NUCLEAR MAGNETIC RESONANCE STUDIES OF THE CATALYTIC MECHANISM OF PROTEOLYTIC ENZYMES

I. IONIZATION BEHAVIOR OF THE HISTIDINE RESIDUE IN THE CATALYTIC TRIAD OF α-LYTIC PROTEASE--IMPLICATIONS FOR THE CATALYTIC MECHANISM OF SERINE PROTEASES

II. IONIZATION BEHAVIOR OF ENZYMIC AND INHIBITOR GROUPS IN THE TETRAHEDRAL ADDUCT BETWEEN α-LYTIC PROTEASE AND A PEPTIDE ALDEHYDE

III. KINETICS OF PEPSIN-CATALYZED HYDROLYSIS OF N-TRIFLUOROACETYL AMINO ACIDS

Thesis by

Michael W. Hunkapiller

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1974

(Submitted April 10, 1974)

ACKNOWLEDGMENTS

I gratefully acknowledge the advice and guidance of my research supervisor, Professor John H. Richards, whose scientific insight has broadened my own. His encouragement and granting of considerable freedom to follow my own inclinations have made my graduate studies thoroughly enjoyable.

I am also thankful to my colleagues in Dr. Richards' research group whose friendship and interest have added immensely to my experiences at Caltech. I am particularly thankful to Dr. Stephen Smallcombe, both for his collaboration on part of the work reported in this thesis and for his invaluable technical assistance in nmr spectrometer operation.

Professors Michael Raftery and Robert Stroud provided many helpful discussions which have contributed much to my research efforts. Professor Donald Whitaker and Mr. Camille Roy provided considerable assistance in imparting to me their painstakingly acquired knowledge of the culturing of Myxobacter 495 and the purification of α-lytic protease.

Finally, I wish to acknowledge the financial support of the National Institutes of Health, in the form of a traineeship, and the California Institute of Technology, in the form of a supplemental research assistantship.

TABLE OF CONTENTS

PART I.

PART II.

PROPOSITIONS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

PART I

Selective ^{13}C enrichment of C-2 of the single histidine residue of the serine protease α-lytic protease has allowed direct study of the Asp-His-Ser catalytic triad by magnetic resonance techniques. Both the chemical shift of C-2 and the coupling between C-2 and its directly bonded hydrogen have been observed as a function of pH. The results indicate that only below pH 3.3 does the histidine imidazole ring become protonated and only above pH 6.7 does the aspartic acid residue lose a proton to generate a carboxylate anion. Over the pH range 3.3-6.7, the catalytic triad contains a neutral aspartic acid and neutral histidine residue--not the ionized forms hitherto assumed. This interpretation of the ionization characteristics of the catalytic triad leads to a proposed catalytic mechanism which avoids any requirement for unfavorable charge separation in the transition state. The histidine residue plays two roles: (i) it provides insulation between water and the buried carboxylate anion, thus ensuring the latter a hydrophobic environment, and (ii) it provides a relay for net proton transfer from serine hydroxyl to carboxylate anion. The aspartate anion acts as the ultimate base which holds a proton during catalysis. An anionic, rather than a neutral, base both avoids the necessity of charge separation and, by giving the catalytic locus an overall negative charge, assists preferential expulsion of product relative to substrate from the

active site. Relaxation measurements (T_1, T_2, and nuclear Overhauser enhancement) indicate that, over the pH range of enzymic activity, the histidine residue is held rigidly within the protein.

PART II

Magnetic resonance techniques have been used to study ionization behavior of enzymic and inhibitor moieties in the tetrahedral adduct (hemiacetal) formed between α-lytic protease and a peptide aldehyde, N-Ac-L-Ala-L-Pro-L-alaninal. Chemical shift, coupling constant, and relaxation measurements of ^{13}C-enriched C-2 of the catalytic histidine residue indicate that at pH $>$ 6.25 the complex contains neutral aspartic acid, neutral histidine, and negatively charged inhibitor. Below pH 6.25, both the inhibitor oxyanion and the histidine become protonated in a cooperative ionization process which forces the histidine from its rigidly-held position as a member of the catalytic triad into a solution-like environment. This behavior by a complex thought to resemble the transition state for serine protease-catalyzed hydrolysis of ester and amide substrates supports proposals for a catalytic mechanism which involves a minimum of charge separation in the transition state. It also attests to the power of the intricate hydrogen-bonding network (previously observed in x-ray diffraction studies) to stabilize an otherwise high-energy intermediate and thereby achieve catalysis.

PART III

The acidic gastric proteases, pepsin and gastricsin, have been found to catalyze hydrolysis of several N-trifluoroacetyl-L-amino acids with aromatic side chains. This catalytic activity is lost when they are chemically modified so as to inactivate their proteolytic activity. Magnetic resonance techniques were used to follow the porcine pepsin-catalyzed hydrolysis of N-trifluoroacetyl-L-phenylalanine in the pH range 1.7-5.4. This study revealed that non-productive binding strongly influences the observed kinetic parameters and that productive enzyme-substrate binding requires an anionic substrate (pK_a 2.8) and an undissociated group (pK_a 3.7) on the free enzyme. Binding is also affected by ionization of a group on the free enzyme with a pK_a near 4.8. A kinetic isotope effect ($k_{H_2O}/k_{D_2O} = 3$) has been observed for the reaction which suggests that proton transfer is involved in the rate-limiting step. A new mechanism--one involving three carboxylic acid groups on the enzyme and an intermediate in which the amino moiety is non-covalently held by the enzyme after release of the acyl moiety--is proposed to explain these and previous observations on catalysis by pepsin.

ABSTRACTS OF THE PROPOSITIONS

PROPOSITION I

A carbon magnetic resonance study of the trypsin/soybean trypsin inhibitor complex with active lysine inhibitor residue enriched in ^{13}C at carbonyl carbon is proposed.

PROPOSITION II

A study of soluble mediators of T cell-B cell interaction in cultures highly enriched in dinitrophenyl-specific lymphocytes is proposed.

PROPOSITION III

A study of the effects of polyanions on the capping phenomena in the phosphorylcholine-sensitive B lymphocyte clone from BALB/c mice is proposed.

PROPOSITION IV

A study of the chemical modification of myeloma proteins using affinity labels with alkylisourea, aryl cyanate, and aryl sulfonate reacting groups is proposed.

PROPOSITION V

An ^{18}O isotopic tracer study of the pepsin-catalyzed hydrolysis of peptides and sulfite esters is proposed.

PART I

Ionization Behavior of the Histidine Residue in the Catalytic Triad of α-Lytic Protease-- Implications for the Catalytic Mechanism of Serine Proteases

INTRODUCTION

From the first discovery (1-2) of a buried carboxylate anion as part of a precisely arranged triad of amino acid residues (consisting of the carboxylate anion of aspartic acid, the imidazole ring of histidine, and the hydroxyl group of serine), the detailed nature of the charge relays and accompanying proton transfers that occur during catalysis by serine proteases has been the focus of considerable interest. This array of three residues occurs in many enzymes which show clear homology and which most likely were derived from a single ancestral protein by a process of divergent evolution. (Members of this family include, for example, chymotrypsin, trypsin, elastase, thrombin, and α-lytic protease (3).) Even in enzymes which possess no other apparent homology, and are therefore probably unrelated in terms of their ultimate origins (such as the chymotrypsin family on the one hand and the subtilisin family on the other), the striking presence of this identical catalytic triad provides evolutionary testimony to its unique catalytic efficacy.

Ambiguity concerning the true microscopic ionization behavior of the members of this triad has, however, hindered realization of a completely satisfactory account of the catalytic act. It has been well established, though, that the initial phase of catalysis involves nucleophilic attack on the carbonyl carbon of the substrate by the serine hydroxyl oxygen to yield a tetrahedral adduct (4-5). This attack is assisted by transfer of the serine hydroxyl hydrogen to

the neighboring N^{τ} nitrogen of the histidine residue. This hydrogen is subsequently transferred to the leaving group during breakdown of the tetrahedral intermediate to yield an acyl enzyme and one of the products (amine or alcohol) of the reaction. Replacement of the first product by a water molecule can then be followed by a reverse of the above sequence to regenerate active enzyme and liberate the free acid product.

