire pH and a final volume of 4.0 ml. Substrates were added either as a solid or predissolved in 0.05 ml of methanol. The sample was placed into a 12 mm nmr tube equipped with a concentrically held locking capillary (5 mm) of deuterium oxide. All samples were equilibrated to probe temperature before final spectra were recorded.

Nmr Spectra

All spectra were recorded on a Varian XL-100-15 nuclear magnetic resonance spectrometer operating in the Fourier transform

mode. The instrument was locked on deuterium oxide held concentrically in a locking capillary. Chemical shifts were measured using the Varian marking routine supplied with the 16K FT program. Generally, a pulse width of 50 μsec ($\sim 50^\circ$ pulse) and an acquisition time of from 0.5 to 1.0 seconds were used to record spectra. Attempts to gain peak resolution by using acquisition times of 2.0 seconds were unsuccessful. The gated decoupling experiments were carried out using a computer program prepared by S. H. Smallcombe.

REFERENCES

1. R. E. Dickerson and I. Geis, "The Structure and Action of Proteins," Harper and Row, New York, 1969, p. 83.
2. C. J. Gray, "Enzyme-Catalyzed Reactions," Van Nostrand Reinhold Company, London, 1971, p. 165.
3. B. S. Hartley and B. A. Kilby, Biochem. J., 56, 288 (1954).
4. H. Gutfreund and J. M. Sturtevant, Biochem. J., 63, 656 (1956).
5. L. Faller and J. M. Sturtevant, J. Biol. Chem., 241, 4825 (1966).
6. C. Niemann, Science, 143, 1287 (1964).
7. M. L. Bender and F. J. Kezdy, Ann. Rev. Biochem., 34, 49 (1965).
8. D. M. Blow , J. J. Birktoft, and B. S. Hartley, Nature, 221, 337 (1969).
9. B. Zerner and M. L. Bender, J. Amer. Chem. Soc., 86, 3669 (1964).
10. B. Zerner, R. P. Bond, and M. L. Bender, J. Amer. Chem. Soc., 86, 3674 (1964).
11. F. J. Kezdy, G. E. Clement, and M. L. Bender, J. Amer. Chem. Soc., 86, 3690 (1964).
12. D. W. Ingles, J. R. Knowles, and T. A. Tomlinson, Biochem. Biophys. Res. Commun., 23, 619 (1966).

13. D. E. Fahrney and D. M. Gold, J. Amer. Chem. Soc., 85, 997 (1963).

14. I. Solomon, Phys. Rev., 99, 559 (1955).

15. J. B. Hendrickson, D. J. Cram, and G. S. Hammond, "Organic Chemistry," McGraw-Hill Book Company, New York, 1970, p. 304.

16. E. W. Bittner and J. T. Gerig, J. Amer. Chem. Soc., 92, 5001 (1970).

17. R. P. Haugland and L. Stizer, in "Conformations of Biopolymers, Vol. I," Academic Press, New York, 1967, p. 321.

18. S. H. Smallcombe, personal communication, 1972.

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

20. A. R. Fersht and Y. Requena, J. Mol. Biol., 60, 279 (1971).

21. R. Lumry and S. Rajendi, J. Phys. Chem., 75, 1387 (1971).

22. C. C. Cuppett, H. Resnick, and W. J. Cenady, J. Biol. Chem., 246, 1135 (1971).

23. Y. Nakagawa and M. L. Bender, Biochemistry, 9, 261 (1970).

24. T. H. Fife, J. E. C. Hutchins,and D. M. McMahon, J. Amer. Chem. Soc., 94, 1316 (1972).

25. H. Gutfreund and J. M. Sturtevant, Biochem. J., 63, 656 (1956).

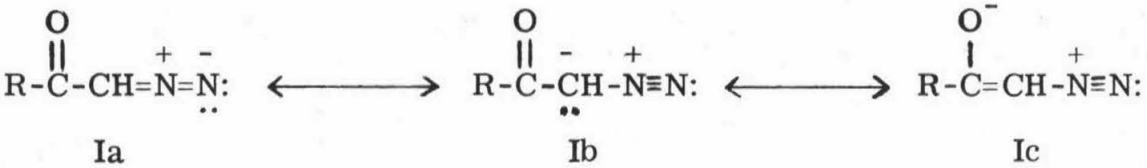
26. H. Gutfreund and J. M. Sturtevant, Proc. Nat. Acad. Sci. U. S., 42, 719 (1956).

27. J. D. Morrisett and C. A. Broomfield, J. Amer. Chem. Soc., 93, 7297 (1971).

PROPOSITION I

The use of copper and copper compounds as catalysts in the reactions of diazoketones is well documented (1). In general, such reactions are carried out by mixing the reagents in organic solvents and heating. Usually these reactions can be followed by nitrogen evolution. Copper catalyzed reactions of diazoketones include addition to alkenes and alkynes (1) and insertion into the O-H bond of alcohols (2) and carboxylic acids (3). These reactions are similar to carbene reactions; however, the more energetic carbene reactions such as C-H insertion are not observed in diazoketone-copper systems. The term carbenoid has been applied to the reactive intermediate in such reactions. The carbenoid is generally assumed to be an organic-copper complex (1).

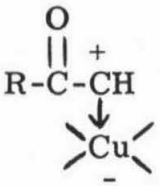
The mechanism of these reactions is not well understood (1), and there is no experimental or theoretical proof for any intermediate. One of the resonance structures of a diazoketone (Ib) places a nonbonded electron pair on the α-carbon (4). Most



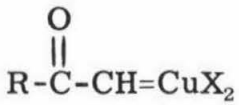
Ia Ib Ic

authors have assumed that the intermediate formed involves

the interaction of this electron pair with the copper followed by immediate loss of nitrogen to produce the reactive species. Structures such as II (2) and III (5) have been proposed for intermediates involving copper (II).



II



III

Recently the copper (II) catalyzed reactions of diazoketones with carboxylic acids to form esters was used in chemical modification studies of the enzyme pepsin (6). Copper (II) was shown to greatly enhance the rate of ester formation (as measured by enzyme inactivation) in an aqueous solution of diazoacetylglycine ethyl ester, copper (II), and pepsin. These authors observed that prior mixing of the copper salt and the diazoketone in ethanol or methanol further enhanced the rate of ester formation. It was suggested that an intermediate like III was formed in the alcohol and reacted rapidly when mixed with aqueous pepsin solutions. A similar rate enhancement by premixing has been observed for diazoacetylnorleucine methyl ester and 2-diazo-4'-bromoacetophenone (7). These studies would certainly indicate that some intermediate between the copper (II) and the diazoketone is formed in alcoholic solution. It has been observed, however, that mixing of a

diazoketone with copper (II) salts in alcohol does not cause nitrogen evolution (2, 8). This obviously eliminates III as the reactive intermediate in the pepsin inactivation reaction. Furthermore, in most other copper catalyzed diazoketone reactions such as addition to double and triple bonds and O-H insertion, the evolution of nitrogen occurs only after heating required for product formation (1-3). It is generally assumed that the nitrogen evolution occurs as the intermediate species is formed and not when the reaction occurs. Based on observation in alcohol, it is likely that a nitrogen-containing intermediate may be formed at lower temperatures and that it is this intermediate which reacts at higher temperatures.

A spectroscopic investigation of the intermediate formed in alcoholic solutions is proposed. It is further proposed that experiments be carried out to investigate the effect of premixing of dizaoketones and copper salts on the rate of reaction with alkenes, aldehydes, ketones, alcohols, and phenols. If such studies indicate the formation of a reactive intermediate, a spectroscopic comparison of this intermediate to the one formed in alcohol is proposed.

2-Diazo-4'bromoacetophenone would be an ideal compound to use for such a study. The ultraviolet spectrum of this compound has been reported (9), and its infrared bands can easily be assigned by analogy to those reported for diazoacetophenone (10). This

compound forms a reactive intermediate in ethanol (6) and like diazoacetophenone (2, 11) should undergo addition to alkenes and insertion into O-H bonds.

Since isolation of the intermediate formed in ethanol may not be feasible (the intermediate may not be very stable and would perhaps decompose explosively like many diazo compounds (1)), changes in the spectrum of the diazo compound in ethanol caused by addition of copper (II) salts must be observed. By using a large excess of copper (II) salt, one should be able to force most of the diazoketone into the complexed form, thereby eliminating interfering absorptions from the uncomplexed diazoketone.

It should be relatively easy to show that the reactive intermediate formed in ethanol does not have a carbenoid structure suggested by structures II and III. The diazo group of diazoketones gives rise to fairly characteristic infrared and ultraviolet bands. Although they will quite possibly be greatly perturbed in the complex, these absorptions should definitely prove that the complex is not formed with loss of nitrogen. The NN stretch of 2-diazo-4'-bromoacetophenone will be a strong absorption at about 2110 cm^{-1} (10). If the complex involves coordination of the terminal nitrogen to the copper, then this band will probably come at lower wave numbers, perhaps in the region of the NN stretch of azo compounds which is in the range 1630-1575 cm^{-1} (12). The weakness of this band (13) should make it possible to distinguish the NN stretch

from a carbonyl absorption which may come in roughly the same region (14). 2-Diazo-4'-bromoacetophenone also exhibits 290 nm ultraviolet absorption (9) which results from an electronic transition from a π-type orbital over the $-C=\overset{+}{N}_2$ group (15). If this band is not lost in the organo-copper intermediate, then it would strongly indicate that the complex still contains the diazo group.

There are numerous possible intermediates which can be drawn for a diazoketone-copper (II) complex including coordination of the oxygen, terminal nitrogen, or both. Changes in the infrared absorption frequencies associated with the carbonyl and diazo functional groups upon addition of copper (II) should be helpful in determining the nature of the complex (16).

Cyclic structures analogous to azo dyes (17) are perhaps also possible and would probably make the infrared spectrum of the complex difficult to interpret. The ultraviolet spectra of diazoketones show absorption from cis and trans isomers due to somewhat restricted rotation about the carbonyl carbon-α-carbon bond (9). Since cyclic complexes should stabilize the cis isomers, absorption from two conformers should not be observed if such cyclic complexes are formed.

Since copper complexes of diazoketones have not been reported, it is rather difficult to predict what the spectra of such a complex will look like; however, it should be possible to draw certain conclusions about the structure of the complex from spectroscopic studies. At worst, the spectra of the intermediate can

be characterized enough to allow a search for a similar intermediate in other systems as proposed below.

Copper catalyzed reactions of diazoketones with aromatic ketones and aldehydes, alcohols, carboxylic acids, alkenes, and alkynes have been carried out in solvents ranging from ethanol to benzene (1-3, 11, 18). It is proposed that a variety of these reactions be run after equilibration of the diazoketone with the copper (II) compound for about ten minutes. The rates of such reactions can be monitored by the evolution of nitrogen or by measuring the percent product formation in a given length of time. The reactions with aldehydes, ketones, and olefins often give several products (11, 18). It would be interesting to measure any change in the product ratios brought about by premixing of the copper (II) salt and the diazoketone.

If it is observed that premixing does produce rate enhancement, it is proposed that a spectroscopic study of the intermediate formed be carried out and a comparison of this intermediate with the one formed in ethanol be made.

If it can be shown that there is a common nitrogen-containing intermediate formed in each of the systems studied, then a generalized mechanism for carbenoid reactions can perhaps be put forth on the basis of structural information obtained from spectroscopic studies. It is of course possible that the pepsin reaction is a special case and that the intermediate formed in

ethanol is reactive only because of the special nature of the enzyme's active site. If this is the case, then the intermediate formed in ethanol may have little connection with copper catalysis in other reactions; however, a kinetic and spectroscopic study of other systems may be useful in determining whether or not structures like II and III are reasonable intermediates.