This simple reaction scheme points to, but does not satisfactorily explain, the apparent high nucleophilicity of the serine hydroxyl group. Robertus et al. (6) have suggested that much of the reactivity arises from stabilization of the transition state (which probably resembles the tetrahedral adduct) by formation of hydrogen bonds between backbone NH groups of the enzyme and the oxyanion of the substrate in the tetrahedral intermediate. However, if the enzyme functions by delocalizing the transient negative charge that forms on the substrate, one would also expect it to have some means to minimize the formation of positive charge on the histidine imidazole ring. Otherwise, the transition state would contain significantly greater charge separation than that which exists in the Michaelis-Menten complex. Such charge separation in an environment from which solvent water is virtually excluded (7) should destabilize the transition state and hence decrease the catalytic efficiency of the protein. The enzyme can minimize the charge separation, however, by positioning a negatively charged base which can accept a proton from the imidazole N^{π} nitrogen (i.e., the one opposite the N^{τ} nitrogen

which accepts the proton from the serine hydroxyl group). Blow et al. (2) were the first to discover the presence of the buried aspartic acid carboxylate side chain next to the imidazole ring, but, although they and others have postulated that proton transfer from the imidazole to the carboxylate anion is critical for catalysis, they have continually overlooked the fact that such a transfer will make a significant contribution to the catalytic process only if the carboxylate anion is a stronger base than is the neutral imidazole group.

Numerous studies (8-11) using several techniques have led to the conclusion, however, that the pK_a of the histidine side chain is around 6.7 while that of the aspartic acid group is < 4. If these assignments are correct, then the proton transfer discussed above will not be a significant means of stabilizing the transition state since the imidazole, being a stronger base than is the carboxylate, will retain its N^{π} proton even after it receives another proton at the N^{τ} position from the serine hydroxyl. Therefore, because of the importance of correct assignment of pK_a's to an understanding of the catalytic process and because of the ambiguity inherent in previous attempts to make the assignments, we began a series of experiments involving nuclear magnetic resonance (nmr) spectroscopy in order to determine the true ionization behavior of the relevant groups.

Nmr is particularly well suited for these studies because it provides information about the characteristics and environment of individual nuclei of macromolecules in solution, and chemists

have recently made increasingly fruitful applications of nmr to the study of problems of biological importance. Meadows et al. (12-13) used proton magnetic resonance to study ionization of the histidine residues in ribonuclease and the effect of inhibitor binding on the two catalytic histidine groups of the enzyme. Wuthrich et al. (14) and Ogawa and Shulman (15) observed those proton resonances of hemoglobin which are shifted by the ring currents of the porphyrin ring and thereby studied the effects on protein conformation of ligand binding to the heme groups. However, the relatively narrow range of proton chemical shifts and the resultant lack of dispersion usually require exchange with deuterium oxide to remove amide protons from the backbone peptide bonds and expose signals from other protons of interest. Groups containing fluorine nuclei have been covalently attached to proteins. Subsequent study by ^{19}F magnetic resonance spectroscopy has been reported, for example, for hemoglobin and ribonuclease by Raftery et al. (16) and Huestis and Raftery (17). Though, in the cases mentioned, appropriate control studies showed only insignificant change in protein function as a result of attachment of label, such techniques may potentially cause unknown alterations in protein conformations.

Carbon magnetic resonance (cmr) offers great potential for studies of proteins because of the wide chemical shift range of ^{13}C (18). However, the low natural abundance and relatively low sensitivity of ^{13}C usually require unattainably long signal accumulation times in order to observe single resonances from proteins

even though recent increases in probe sizes have dramatically improved achievable signal-to-noise levels for dilute solutions of proteins (19). Nevertheless, natural abundance cmr studies have been productively applied to protein studies (20).

Specific ^{13}C enrichment of functionally important nuclei has great promise and is the technique used in the work reported in this paper. In this case, the low natural abundance ^{13}C background becomes an advantage as it usually allows the unambiguous observation of the enriched site(s). Moreover, replacement of ^{12}C by ^{13}C leads to only negligible changes in the structure or action of a protein. Chaiken et al. (21) synthesized the S-peptide of ribonuclease with uniformly ^{13}C-enriched phenylalanine and studied the complex between this S-peptide and the remaining fragment of the enzyme. (Though not the native enzyme, the complex possesses 15% catalytic activity.) Browne et al. (22) enriched the C-2 carbon of the imidazole ring in each of the four histidine residues of the α subunit of tryptophan synthetase from E. coli but were unable to resolve the overlapping signals from the four ^{13}C nuclei in the four histidine residues.

The study reported in this paper represents the first specific ^{13}C enrichment of a single carbon atom in a native protein together with a study of the magnetic resonance parameters of this carbon (23-24). The C-2[1] carbon of the imidazole ring of the single histidine residue of the bacterial serine protease

---

[1]This carbon can also be designated the $C^{\epsilon 1}$ of histidine.

α-lytic protease from Myxobacter 495 (25) was enriched in ^{13}C by growing the bacteria in a medium containing L-[2-^{13}C]histidine.

We chose α-lytic protease as the subject of this study for four reasons. (i) α-Lytic protease has a bacterial origin. This facilitates biosynthetic incorporation of labeled amino acids. (ii) α-Lytic protease possesses only a single histidine residue. In contrast, chymotrypsin has two, trypsin has three, and elastase and subtilisin have six histidine residues. Therefore, the behavior of the single histidine of the catalytic triad of α-lytic protease can be observed without possibly confusing complications caused by the presence of other histidine residues. (iii) α-Lytic protease exhibits remarkable stability toward denaturation and autolysis even in concentrated solutions and under conditions of pH and temperature where enzymic activity is maximal. This stability is unparalleled by that of other well-studied proteolytic enzymes and is essential for cmr experiments because of the time required for signal accumulation. (iv) The enzyme is a homolog of mammalian serine proteases, a family of proteolytic enzymes each member of which possesses a catalytic triad of side-chain groups consisting of the carboxyl group of an aspartic acid (residue 102 in the amino acid sequence of chymotrypsinogen, the imidazole ring of a histidine (residue 57 in chymotrypsinogen), and the hydroxyl group of a serine (residue 195 in chymotrypsinogen). As such, α-lytic protease serves as a paradigm for this great group of enzymes.

The concept of the close relationship of α-lytic protease to the mammalian serine proteases finds support in the extensive sequence homology between α-lytic protease on the one hand and chymotrypsin, trypsin, and elastase on the other (26). In fact, McLachlan and Shotton (27) were able to construct a tentative three-dimensional model of α-lytic protease based on the sequence homology between α-lytic protease and elastase and the known three-dimensional structure of elastase. In so doing, they formed a catalytic core which displayed the same relative orientation of the three components of the catalytic triad that characterizes the mammalian enzymes. The sequence homology which α-lytic protease shares with the mammalian proteases distinguishes it from another bacterial enzyme, subtilisin, which, though having a structurally and functionally identical catalytic locus (6) lacks any discernible sequence homology with the mammalian enzymes.

In specificity, α-lytic protease mimics elastase. Both possess elastolytic and bacteriolytic activity, and both preferentially hydrolyze amide bonds whose carbonyl groups belong to alanine or valine (28). Also, both enzymes bind neutral specific substrates independently of pH over the range pH 5-10 (28). In contrast, substrate binding to trypsin and chymotrypsin decreases drastically at higher pH with an apparent pK_a of 8.5-9.5 (29-30). This decrease in binding at high pH in chymotrypsin reflects deprotonation of the N-terminal amino group of Ile 16 which forms a salt bridge with Asp 194; disruption of this

Ile-Asp salt bridge leads in turn to destruction of the specificity binding picket. Elastase and α-lytic protease, on the other hand, show no such dependence of binding on ionization of an amino terminus presumably because, in these enzymes, the region analogous to the specificity pocket in trypsin and chymotrypsin is filled with side-chain groups of the enzymes themselves. As a result of this absence of a specificity pocket in elastase and α-lytic protease, binding is relatively weak over the entire pH range, and this binding is, therefore, not significantly affected by deprotonation of the N-terminal amino group.

α-Lytic protease also reacts with irreversible inhibitors as do other serine proteases. Thus, the active serine residue of α-lytic protease reacts irreversibly with diisopropyl fluorophosphate, a typical inhibitor of serine proteases, to yield an inactive enzyme (31). In contrast to the active site histidine residues of chymotrypsin and trypsin (which react with the chloromethyl ketone derivatives of tosyl-L-phenylalanine and tosyl-L-lysine, respectively), those of elastase and α-lytic protease fail to react with tosyl-L-alanine chloromethyl ketone (28). However, Thompson and Blout (32) have recently reported that the active site histidine of elastase does react with Ac-L-Pro-L-Ala-L-Pro-L-AlaCH_2Cl.