REFERENCES

1. D. Vinod and E. W. Warnoff, Chapter 3 in "Organic Reactions, Vol. 18," John Wiley and Sons, Inc., New York, 1970.
2. M. Takebayashi, T. Ibata, H. Kohara, and B. U. Kim, Bull. Chem. Soc. Japan, 40, 2392 (1967).
3. J. L. E. Erickson, J. M. Dechary, and M. R. Kesling, J. Amer. Chem. Soc., 73, 5301 (1951).
4. H. Zollinger, "Azo and Diazo Chemistry," Interscience Publishers, Inc., New York, 1961, p. 43.
5. D. O. Cowan, M. M. Couch, K. R. Kopechy, and G. S. Hammond, J. Org. Chem., 29, 1922 (1964).
6. R. L. Lundblad and W. H. Stein, J. Biol. Chem., 244, 154 (1969).
7. M. Hunkapiller, J. E. Heinze, and J. N. Mills, Biochemistry, 9, 2897 (1970).
8. M. Hunkapiller, personal communication, 1971.
9. L. L. Veveson and C. W. Thomas, Tetrahedron, 22, 209 (1966).
10. C. Pecile, A. Fobbani, and S. Ghersetti, Tetrahedron, 20, 823 (1964).
11. M. Takebayashi, T. Ibata, H. Kohara, and K. Ueda, Bull. Chem. Soc. Japan, 42, 2938 (1969).

12. J. R. Dyer, "Applications of Absorption Spectroscopy of Organic Compounds," Prentice-Hall, Inc., Englewood Cliffs, 1965, p. 37.

13. N. B. Colthup, L. H. Daly, and S. E. Wiberley, "Introduction to Infrared and Raman Spectroscopy," Academic Press, New York, 1964, p. 288.

14. K. Nakanishi, "Infrared Absorption Spectroscopy--Practical," Holden-Day, Inc., San Francisco, 1962, p. 28.

15. I. G. Csizmadia, S. A. Houlden, D. Meresy, and P. Yates, Tetrahedron, 25, 2121 (1969).

16. M. M. Jones, "Ligand Reactivity and Catalysis," Academic Press, New York, 1968, pp. 213-216.

17. M. Zollinger, Chapter 14.

18. M. Takebayashi, T. Ibata, and K. Ueda, Bull. Chem. Soc. Japan, 43, 1500 (1970).

PROPOSITION II

Recently, work has been reported on the decomposition of high energy species produced by the pyrolysis of alkylsilanes (1-8). These studies have suggested that such compounds decompose by a molecular elimination process in addition to the unimolecular bond fission mechanism exhibited by acyclic hydrocarbons (9). For example, disilane, Si_2H_6, seems to decompose by the molecular elimination process (5, 6), but Si-C and Si-H bond fission are favored in trimethylsilane, $(CH_3)_3SiH$ (2). The mechanisms apparently compete with one another in the thermal decomposition of methylsilane, CH_3SiH_3 (4, 8). Due to the wide variety of products and side reactions found in pyrolysis experiments, sufficiently good data have not been obtained to allow unambiguous distinction between the two mechanisms in all cases. Rate parameters and activation energies for various decomposition pathways have consequently been difficult to determine reliably (10).

Another approach to understanding the decay modes of vibrationally excited alkylsilanes has therefore been taken (10, 11). This method involves the insertion of methylene ($:CH_2$) into alkylsilanes to produce high energy species which usually decompose to yield fewer products than are observed in pyrolysis experiments. For example, the addition of methylene to dimethylsilane produces an

excited form of trimethylsilane (as well as some methylethylsilane). Product analysis yields information about the decomposition pathways and their associated rates for trimethylsilane. Although several products may result, an improvement over the large number of compounds formed in pyrolysis experiments has been realized.

One complication in such experiments is the competing reaction of hydrogen abstraction by methylene from Si-H bonds by methylene to produce methyl and alkylsilyl radicals which may recombine to yield a lower energy form of the same compound obtained from insertion or may react in other ways to produce any of several products (10, 11). It has been experimentally shown for hydrocarbons that singlet methylene ($^1CH_2$) is responsible for insertion into C-H bonds and that triplet methylene ($^3CH_2$) is the species involved in hydrogen abstraction from C-H bonds (12-15). In the case of silicon compounds, there are no quantitative measurements to confirm that a similar situation exists (10, 16). Generally, it is assumed that the species responsible for insertion is $^1CH_2$ since the pressure dependence of such reactions is in agreement with this assumption (11). For the abstraction reaction, however, none of the experimental data obtained so far allows for a conclusive statement about the nature of the abstracting species, and generally all methylene involved in abstraction reactions is taken as one species (10, 11).

Usually, the methylene used in these experiments is prepared by photolysis of ketene or diazomethane. Initially, this produces only ^{1}:CH_2 (12, 17), but molecular collisions produce intersystem crossing resulting in the formation of the ground state ^{3}:CH_2 (15, 18). Since this conversion requires collisional activation, the rate observed is pressure sensitive, and varying amounts of ^{1}:CH_2 and ^{3}:CH_2 would be expected at different total pressures. It therefore seems risky to calculate rate parameters from pressure dependent experiments without distinguishing between the two species, especially if the two forms effectively compete in hydrogen abstraction reactions. Since as much as 27% abstraction is observed in methylene-alkylsilane systems (16), quantitative analysis requires distinction between the two species if both are involved in abstraction reactions.

There is some evidence to indicate that the amount of hydrogen abstraction observed in the reaction of monosilane with methylene is perhaps greater than would be expected from the amount of triplet methylene produced under the experimental conditions (11). Thus, it is possible that both forms of methylene can react by an abstraction mechanism at competing rates. The solution to this problem would have bearing on the analysis of experiments such as those described above as well as the theoretical understanding of singlet and triplet methylene.

It is therefore proposed that a study be made to determine the way in which singlet and triplet methylene react with Si-H bonds

especially with regard to hydrogen abstraction. Experimentally, this can be accomplished by examining the effect of varying singlet to triplet ratios on the products formed in methylene-alkylsilane systems.

The obvious way to vary the singlet to triplet ratio is by changing the total pressure of the system since higher pressures produce greater amounts of the triplet form. Unfortunately, the products obtained from methylene-alkylsilane systems are also quite pressure sensitive. At higher pressures an excited species produced by methylene insertion is more likely to be collisionally deactivated before decomposing along one of several pathways (10, 11). These competing effects of pressure would make interpretation of product analysis very difficult.