α-Lytic protease exhibits kinetic properties remarkably similar to those of mammalian enzymes (25, 33). These enzymes all manifest a similar deuterium isotope effect. In deuterium oxide, the value of k_{cat}/K_M is only one-third that observed in

aqueous solution for enzyme-catalyzed hydrolysis of appropriate N-acetylamino acid esters. (For ester hydrolysis catalyzed by these enzymes, deacylation is rate limiting.) Moreover, in all these cases, k_{cat}/K_M depends upon an ionization of a group of the free enzyme with an apparent pK_a of 6.7.

Based on these striking similarities in sequence homology, specificity, reactivity toward irreversible inhibitors, and catalytic behavior, we conclude that α-lytic protease is a representative member of the family of serine proteases. Findings about its mechanism of action will, therefore, be generally applicable to the other serine proteases.

Myxobacter 495 also produces, in addition to α-lytic protease, an enzyme containing zinc β-lytic protease, an endopeptidase possessing eight histidine residues. This provides another protein which becomes enriched with ^{13}C during growth of the bacteria on a medium containing L-[2-^{13}C]histidine and allows study of the C-2 of histidine residues in a random-coil polypeptide when the protein is denatured.

EXPERIMENTAL SECTION

Materials. L-[2-^{13}C]Histidine (lot CFA 137-56, 50 Ci/mol) was purchased from Amersham-Searle. Potassium [91.4% ^{13}C]-cyanide (lot 8x43) was obtained from Prochem. Amberlite IR-45 (20-50 mesh), IR-120 (20-50 mesh), and CG-50 (200-400 mesh) were purchased from Mallinckrodt, Bio-Rad AG11A8 (50-100 mesh) was obtained from Bio-Rad, and Rexyn I-300 (10-50 mesh) was obtained from Fisher. Monosodium glutamate, sucrose, and salt-free casein hydrolysate were purchased from Nutritional Biochemicals.

N-Benzoyl-L-alanine Methyl Ester. L-Alanine (8.9 g, 0.1 mol) was suspended in 250 ml of absolute methanol, and dry hydrogen chloride was bubbled through the suspension until all of the alanine dissolved. The solvent was removed by rotary evaporation, and the residue (syrup) was dried in vacuo (0.1 Torr) over NaOH pellets for 24 hr. The alanine methyl ester hydrochloride was then dissolved in 50 ml of water, and the solution was titrated to pH 8 with 2 N NaOH. Benzoyl chloride (24.9 g, 0.1 mol) was added dropwise over a period of 30 min to the vigorously stirred solution while the pH was maintained at pH 8 by addition of 1 N NaOH. The aqueous solution was extracted twice with 100 ml portions of ethyl acetate, and the ethyl acetate solution was washed with 5% aqueous Na_2CO_3, 1 N HCl, and water. It was then dried over Na_2SO_4, filtered, and rotary

evaporated to yield a white solid. Two crystallizations from ethyl acetate/n-hexane gave 12.5 g (60%) of N-benzoyl-L-alanine methyl ester (mp 58.5-59°; lit. mp 58-59° [25]).

L-[2-^{13}C]Histidine. The procedures used for synthesis of this compound were those (with slight modifications) described by Ashley and Harrington (34) and Heath et al. (35).

(i) L-Histidine Methyl Ester Dihydrochloride. L-Histidine·HCl·H_2O (200 g, 0.95 mol) was suspended in 5 l. of absolute methanol. The mixture was heated on a steam bath, and dry hydrogen chloride was bubbled through the suspension until all of the histidine dissolved. The solvent was removed by rotary evaporation, and the white residue was washed on a Buchner funnel with 200 ml of cold methanol. The solid was then dried in vacuo (0.1 Torr) over NaOH pellets overnight. Yield: 212 g (92%).

(ii) Methyl 2,4,5-Tribenzamidopent-4-enoate. L-Histidine methyl ester dihydrochloride (212 g, 0.88 mol) was dissolved in 6 l. of water and cooled in an ice bath. Benzoyl chloride (960 ml, 7.92 mol) in 4 l. of benzene was added with vigorous mechanical stirring. Solid Na_2CO_3 (1026 g, 9.68 mol) was added in small portions over 2 hr, and the cold mixture was stirred vigorously for an additional 6 hr. Two liters of water were added to dissolve the remaining solid Na_2CO_3, and the phases were separated. The aqueous phase was extracted with 1 l. of benzene. The combined benzene solutions were dried over Na_2SO_4

and concentrated by rotary evaporation to remove most of the benzene. Two liters of ether and 2 l. of n-hexane were added, and the precipitated, resinous product was allowed to settle out at 4° overnight. The solvent was decanted, and the residue was washed with 2 l. of ether. This crude product was suspended in 2 l. of methanol and heated on a steam bath. It first dissolved, but it rapidly crystallized when the solution began to boil. Two liters of ether were added, and the solution was left at 4° overnight to complete crystallization. The product was filtered off, washed with 2 l. of ether, and dried in vacuo (0.1 Torr). Yield: 320 g (62%).

(iii) Methyl 2,5-Dibenzamido-4-ketopentanoate. Methyl 2,4,5-tribenzamidopent-4-enoate (320 g, 0.68 mol) was suspended in 4 l. of methanol, and the solution was heated to boiling on a steam bath. Concentrated HCl (900 ml) was added, and boiling was continued until all of the solid dissolved (6 hr). Methanol was added occasionally to replace that which boiled off. The solution was concentrated by rotary evaporation to remove most of the solvent. Five liters of distilled water were added, and the product was allowed to settle out at 4° overnight. It was collected on a Buchner funnel and washed with 20% methanol, water, and ether. It was dried in vacuo (0.1 Torr) over NaOH pellets overnight. Yield: 245 g (59%).

(iv) 2,5-Diamino-4-ketopentanoic Acid Dihydrochloride. Methyl 2,5-dibenzamido-4-ketopentanoate (245 g, 0.39 mol) was suspended in 600 ml of water and 700 ml of conc. HCl.

The mixture was heated at reflux on an oil bath until 1 hr after all of the solid dissolved (6 hr). The solution was chilled in an ice bath, the precipitated benzoic acid was removed by filtration, and the solvent was removed by rotary evaporation. The residue was dissolved in 100 ml of water, and the solution was extracted several times with ether to remove the last traces of benzoic acid. The aqueous solution was treated with charcoal, filtered, and freeze dried to give a light brown, resinous product. Yield: 83 g (97%).

(v) Potassium [^{13}C]Thiocyanate. A mixture of potassium [91.4% ^{13}C] cyanide (10 g, 0.15 mol), sulfur (5.4 g, 0.17 mol), and 100 ml of acetone was refluxed for 1 hour. The solution was drawn off, the residue was mixed with sulfur (2.7 g, 0.08 mol) and 50 ml of acetone, and the mixture was refluxed another hr. The combined acetone solutions were filtered and then rotary evaporated at room temperature. The solid residue was dissolved in a few ml of water and filtered. Yield: quantitative.

(vi) L-2-Thiol-[2-^{13}C]histidine. 2,5-Diamino-4-ketopentanoic acid dihydrochloride (35 g, 0.16 mol) dissolved in 75 ml of water was heated on a steam bath. Potassium [^{13}C]thiocyanate (14.8 g, 0.15 mol) in 15 ml of water was added dropwise over a period of 1 hr. The solution was heated for an additional hour, concentrated to 50 ml on a rotary evaporator, treated with charcoal, and filtered. It was then titrated to pH 6 by addition of solid Na_2CO_3 and left at 4° for 3 days to crystallize. The product was collected on a Buchner funnel, washed with a few ml of 50%

methanol, and dried in vacuo (0.1 Torr). Yield: 14.1 g (50%).

(vii) L-[2-^{13}C]Histidine. L-2-Thiol-[2-^{13}C]histidine (14.1 g, 0.075 mol) was added to a solution of ferric sulfate (280 g, 0.5 mol) in 1.5 l. of water, and the solution was heated on a steam bath for 1 hr. Water (1.5 l.) was added, the solution was heated to boiling, and $Ba(OH)_2$ (260 g, 1.5 mol) was added to the stirred solution. The mixture was filtered and washed with 1 l. of boiling water. The combined filtrates were titrated to pH 6.0 with 10% H_2SO_4, and the precipitated $BaSO_4$ was filtered off. The solution was concentrated to 50 ml on a rotary evaporator, treated with charcoal, and filtered. The clear solution was then freeze dried to give a pale brown solid. This was dissolved in a few ml of water and passed down a column (2.5 x 60 cm) of Bio-Rad AG 11A8 ion retardation resin with water as eluant. The fractions containing histidine were rotary evaporated, and the residue was dissolved in a few ml of water, treated with charcoal, and filtered. Four volumes of hot methanol were added, the solution was seeded with a few crystals of L-histidine, and the product was allowed to crystallize at 4° for 2 days. It was then collected on a Buchner funnel, washed with methanol, and dried in vacuo (0.1 Torr). Both proton and carbon magnetic resonance spectra were consistent with L-[2-91% ^{13}C]histidine. Yield: 5.8 g (50%).

Growth of Myxobacter 495. The procedure described by Whitaker (25) for culturing Myxobacter 495 was modified to increase enzyme production and decrease dilution of L-[2-^{13}C]-histidine by histidine in the casein hydrolysate used as nutrient. The casamino acid concentration of the culture medium was reduced from 20 to 0.5 g/l., and monosodium glutamate (20 g/l.) served as the new nitrogen source. The inorganic salt content of the medium consisted of $K_2HPO_4 \cdot 3H_2O$ (2 g/l.), NaCl (2 g/l.), $MgSO_4 \cdot 7H_2O$ (2 g/l.), ferric sulfate (25 mg/l.), $ZnSO_4 \cdot 7H_2O$ (25 mg/l.), and $MnSO_4 \cdot H_2O$ (3 mg/l.). Sucrose (10 g/l.) replaced the glucose used with the casamino acid medium. Solutions of the amino acids and inorganic salts in tap water were mixed in 2800 ml Fernbach culture flasks fitted with cotton plugs and autoclaved at 121° for 20 min. Sucrose solutions in distilled water were autoclaved separately and added to the culture flasks before inoculation.

Freeze-dried cultures of Myxobacter 495 were stored at -20°. They were transferred to flasks containing 50 ml of culture medium and incubated at 27° for 48 hr on a rotary shaker describing a circle of 1 inch radius at 100 rpm. These 50-ml cultures were used as inocula for the Fernbach flasks containing 1 l. of medium. The Fernbach cultures were then incubated with shaking for 84 hr. For preparation of ^{13}C- and ^{14}C-enriched enzyme, 1 ml of a sterile 5% solution of labeled histidine was added to each Fernbach flask at 0, 20, 40, and 60 hr after inoculation.

Purification of α- and β-Lytic Protease. The procedure for enzyme purification described by Whitaker (25) was modified to increase the ease and efficiency of isolating enzyme from a small volume of culture medium. All purification procedures were carried out in a cold room at 4°.

Six liters of 84-hr culture were centrifuged (8000 rpm, 30 min) to remove bacteria. Sixty milliliters (settled volume) of Amberlite IR-45 (acetate form) and 50 ml of Amberlite IR-120 (ammonium form) were added to the supernatant, the mixture was stirred for 2 hr, and the solution was filtered through nylon mesh to remove the mixed-bed ion exchange resins. The filtrate was titrated to pH 4.95 by addition of 20% acetic acid, and 100 ml of Amberlite CG-50 equilibrated with 0.10 M sodium acetate buffer (pH 4.95) was added to it. The mixture was stirred for 24 hr, and the resin was collected by filtration on a coarse-frit, sintered-glass funnel. The resin was washed on the funnel with 2 l. of pH 4.95 acetate buffer and transferred to a beaker containing 100 ml of 0.033 M sodium citrate buffer (pH 6.25). It was then titrated to pH 6.25 by slow addition (with stirring) of 0.5 N NaOH. The resin was washed on the funnel with 1 l. of pH 6.25 citrate buffer and added to a 2 cm diameter chromatography column previously packed with 100 ml of Amberlite CG-50 equilibrated with pH 6.25 citrate buffer. The column was washed successively with 250 ml of pH 6.25 citrate buffer, 1 l. of 0.16 M sodium citrate buffer (pH 5.88), and 1 l. of 0.27 M sodium citrate buffer (pH 6.20). β-Lytic protease and α-lytic protease were eluted by the pH 5.88

buffer and pH 6.20 buffer, respectively. The two enzyme fractions were then titrated to pH 4.95, adsorbed onto 20 ml of Amberlite CG-50, and chromatographed as before. The rechromatographed fractions were exhaustively dialyzed against distilled water and lyophilized. Typical yields of freeze-dried enzyme were 600 mg of α-lytic protease and 125 mg of β-lytic protease. Each enzyme gave a single, sharp band on polyacrylamide disc gel electrophoresis at pH 8.3 (36). The α-lytic protease was assayed for esterase activity against N-benzoyl-L-alanine methyl ester (0.01 M in 0.10 M KCl at 25°) and showed a value of k_{cat}/K_M = 740 $M^{-1}sec^{-1}$ (based on enzyme $E^{1\%}_{278}$ = 8.9) compared to the previously reported value of 723 $M^{-1}sec^{-1}$ (25).

L-[2-^{14}C]Histidine Incorporation into α-Lytic Protease. Solutions of enzyme (20 mg/ml) produced in a medium supplemented by 200 mg of L-[2-^{14}C]histidine (24.6 mCi/mol; 5.42×10^7 dpm/mmol) were added to Scintisol Complete scintillation fluid and counted in a Packard TriCarb liquid scintillation spectrometer, Model 3375. The counts were corrected for background and counting efficiency and were used to calculate the specific radioactivity of the enzyme (2.1×10^7 dpm/mmol).

Samples of enzyme (5 mg) were dissolved in 1 ml of constant-boiling hydrochloric acid, degassed, sealed under vacuum, and heated at 110° for 24 hr. The hydrolyzed samples were diluted with water and dried in vacuo over potassium hydroxide pellets to remove excess acid. The hydrolysate was then subjected to high

voltage paper electrophoresis at pH 6.5. The amino acids were separated into five bands: aspartic acid, glutamic acid, neutral amino acids, histidine, and lysine + arginine. The bands were eluted with 1% acetic acid, and the amino acid concentration and specific radioactivity of each were determined. Only the histidine band showed significant radioactivity (2.2×10^7 dpm/mmol); all other bands had less than 250 dpm/mmol.

Nmr Spectra. Nmr spectra were recorded on a Varian XL-100-15 nmr spectrometer operating at 25.17 MHz in the Fourier transform mode. Data accumulation and Fourier transformation of the free induction decay were carried out by a Varian 620/i computer interfaced to the spectrometer. In general, spectra were taken at a 2000 Hz sweep width using a 90° pulse (150 or 90 μsec) and an acquisition time of 0.15 sec. A sensitivity enhancement of 0.04-0.10 sec was applied to the free induction decay before the Fourier transformation was carried out. Proton noise decoupling was used in all cases except determination of coupling constants and nuclear Overhauser enhancements.

Study of Titration of Imidazole Groups by Cmr. α-Lytic protease solutions (5-6 mM) were made up in 0.2 M KCl and transferred to a 12 mm o.d. nmr tube fitted with vortex plugs and a D_2O locking capillary. The pH was measured with a Radiometer Model PM26 pH meter equipped with a Radiometer Model GK2322C combination electrode that could be inserted into the nmr tube. The pH was checked before and after each spectrum was recorded, and the two values

always agreed to within ±0.05 pH. The pH was changed by adding 1 N KOH or 1 N HCl. Enzyme activity against N-benzoyl-L-alanine methyl ester was unchanged (±5%) during recording of the spectra.

The low water solubility of native β-lytic protease precluded cmr studies of the active enzyme. However, the protein was denatured and solubilized by suspending it in 1 N KOH for 24 hr at room temperature. The solution was titrated to pH 8 with 2 N HCl, dialyzed exhaustively against distilled water, and freeze dried. The protein (95 mg) was then dissolved in 1.5 ml of 0.2 M KCl, and the cmr spectrum of this solution was recorded at several values of pH over the range pH 4.6-8.2 (pH adjusted with 1 N KOH or 1 N HCl).

T_1 Measurements. The spin-lattice relaxation time, T_1, was determined by the progressive saturation method (37). T_1 values were calculated by an iterative computer fit of the relative peak intensities, S_a/S_b, and the pulse intervals, a and b, according to the formula

$$S_a/S_b = (1 - e^{-a/T_1})/(1 - e^{-b/T_1})$$

Nuclear Overhauser Effect Measurements. NOE values (38) were measured from the relative areas of the proton coupled and decoupled spectra. A 90° pulse and a pulse interval of ~4 T_1 were used. Spectra were plotted using the absolute intensity scale of the Varian 16K Fourier transform program. Peak areas were determined by computer integration or by Xeroxing the spectra, cutting out the peaks, and weighing them.