It is possible to change the pressure dependence of the singlet to triplet ratio produced by the photolysis of diazomethane and ketene by using the mercury photosensitized decomposition of ketene as the source of methylene (19). This process produces essentially pure triplet species at low ketene partial pressures by the following pathway.

$$Hg(^1S_0) + h\nu(2537\ \mathring{A}) \rightleftharpoons Hg(^3P_1)$$

$$Hg(^3P_1) + CH_2CO \rightleftharpoons Hg(^1S_0) + {}^3(CH_2CO)$$

$${}^3(CH_2CO) \rightleftharpoons {}^3(CH_2) + CO$$

At higher partial pressures of ketene (50 Torr), direct photon capture by ketene becomes important, and singlet methylene is produced in increasing amounts up to about 25% at 700 Torr. It is thus possible to vary the ratio of singlet to triplet methylene in a different manner than has been used with previous studies on alkylsilanes. Methylene produced by this mechanism seems to behave normally in reactions with propane (19).

It is proposed that the reaction of methylene (produced by mercury photosensitized decomposition of ketene) with silane be studied to examine the differences between the reactions of the singlet and triplet forms. This is the simplest system possible and should yield the desired information about hydrogen abstraction. The reaction between monosilane and methylene produced by the photodecomposition of diazomethane has been studied over a wide pressure range (11). This study concluded only that there was a good deal of hydrogen abstraction involved and that the amount perhaps exceeded that expected from the amount of triplet methylene present. This study reported the ratio of observed products such as disilane, ethylsilane, and dimethylsilane relative to methylsilane (the product expected from methylene insertion into SiH_4 followed by collisional deactivation before decomposition) over a pressure range from 0.1 Torr to 2400 Torr. Below about 20 Torr the amount of decomposition products was quite large indicating mostly unimolecular decomposition of excited species before collisional deactivation. Above 20

Torr product ratios rapidly decrease to a small constant value (equivalent to about 20% of the total products formed) from 50 to 2400 Torr. Since all excited CH_3SiH_3 formed at the higher pressures was deactivated to ground state CH_3SiH_3 before decomposition to other products, the residual products other than CH_3SiH_3 obtained in this pressure range were the result of hydrogen abstraction by methylene. No attempt was made to distinguish between collisional deactivation of excited species and varying singlet to triplet ratios as the cause of the pressure dependence of the reaction products.

First, it is proposed that the reaction of SiH_4 with $^3:CH_2$ produced by the mercury photosensitized decomposition of ketene (maintained at a low partial pressure) be carried out over the pressure range from 0.1 to 2400 Torr. An inert gas such as helium can be used to vary the total pressure while maintaining the pressure of ketene and silane at a constant value. If there is no insertion by $^3:CH_2$, the product ratios (relative to CH_3SiH_3) should be constant with pressure since only hydrogen abstraction to produce radicals will occur. Any pressure dependence, such as decreasing amounts of CH_3SiH_3 at low pressures, will indicate that $^3:CH_2$ is undergoing insertion into Si-H bonds to produce excited states of CH_3SiH_3 which decompose before undergoing collisional deactivation.

Secondly, it is proposed that the reaction of $^3:CH_2$ with SiH_4 at some high pressure (200 Torr) be studied as a function of the

partial pressure of ketene. Since the amount of singlet methylene formed will be essentially linear with the partial pressure of ketene (above 5 Torr), the amount of CH_3SiH_3 produced should increase with the ketene partial pressure if only insertion occurs (19). If less CH_3SiH_3 than expected is produced, it will indicate that some of the singlet methylene formed is undergoing hydrogen abstraction reactions to produce products other than CH_3SiH_3. If appreciable hydrogen abstraction is observed, then reanalysis of some previously reported rate constants based on data obtained in pressure dependent experiments would be necessary.

REFERENCES

1. H. Sakurai and M. Kumada, J. Chem. Soc. D, 4 (1969).
2. I. M. T. Davidson and C. A. Lambert, J. Chem. Soc. D, 1276 (1969).
3. E. M. Tebben and M. A. Ring, J. Inorg. Chem., 8, 1787 (1969).
4. J. J. Kohanek, P. Estacio, and M. A. Ring, J. Inorg. Chem., 8, 2516 (1969).
5. M. A. Ring, R. B. Baird, and P. Estacio, J. Inorg. Chem., 9, 1004 (1970).
6. P. Estacio, M. D. Sefcik, E. K. Chan, and M. A. Ring, J. Inorg. Chem., 9, 1068 (1970).
7. R. B. Baird, M. D. Sefcik, and M. A. Ring, J. Inorg. Chem., 10, 883 (1971).
8. M. A. Ring, M. J. Puentes, and H. E. O'Neal, J. Amer. Chem. Soc., 92, 4845 (1970).
9. H. M. Frey and R. Walsh, Chem. Rev., 69, 103 (1969).
10. W. L. Hase, W. G. Breiland, P. W. McGrath, and J. W. Simons, J. Phys. Chem., 76, 459 (1972).
11. J. W. Simons and C. J. Mazac, Can. J. Chem., 45, 1717 (1967).

12. D. F. Ring and B. S. Rabinovitch, J. Phys. Chem., 72, 191 (1968).

13. D. F. Ring and B. S. Rabinovitch, Int. J. Chem. Kinet., 1, 11 (1969).

14. W. Braun, A. M. Bass, and M. Pilling, J. Chem. Phys., 52, 5131 (1970).

15. J. A. Bell, J. Phys. Chem., 75, 1537 (1971).

16. C. J. Mazac and J. W. Simons, J. Amer. Chem. Soc., 90, 2484 (1968).

17. F. B. Gravcock, W. L. Hase, and J. W. Simons, J. Phys. Chem., 76, 607 (1972).

18. B. S. Rabinovitch, K. W. Watkins, and D. F. Ring, J. Amer. Chem. Soc., 87, 4960 (1965).

19. H. E. Avery and R. J. Cvetanovic, J. Chem. Phys., 48, 380 (1968).

PROPOSITION III

Chemical modification studies have been widely used as a tool for identifying which groups of an enzyme are involved in catalysis (1, 2). One such modification is the methylation of α-chymotrypsin with methyl p-nitrobenzenesulfonate (3). The aromatic moiety of this reagent directs the sulfonic acid ester to the active site by hydrophobic interaction with the specificity pocket of the enzyme. Subsequent displacement of the p-nitrobenzenesulfonate group by the $N^{\xi 3}$-nitrogen of His 57 results in methylation of the histidine. This type of modification has the advantage of producing only a small perturbation in the overall enzyme structure while greatly perturbing one particular residue. Other modifying reagents such as L-(1-tosylamido-2-phenyl)ethyl-chloromethyl ketone are somewhat ambiguous in their effect in that they react chemically with His 57 and also place a large hydrophobic moiety at the active site (4). It is not clear whether the loss of activity associated with such a modification is the result of perturbation of the chemical nature of the histidine residue or the presence of a bulky group which blocks access to the active site. Loss of activity caused by methylation can, however, be directly associated with chemical modification of His 57 and strongly implicates this group as being important in catalysis.

Crystallographic (5) and kinetic (6) studies on methylated α-chymotrypsin have shown that it has structural and binding properties which are very similar to those of the native protein. Except for expected changes caused by the modifying methyl group, the tertiary structure of the two proteins seems to be identical. Although the catalytic activity of the modified enzyme toward specific ester substrates is reduced 5000 to 200, 000 fold, it is still a much better catalyst than other α-chymotrypsin derivatives in which His 57 has been modified.

The results presented in Part III of this thesis have indicated that methylated α-chymotrypsin is readily studied by ^{13}C-nmr techniques. The low activity of this protein allows long term spectra to be taken without autolysis. Identification of the ^{13}C-resonance of the modifying methyl group by selective enrichment has been accomplished. Studies of the effect of pH, salt concentration, and chemical modifications on this resonance should yield information about the native protein.

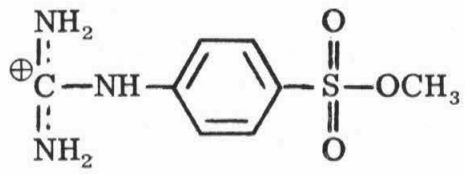
The proteolytic enzyme trypsin is very similar to α-chymotrypsin in many ways. Sequence studies have shown that the two proteins have homologous primary structures (7, 8). From kinetic evidence (9-11) and chemical modification studies (12-14), the mechanism of the two proteins has been shown to be very similar. Crystallographic studies have indicated that the charge transfer system seen in α-chymotrypsin could also be present in trypsin with the residues Asp 90, His 46, and Ser 183 forming a hydrogen bonded

network (15). Trypsin differs from chymotrypsin in that it is specific for the side chains of arginine and lysine instead of those of tryptophan, phenylalanine, and tyrosine (16). This is a result of a favorable interaction of the positive charge of arginine and lysine side chains with the negatively charged carboxyl group of Asp 177 on the trypsin molecule.

Chemical modification studies involving His 46 have been carried out on trypsin. Just as L-(1-tosylamido-2-phenyl)ethyl-chloromethyl ketone reacts with His 57 of α-chymotrypsin, tosyl-L-lysine chloromethyl ketone reacts with His 46 of trypsin resulting in inactivation of the enzyme (12-14). As in the case of the analogous α-chymotrypsin modification, it is not possible to conclusively say that the inactivation is a result of a chemical change in the histidine residue rather than a steric effect caused by the bulky modifying group.

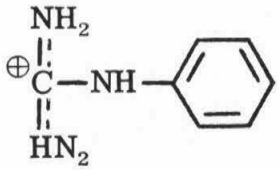
It seems that this ambiguity could be resolved by selective methylation of His 46 of trypsin. It is therefore proposed that trypsin be methylated and that the modified protein be studied and compared to methylated α-chymotrypsin.

The methylation of trypsin with methyl p-nitrobenzenesulfonate has been attempted (3), but the attempt proved unsuccessful presumably because this reagent is not strongly bound in the vicinity of the active site histidine residue. It should be possible to carry out the methylation by using the reagent methyl p-guanidinobenzene-sulfonate (1). The positive charge of this compound should direct



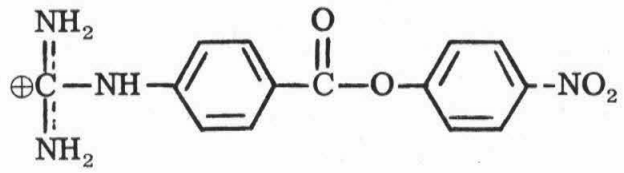
1

the sulfonic acid ester to the active site of the enzyme where reaction with His 46 can occur. The compound phenylguanidine (2) has been shown to be a strong competitive inhibitor of trypsin



2

with a kinetically determined dissociation constant of 7.25×10^{-5} M (17). p-Nitrophenyl p-guanidinobenzoate (3) is used as an active



3

site titrant for trypsin (18). It therefore seems reasonable that compound 1 should be directed to the active site of trypsin and that subsequent methylation of His 46 should occur if the active sites of trypsin and α-chymotrypsin are identical as has been indicated (7, 8, 10-12, 15).

A comparison of the rate of inactivation of trypsin by this molecule as a function of pH with the rates observed for inactivation

of α-chymotrypsin by methyl p-nitrobenzenesulfonate is of interest since a pK_a of about 7.0 has been shown to be involved in the latter reaction. A similar pK should be observed with trypsin if the two active sites are equivalent. Kinetic studies with the modified protein would determine whether or not the residual catalytic activity found in methylated α-chymotrypsin (6) is also present in methylated trypsin.

^{13}C-nmr studies on methylated trypsin could be conducted since the molecule would presumably be stable to autolysis for the long time periods required to obtain such spectra. Such studies would allow observation of conformational changes associated with changes in pH and ionic strength.