RESULTS

Incorporation of Histidine into α-Lytic Protease. As a preliminary test for (i)dilution of added histidine by endogenous sources and (ii)significant operation of metabolic pathways which transfer label originally present in C-2 of added histidine into other amino acids, we studied the incorporation of L-[2-^{14}C]-histidine into α-lytic protease. Addition of labeled histidine to the culture medium of wild Myxobacter 495 in several aliquots at intervals during the growth cycle gave reasonable incorporation of label without significant transfer to other amino acid residues. Specifically, α-lytic protease isolated from a medium supplemented by 200 mg of L-[2-^{14}C]histidine (5.42×10^7 dpm/mmol; added in four equal aliquots 0, 20, 40, and 60 hr after inoculation) had a specific activity of 2.08×10^7 dpm/mmol (dilution to 40% of the original activity). The resulting protein was hydrolyzed with hydrochloric acid, and the hydrolysate was separated into different amino acid fractions by paper electrophoresis. Appreciable radioactivity appeared only in the histidine band (2.2×10^7 dpm/mmol); the other bands all showed less than 250 dpm/mmol. We accordingly conclude that no label originally present at C-2 of the added histidine is transferred to any other amino acid residue (at least not without very extensive dilution) by the metabolic activities of the microorganism.

In the experiments involving addition of ^{13}C-labeled histidine, label in the isolated α-lytic protease was typically diluted only to

about 55% of its original concentration (91% enriched L-[2-^{13}C]-histidine was added, and the isolated α-lytic protease was judged to have a ^{13}C enrichment in C-2 of the histidine residue of about 50%).

Magnetic Resonance Parameters. Figure 1 shows two proton noise-decoupled natural abundance cmr spectra in the low-field region of α-lytic protease at pH 5.8 and 8.1. The resonances can be assigned from the data of Horsley et al. (18). The signals from the ring carbons of the six phenylalanine and four tyrosine residues of α-lytic protease are evident 129 ppm downfield from external tetramethylsilane (TMS). The guanidinium carbons of the 12 arginine residues produce the sharp signal at -157.25 ppm. The broad signal centered at -173 ppm represents the carbonyl carbons of the peptide backbone.

Figure 2 shows three comparable cmr spectra (at pH 5.7, 6.6, and 8.5) for α-lytic protease containing histidine enriched with ^{13}C at C-2 (approximately 50% enrichment). A new peak, which we assign to the C-2 carbon of the single histidine residue, appears at -136 ppm ($\nu_{\frac{1}{2}}$ = 30 Hz). In other aspects, the spectra of natural abundance and L-[2-^{13}C]histidine α-lytic protease are identical.

Incorporation of L-[2-^{13}C]histidine into α-lytic protease by Myxobacter 495 also yields enriched β-lytic protease which, when denatured, provides a useful comparison for the magnetic resonance parameters of histidine residues in a random-coil polypeptide.

FIGURE 1. Proton Noise Decoupled Cmr Spectra of α-Lytic Protease. 6 mM enzyme, 34°, 0.2 M KCl. Each spectrum represents 250,000 transients at 0.20 sec acquisition time, 2000 Hz sweep width, and 90° pulse.

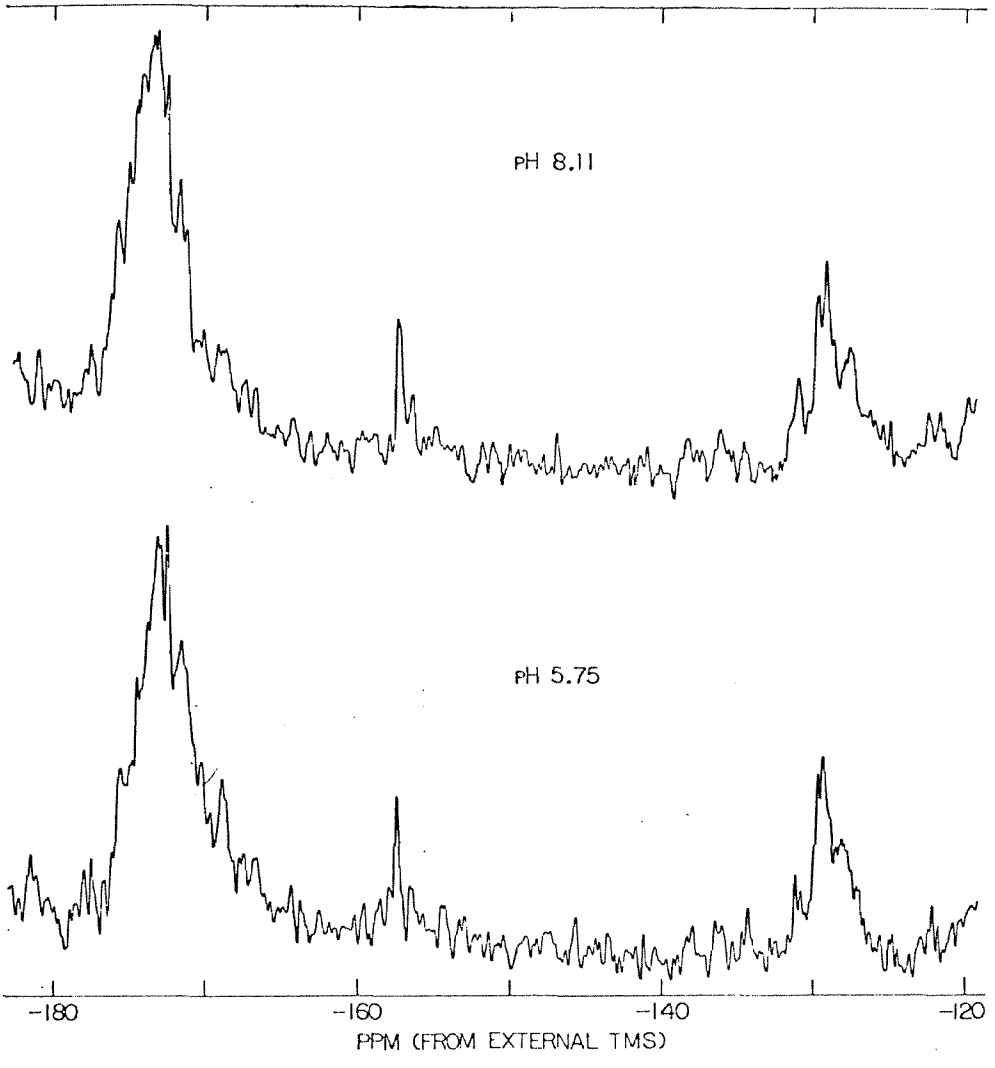
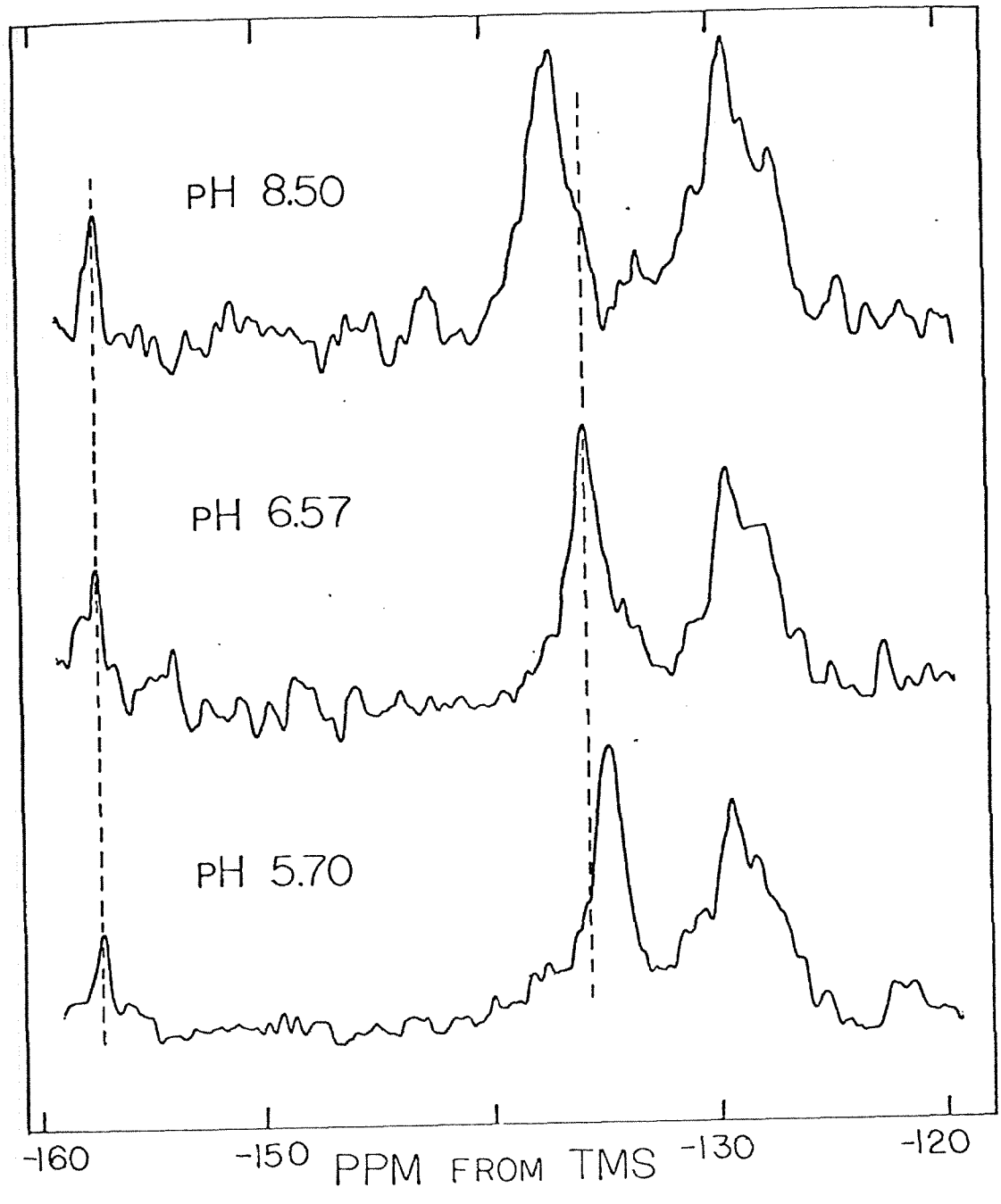