It is also proposed that methylated trypsin be prepared with a ^{13}C-enriched methyl group. Based on the results reported in Part III of this thesis, it should be possible to easily identify the enriched methyl resonance. The pH dependent chemical shift can be measured and compared to results reported for methylated α-chymotrypsin. If methylated trypsin retains any catalytic activity, it might also undergo a conformational change with ionization of His 46. This should lead to the same chemical shift observed for the enriched resonance of methylated α-chymotrypsin. Since the primary effect on the environment seen by the modifying methyl group is controlled by the active site region, a comparison of the chemical shifts observed in methylated trypsin with those

seen in methylated α-chymotrypsin would be a very sensitive measure of the similarities of the two active sites. Measurements of T_1, T_2, and nuclear Overhauser enhancements for the enriched methyl group can be related to correlation time of the methyl group. A comparison of these values after ionization with those before might yield further evidence for a conformational change upon ionization if such a change affects the mobility of the methyl group.

It is proposed that the synthesis of methyl p-guanidinobenzenesulfonate be carried out as follows. By analogy with p-aminobenzoic acid (19), paminobenzenesulfonic acid can be converted to p-thioureidobenzenesulfonic acid by reaction with ammonium thiocyanate. Subsequent reaction with methyl iodide should yield p-(S-methylisothioureido)benzenesulfonic acid which can be converted to p-guanidinobenzenesulfonic acid by ammonolysis. Conversion of the sulfonic acid to its silver salt followed by reaction with methyl iodide (21) should yield the desired compound in high yield. A similar procedure for preparing the methyl ester worked very well with p-nitrobenzenesulfonic acid. ^{13}C-enriched methyl iodide can be used for preparing the ^{13}C-enriched ester.

REFERENCES

1. T. F. Spande, B. Witkop, Y. Degani, and A. Patchornik, Adv. Protein Chem., 24, 97 (1970).
2. G. R. Stark, Adv. Protein Chem., 24, 261 (1970).
3. Y. Nakagawa and M. L. Bender, Biochemistry, 9, 259 (1970).
4. K. J. Stevenson and L. B. Smillie, J. Mol. Biol., 12, 937 (1965).
5. C. S. Wright, G. P. Hess, and D. M. Blow, J. Mol. Biol., 63, 295 (1972).
6. R. Henderson, Biochem. J., 124, 13 (1971).
7. B. S. Hartley, J. R. Brown, D. L. Kaufmann, and L. B. Smillie, Nature, 207, 1157 (1965).
8. B. S. Hartley, Phil. Trans. Roy. Soc. London B, 257, 77 (1970).
9. M. L. Bender and F. J. Kezdy, J. Amer. Chem. Soc., 86, 3704 (1964).
10. M. L. Bender, J. V. Killheffer, Jr., and F. J. Kezdy, J. Amer. Chem. Soc., 86, 5330 (1964).
11. M. L. Bender, J. V. Killheffer, Jr., and F. J. Kezdy, J. Amer. Chem. Soc., 86, 5337 (1964).
12. M. Mares-Guia and E. Shaw, Fed. Proc., 22, 528 (1963).

13. V. Tomasek, E. Severin, and F. Storm, Biochem. Biophys. Res. Commun., 20, 545 (1965).

14. E. Shaw and S. Springhorn, Biochem. Biophys. Res. Commun., 27, 391 (1967).

15. R. M. Stroud, L. M. Kay, and R. E. Dickerson, Cold Spring Harbor Symposia on Quantitative Biology, 36, 125 (1971).

16. R. L. Smith and E. Shaw, J. Biol. Chem., 244, 4704 (1969).

17. M. Mares-Guia and E. Shaw, J. Biol. Chem., 240, 1579 (1964).

18. T. Chase, Jr., and E. Shaw, Biochem. Biophys. Res. Commun., 29, 508 (1967).

19. H. C. Beyerman and J. S. Bontekoe, Rec. Trav. Chim., 72, 643 (1953).

20. N. Kornblum, W. J. Jones, and G. J. Anderson, J. Amer. Chem. Soc., 81, 4113 (1959).

PROPOSITION IV

Although the crystal structure of α-chymotrypsin (1) has indicated that His 40 is perhaps close enough to the active site of the enzyme to affect the conformation of the hydrolytic locus and specificity pocket, this residue has not been directly observed in any previously reported kinetic or binding studies. Although drastic changes in binding and catalytic activity do occur over the pH range in which His 40 would be expected to ionize, these effects have in the past been attributed to the ionization of His 57 which is at the active site of the enzyme. Due to the large effect of His 57, it is possible that any conformational changes produced by His 40 might have been masked (2, 3).

Studies at 340 nm have indicated that an ionization with a pK_a of about 6.0 is responsible for quenching the fluorescence of a tryptophan residue. Chemical modification studies indicated that the ionization probably was not associated with His 57. Since His 40 is also near a tryptophan residue, it was proposed that the observed pK_a was associated with this group (4). Competitive labeling studies at 20° have indicated that His 40 has a pK_a of about 6.7 (5). Thus, it seems reasonable that this residue ionizes in the same pH range as His 57. Careful measurements might therefore be able to detect this ionization if the effects of His 57

could somehow be minimized.

The nmr studies reported in Part II of this thesis have indicated that the ionization of His 40 might affect the binding of neutral inhibitor molecules such as N-TFA-D-tryptophan semicarbazide. Since the ionization of His 57 should not produce strong unfavorable electrostatic interactions with such neutral molecules, the effect of His 40 might well be observed if it exists. Unfortunately, the very small chemical shifts experienced by the trifluoroacetyl fluorines of such inhibitors when bound to the enzyme resulted in rather small experimentally observable chemical shifts. At pH 5.0 a total shift range of only 3 Hz was observed. At best no more than about a 10 Hz shift was seen.

Although the data strongly indicated that two ionizations were involved (and in fact could not be reasonably fit with only one ionization), the scatter in the results was too large to determine the pK_a of the two ionizations or their relative effects on Δ and K_I. Therefore, a reasonable explanation based on what is known from previous nmr studies and several assumptions was put forth to show that the experimental results could be explained quite well with two ionizations.