pH 8.11
pH 5.75
-180
-160
-140
-120
PPM (FROM EXTERNAL TMS)

FIGURE 2. ^{13}C-enriched, Proton Noise Decoupled Cmr Spectra of α-Lytic Protease. 5 mM enzyme, 34°, 0.2 M KCl. Each spectrum was recorded using a 0.15 sec acquisition time, 5000 Hz sweep width, and 90° pulse. pH 5.70 spectrum, 220,000 transients; pH 6.57 spectrum, 90,000 transients; pH 8.50 spectrum, 55,000 transients.


pH 8.50
pH 6.57
pH 5.70
-160
-150
PPM FROM TMS
-130
-120

The cmr spectrum of denatured β-lytic protease shows a strong resonance ($\nu_{\frac{1}{2}}$ = 10 Hz) around -135 ppm which we assign to the eight C-2 carbons of the eight histidine residues of the protein.

The upfield resonance of the doublet from the histidine C-2 carbon in proton coupled cmr spectra of α-lytic protease is partially obscured by the signals from the other aromatic carbons (Figure 3). This interference can be eliminated by computer subtraction of a proton coupled natural abundance ^{13}C spectrum taken under identical conditions from the ^{13}C-enriched spectrum. This process yields a simplified spectrum consisting only of the doublet from the histidine C-2 from which $^{1}J_{CH}$ can be measured directly. Alternatively, $^{1}J_{CH}$ values for the C-2 carbon can be calculated from the difference between the position of the resonance in the decoupled spectrum and the position of the downfield peak of the doublet in the coupled spectrum taken of the same sample. Both positions can be measured accurately relative to the sharp arginine signal whose position never varied more than 1 Hz from external D_2O lock in any of the spectra we have recorded. Although this alternate method of measurement yields a somewhat larger error (since the difference in peak positions must be multiplied by two to yield $^{1}J_{CH}$), six determinations in the pH range 5-6 have yielded a reproducible value for $^{1}J_{CH}$ (203, 204, 205, 205, 206, and 208 Hz). Figure 3 shows the spectra used for calculation by both of the above methods for $^{1}J_{CH}$ at pH 5.98.

Table I collects the carbon chemical shifts (δ) and coupling constants ($^{1}J_{CH}$) for the C-2 carbons of imidazole and some of its

FIGURE 3. Measurement of $^{1}J_{CH}$ for Histidine C-2 in α-Lytic Protease at pH 5.98. (a)Proton decoupled cmr spectrum. ^{13}C-enriched, 50,000 transients; (b) (—) proton coupled cmr spectrum, same sample as in (a), 250,000 transients; (---) proton coupled cmr spectrum, natural abundance ^{13}C, 250,000 transients; (c)difference spectrum obtained by computer subtraction of the natural abundance spectrum (---) from ^{13}C-enriched spectrum (—) of (b). $^{1}J_{CH}$ can be calculated either directly from the difference spectrum (c), $^{1}J_{CH}$ = 205 Hz, or by multiplying by a factor of 2 the difference between the chemical shifts of the decoupled resonance (a) and the downfield resonance of the coupled spectrum (b), $^{1}J_{CH}$ = 2(563 - 460) = 206 Hz.

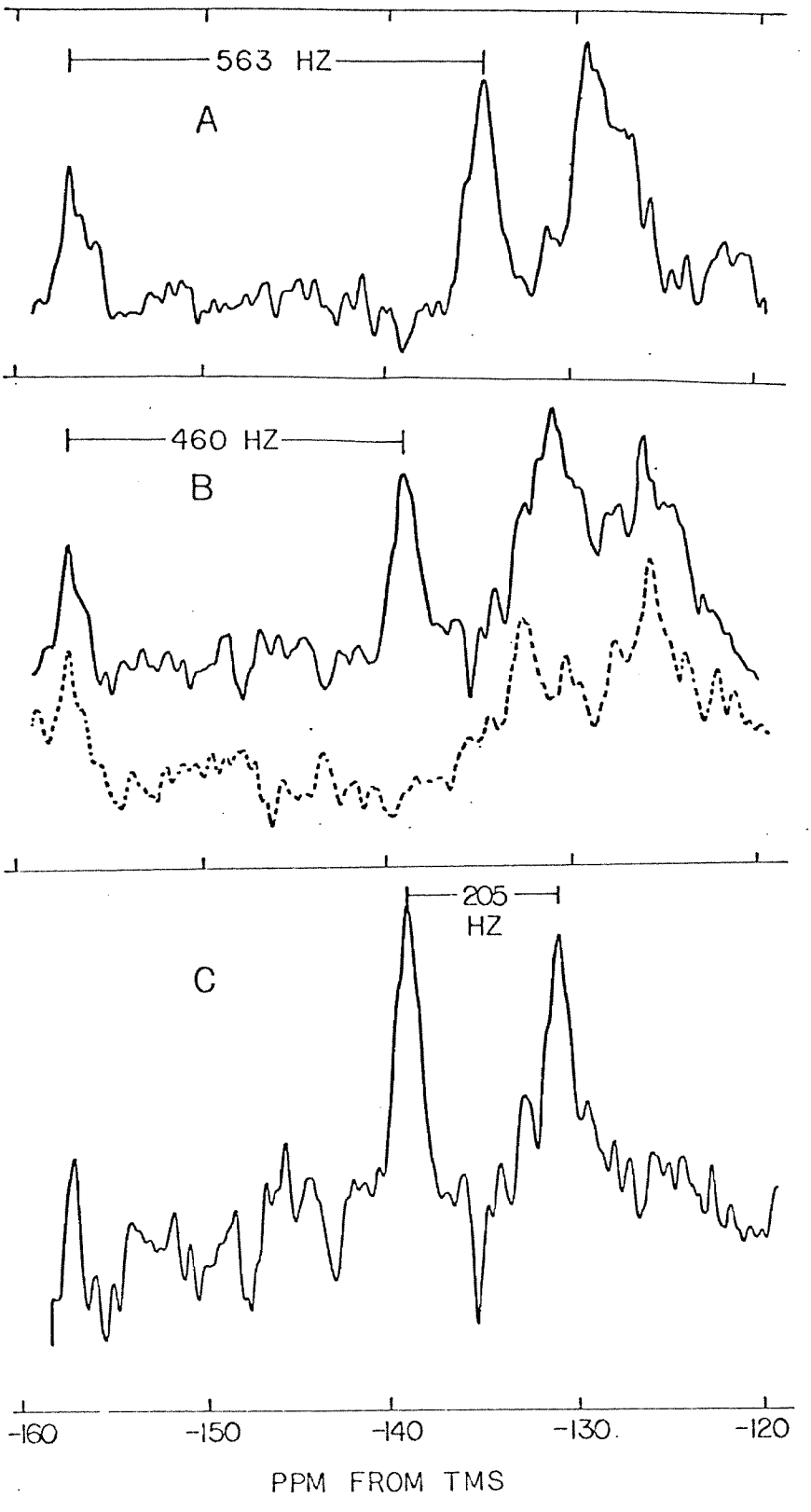

563 HZ
A
460 HZ
B
205
HZ
C
-160
-150
-140
-130
-120
PPM FROM TMS

TABLE I: Chemical Shifts and Directly Bonded Carbon-Hydrogen Coupling Constants for C-2 Carbon in Imidazole Derivatives [a]