Since this study is the first reported observation of the effect of His 40 on the binding of inhibitor molecules, further experimentation seems justified. It is therefore proposed that nmr binding studies be carried out with the inhibitor N-TFA-D-p-fluorophenylalanine semicarbazide. This inhibitor would serve not

only to substantiate the proposal of two ionizations but would offer valuable new information since the aromatic side chain could be monitored by nmr.

Based on previous measurements with N-TFA-D-p-fluorophenylalanine, the aromatic fluorine of the semicarbazide derivative of this inhibitor should experience a downfield shift of about 100 Hz when bound to α-chymotrypsin. This is based on the reasonable assumption that the aromatic moiety of both inhibitors will bind in the hydrophobic specificity pocket. Assuming the binding constant for N-TFA-D-p-fluorophenylalanine semicarbazide vary with pH in a manner similar to that for the tryptophan analog, chemical shifts of 25 to 50 Hz should be experimentally observable. Such measurements would greatly increase the reliability of K_I and Δ values obtained, and pK's for the two ionizations involved might be determined. Although the observed chemical shift for the trifluoroacetyl fluorines would probably be small, Δ for these fluorines can be accurately determined if good K_I values are calculated from the aromatic fluorine results. The use of Fourier transform instrumentation now available will allow observation of the aromatic fluorine at very low ($\sim 10^{-4}$ M) concentrations of inhibitor molecule. Since the semicarbazide derivative will be quite soluble (probably at least 0.1 M), ratios of substrate to enzyme concentrations of from 100/1 to 0.1/1 will be observable. This range coupled with the large expected value of Δ for the aromatic fluorine should allow accurate determination of K_I and Δ for

both types of fluorines.

A comparison of Δobtained for the trifluoroacetyl fluorines of N-TFA-D-tryptophan semicarbazide with the values found for the p-fluorophenylalanine compound would yield information about the similarity of the binding locus of these fluorines. The results obtained in Part I indicate that very similar Δ values should be obtained. Likewise, the aromatic fluorine of the acid and semicarbazide should exhibit quite similar chemical shifts when bound to the enzyme. Any difference can be interpreted in terms of differences in the orientation of the aromatic side chain in the specificity pocket.

The effect of the two ionizations on the chemical shift of the aromatic fluorine can also be measured quite accurately if reliable numbers for K_I are obtained. If His 40 does affect binding affinity, then a conformational change at the hydrophobic pocket may accompany the ionization of this group. Such a change may well be reflected by a change in Δ for the aromatic fluorine of the semicarbazide inhibitor.

If it is possible to accurately measure the effect of the two ionizations on binding and the chemical shift of fluorines on two groups attached to the asymmetric carbon of the inhibitor, it should be feasible to put forth a good working model of the relative importance of the two ionizations in determining the conformation of various parts of the active site. This information along with

the previously reported results obtained from nmr studies should well characterize the pH dependent conformation of the active of α-chymotrypsin. Most importantly, it may prove possible to conclusively show that His 40 does play a role in determining the active site conformation of the enzyme.

REFERENCES

1. (a) B. W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, Nature, 214, 652 (1967).

   (b) P. B. Sigler, D. M. Blow, B. W. Matthews, and R. Henderson, J. Mol. Biol., 35, 143 (1968).

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

   (d) R. Henderson, J. Mol. Biol., 54, 341 (1970).

2. B. Zeiner and M. L. Bender, J. Amer. Chem. Soc., 86, 3669 (1969 and references cited therein.

3. J. Hein and C. Niemann, J. Amer. Chem. Soc., 84, 4495 (1962).

4. G. P. Hess, J. McConn, E. Ku, and G. McConkey, Phil. Trans. Roy. Soc. London B, 257, 89 (1970).

5. H. Kaplan, unpublished results, 1972.

PROPOSITON V

The effect of the nature and concentration of counterions in aqueous solutions of cationic and anionic surfactants which form micelles has been the subject of several papers (1-5). These studies have shown that counterions play a positive role in the formation and stabilization of micelles. The critical micelle concentration (cmc) for a surfactant is found to decrease and the number of monomer units per aggregate is found to increase in the order IO_3^-, CHO_2^-, BrO_3^-, F^-, Cl^-, NO_3^-, Br^-, ClO_3^-, SCN^-, I^-, ClO_4^- for anionic counterions (2) and in the order Li^+, Na^+, tetramethylammonium for cationic counterions (3). If only charge densities are considered, the number of units per micelle would be expected to increase for the halide ions in the order I^-, Br^-, Cl^-, F^-. This is the order of increasing ability for neutralization of the micellular charge which allows more like-charged surfactant molecules to be assembled (2). Since the observed order is just the reverse (2), it has been concluded (1) that increased hydration in the order I^-, Br^-, Cl^-, F^- decreases the ability of a counterion to cause micellation by decreasing the tendency of the counterion to form an ion pair with a charge on the surface of the micelle. Since BrO_3^- and ClO_3^- are thought to hydrate almost identically (6), the difference in their behavior (2) indicates that ion size also exerts an effect on the ability of a counterion to promote micelle

formation.

In general, increasing the concentration of a certain counterion lowers the cmc and increases the number of monomeric surfactant molecules per micelle (2, 3). In some cases, increasing counterion concentration can cause abrupt changes in the size and shape of the micelle formed by a certain surfactant molecule. For example, increasing the sodium concentration of micellular caprylate solution causes an abrupt increase in the number of counterions bound from about 60% to about 80% of the number required for complete charge neutralization (5). Still larger concentrations of sodium eventually cause a change in the shape of caprylate micelles. These changes are as abrupt as the initial transition from monomer to micelle and have been called the "second and third cmc."

A useful method for studying the interaction of charged species is the technique of ultrasonic absorption (7). Numerous studies of aqueous solutions of electrolytes have shown that ion interactions can lead to energy absorption from high frequency mechanical waves being propagated through the solution (7). The pressure differentials accompanying such waves disturb the equilibrium between ion pairs and their water of hydration and produce a relaxational energy absorption phenomenon provided there is a volume change associated with the equilibrium being perturbed. It can be shown (7) that the change in absorption, $\delta\alpha$, associated with the perturbation of an equilibrium involving ions and their water of

hydration is given by

$$\delta\alpha = \frac{AN^2}{1 + \omega^2\tau^2} = \frac{AN^2}{1 + N^2/N_R^2}$$

where $\delta\alpha$ is the difference between the absorption of solvent in the absence and presence of ion equilibria, N is the frequency of the sound wave in Hz, ω (= $2\pi N$) is the angular frequency of the sound wave, N_R is the relaxational frequency of the equilibrium under study, and τ (= $2\pi N_R)^{-1}$ is the relaxational time associated with the relaxation under study. A is a parameter which does not depend on N and has the following form:

$$A = \Delta V_o^2 \tau f(k_1, k_{-1}, c_i)$$

where ΔV_o is the volume change associated with the perturbed equilibrium and $f(k_1, k_{-1}, c_i)$ denotes some functional dependence of A on the rate constants describing the equilibrium under study and the concentration of ions involved in the equilibrium. The absorption change is therefore zero unless perturbation of an equilibrium is accompanied by a volume change. The close interaction of ionic species requires some change in the extent of their hydration and is expected to be accompanied by a volume change since the overall order and density of hydrated water differs from that of bulk water (8).

A recent study (9) has revealed that valuable information about the binding of counterions to polyelectrolytes can be obtained by using ultrasonic absorption techniques. It was shown that this method could be used to determine whether a given ion exists as a site bound complex (10) with the polyion or as a part of the "ionic atmosphere" of the polyion. In the former case (site binding), the counterion must be close enough to one or more charges on the polyion to decrease the net charge in the space around the ions sufficiently to allow partial release of electrostricted water of hydration. There are two types of site bound complexes (10) determined by whether the counterion is bound to one (ion pairing) or more that one (chelation) charge on the polyion. Both types of site binding lead to changes in volume due to displacement of water of hydration and therefore produce energy absorption from ultrasonic waves. Chelation involves a greater loss of hydration and subsequently leads to larger absorption values. Chelation requires that one or more charges on the polyion be close enough together to allow their simultaneous interaction with a single ion. Larger counterions are thus more able to participate in chelation with a polyion than are smaller ions. Counterions which are bound in the "ionic atmosphere" of a polyion are left with their hydration sphere intact (10), and such binding therefore does not lead to excess absorption over that of the solvent (9).

These results indicate that ultrasonic absorption would be a useful technique for investigating counterion binding to micelles. Such a study is proposed, and it is felt that this could prove to be a general method for investigating some of the counterion associated phenomena which have been observed with micelles (1-5).

Although some experimental (11) and theoretical work (12,13) has been done to elucidate the nature of counterion binding, there is disagreement on the extent to which counterions exhibit site binding with the micelle and to what extent the counterion is simply held in the electric field produced by the micellular unit. Ultrasonic absorption experiments should help resolve this problem since only site binding will lead to excess absorption above that of the solvent (9). In particular, a comparison of the absorption of solutions containing an anionic micelle (such as n-dodecylsulfate (3)) with Li^+ or $(CH_3)_4N^+$ as counterions should provide insight into the extent of site binding on the micelle. Since the tetramethylammonium cation has such a large radius, it will not participate in ion pair formation (10) and does not lead to excess ultrasonic absorption (9). Because of its smaller size, the lithium cation participates in ion pair formation and produces excess absorption in anionic polyelectrolyte solutions (9). The absorption of a solution containing lithium counterions relative to one containing tetramethylammonium cations is a measure of the extent to which site binding is involved

with normal inorganic ions.

Perturbation of chelation equilibria (that is binding of one counterion to more than one charged group on the polyion) produces greater energy absorption than does ion pairing (9). Based on reported absorption values for the interaction of ions in simple electrolytic solutions (7), the amount of absorption expected for interaction of a given counterion with one of the charged groups on a micelle can be estimated. Since the number of monomers per micelle and the extent of counterion binding is known for many systems (3-5, 13), it is possible to estimate the amount of absorption expected from various micelle-counterion systems if only ion pair formation is assumed to occur. If considerably larger values are measured, the occurrence of chelation will be indicated. Since there are distance requirements which must be satisfied before chelation can occur (9), information about the distance between charges on the surface of a micelle can be obtained from such studies.

The unusual effects of increasing counterion concentration which have been reported for caprylate micelles (4, 5) can also be productively studied by ultrasonic absorption. Changes in the extent of counterion binding and micelle shape produced by increasing concentrations of sodium should lead to changes in the absorption of such solutions. Changes in absorption associated with transitions between the three forms of caprylate micelles can perhaps be related to the type of counterion binding (ionic atmosphere, ion

pairing, or chelation) which exists in each. If differences are found, correlation to the positive role which the counterion plays in producing the transitions can be attempted.

REFERENCES

1. K. J. Mysels and L. H. Princen, J. Phys. Chem., 63, 1969 (1959).
2. E. W. Anacker and H. M. Ghose, J. Phys. Chem., 67, 1713 (1963).
3. M. J. Schick, J. Phys. Chem., 68, 3585 (1964).
4. P. Ekwall and P. Stenius, Acta Chem. Scand., 21, 1767 (1967).
5. P. Stenius and P. Ekwall, Acta Chem. Scand., 21, 1643 (1967).
6. O. K. Rice, "Electronic Structure and Chemical Bonding," McGraw-Hill, New York, 1940, p. 440.
7. J. Stuehi and E. Yeager, "Physical Acoustics," Vol IIA, W. P. Mason, Ed., Academic Press, New York, 1965, p. 351.
8. H. Frank and W. Wen, Discuss. Faraday Soc., 24, 133 (1957).
9. C. Tondre and R. Zana, J. Phys. Chem., 75, 3367 (1971).
10. U. P. Strauss and Y. P. Leung, J. Amer. Chem. Soc., 87, 1476 (1965).
11. J. Clifford and B. A. Pethica, Trans. Faraday Soc., 60, 216 (1964).
12. S. Lifson and J. L. Jackson, J. Chem. Phys., 36, 2410 (1962).

13. G. M. Bell and A. J. Dunning, Trans. Faraday Soc., 66, 500 (1970).