Compound	δ (ppm from TMS ±0.04)		$^1J_{CH}$ (Hz ±1)	
	Cation	Neutral	Cation	Neutral
Imidazole	-134.05	-136.23	219	209
4-Methylimidazole	-133.17	-135.40	221	208
4-Methylimidazole (dioxane)	-133.13	-134.49	219	205
1-Methylimidazole	-135.52	-138.38	220	207
L-Histidine methyl ester	-135.08	-136.71	222	208
N-Acetyl-L-histidine	-134.17 -133.85	-139.45	221 220	204
(4-Imidazolyl)-acetic acid	-134.25 -133.65	-136.47	220 221	205
β-Lytic protease [b] (denatured)	-134.09	-136.67	220	206

[a] Measured for 1-2 M aqueous solutions unless otherwise indicated. [b] 1-2 mM solution in 0.2 M KCl.

derivatives. Table II lists the δ and $^1J_{CH}$ values for the C-2 carbon of the histidine residue in α-lytic protease at several values of pH. Table III lists the relaxation parameters for the C-2 carbon of the histidine residue of α-lytic protease at pH 5.8 and 8.2.

Titration Results. The chemical shifts and coupling constants of the ring carbons depend on the state of protonation of the nitrogen heteroatoms of the ring and accordingly vary with pH. The chemical shift may also be significantly affected by the external environment experienced by the carbon nuclei. If the rate of proton acquisition or proton loss by the nitrogen atom or changes in the environment around the ring occur rapidly on the nmr time scale, the observed spectrum will represent the average of spectra for the relative populations of each species (40). The condition for this "fast exchange" is that $\tau_A < \sqrt{2}/2\pi\Delta_{AB}$ where τ_A is the mean lifetime of the shorter lived species. For Δ_{AB} = 60 Hz, this requires $\tau_A < 3.5 \times 10^{-3}$ sec, a condition easily satisfied for simple ionization of imidazole and its derivatives in aqueous solution and one that also characterizes the enzyme spectra reported here except for those of α-lytic protease below pH 4. Thus, we observe a single resonance of essentially constant line width that shifts as a function of pH for denatured β-lytic protease and for α-lytic protease between pH 5 and 9.

Under conditions of fast exchange, the observed chemical shift, δ, will vary with pH in the following manner:

$$(\delta_{obsd} - \delta_a)/(\delta_b - \delta_a) = K_a/([H^+] + K_a),$$

TABLE II: Chemical Shift and Coupling Constant Values for C-2 Carbon in Histidine Residue of α-Lytic Protease

pH	Chemical Shift (ppm ± 0.12 from TMS)	$^1J_{CH}$ (Hz ± 3)
8.2	-137.26	205
5.2	-134.79	205 [a]
3.3	-134.81	206
	-134.05	222
	-132.46	220

[a] Six determinations of $^1J_{CH}$ around pH 5-6 yielded values of 203, 204, 205, 205, 206, and 208 Hz.

TABLE III: Nmr Relaxation Parameters for His 57 C-2 Carbon in α-Lytic Protease [a]

pH	$\nu_{\frac{1}{2}}$ (Hz)	T_1 (sec)	NOE	τ_c (sec)
5.8	28 ± 3	0.063 ± 0.002	1.16 ± 0.10	$1.8 \pm 0.1 \times 10^{-8}$
8.2	32 ± 3	0.060 ± 0.015	1.18 ± 0.10	$1.7 \pm 0.2 \times 10^{-8}$

[a] ± Enzyme concentration, 5 mM; temperature, 34°; 0.2 M KCl. τ_c calculated according to Doddrell et al. (39). T_1 values represent average of three determinations.

where δ_a and δ_b are the chemical shifts of the two exchanging species, respectively, and K_a is the acid ionization constant of some ionization (either that of the imidazole itself or a neighboring group) which perturbs the environment experienced by the imidazole C-2. Figure 4 collects the observations of the chemical shifts as a function of pH of the C-2 carbon of the single histidine residue of native α-lytic protease between pH 4 and 10. The solid line in this figure represents a theoretical curve calculated by an iterative computer fit of the experimental points to the above equation. The excellent fit between the experimental points and the theoretical curve based on a simple one group ionization (pK_a = 6.7) suggests that such an ionization does occur in this pH range. However, we observe that $^1J_{CH}$ for C-2 of the histidine residue has a value characteristic of a neutral imidazole ring from pH 4.7 to 8.7 ($^1J_{CH}$ = 205 Hz). Therefore, the ionization (pK_a = 6.7) affecting the C-2 chemical shift does not represent ionization of the histidine itself but rather, most likely, that of the neighboring aspartic acid residue (Asp 102).

Indeed, our interpretation of the spectra of α-lytic protease at pH $<$ 4 suggests that only in this pH range does the histidine become protonated. At pH 3.25, three distinct signals are evident which we assign to the histidine C-2 carbon, and they indicate that at this pH the histidine exists in three, slowly exchanging, states (Figure 5). One, which disappears below pH 3, has the same chemical shift (-134.8 ppm), $^1J_{CH}$ (206 Hz), and line width (30 Hz) as the single resonance observed around pH 5. This resonance

FIGURE 4. Chemical Shift of Histidine C-2 Resonance of α-Lytic Protease as a Function of pH. (○) the chemical shift of C-2 in the neutral histidine residue (see text); (—) theoretical titration curve calculated using pK_a of 6.75 and $\Delta = 62$ Hz.

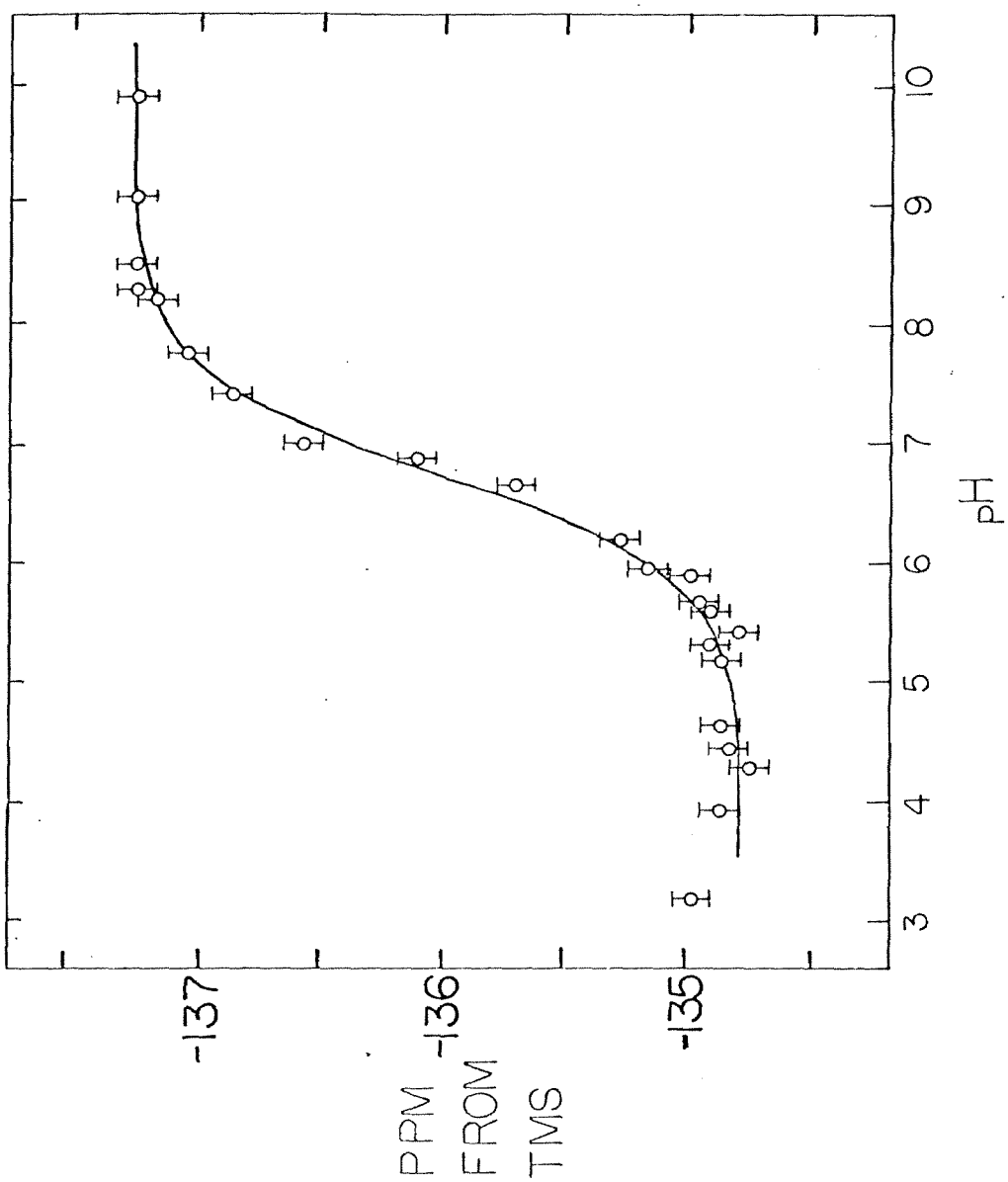
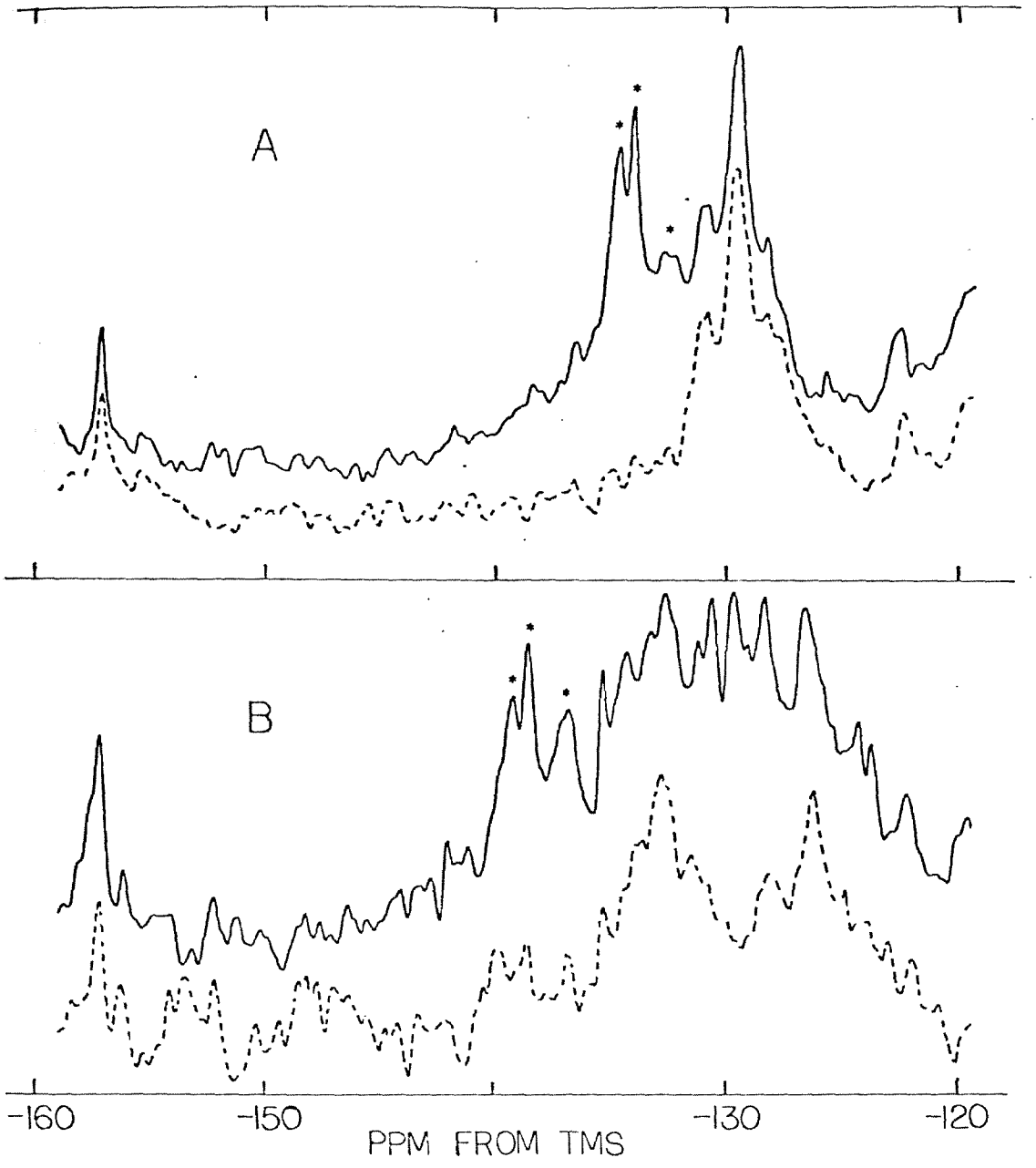

-137
-136
-135
PPM FROM TMS
3
4
5
6
7
8
9
10
pH

FIGURE 5. Cmr Spectra of α-Lytic Protease at pH 3.25. (—) ^{13}C-enriched; (---) natural abundance ^{13}C. (a) Proton noise decoupled; (b) proton coupled. The resonances assigned to C-2 of the histidine residue are marked by an asterisk. Each spectrum represents 250,000 transients at 0.15 sec acquisition time.

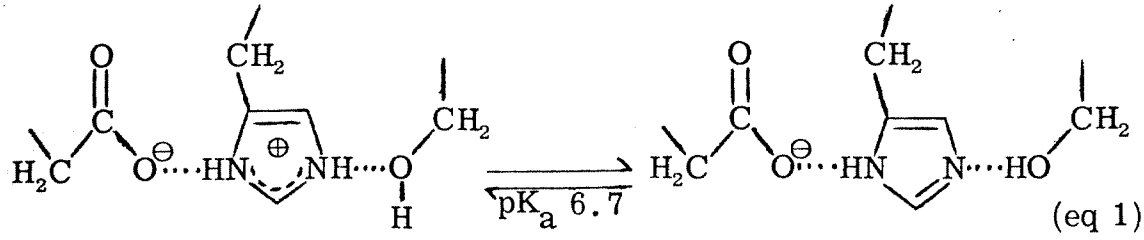

A
B
-160
-150
-130
-120
PPM FROM TMS

probably represents a neutral histidine within the catalytic triad. A second signal has virtually the same chemical shift (-134.1 ppm) and $^{1}J_{CH}$ (222 Hz) values as the C-2 carbons of the histidine residues in denatured β-lytic protease at low pH. The line width (< 12 Hz) of this signal is considerably narrower than that of the other two (indicating significantly more side chain mobility), and we assign this resonance to a protonated histidine that has been ejected into solution from its normal, partially buried position as a member of the catalytic triad. The third resonance has a chemical shift of -132.46 ppm (2.35 ppm upfield of the first mentioned signal) and a $^{1}J_{CH}$ (220 Hz) typical of a protonated histidine. This, along with its line width (30 Hz), suggests that it represents the protonated histidine near its normal location in the catalytic triad. In summary, only below pH 4 does the histidine exist in a protonated state, and the two cationic species (one in and one out of the catalytic triad) are in slow exchange with the single neutral form (in the catalytic triad).

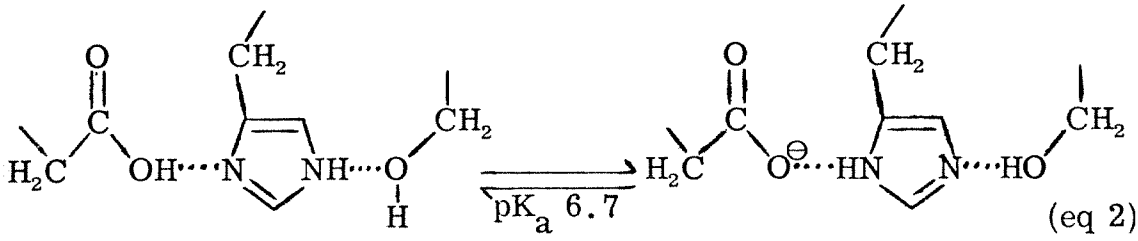
DISCUSSION

Selective ^{13}C enrichment of the histidine residue at the active site of α-lytic protease, a homolog of the mammalian serine proteases, has allowed study of the ionization behavior of this residue as a function of pH. Changes in such nmr parameters as chemical shift, coupling constant, relaxation time, and nuclear Overhauser enhancement shed light on the ionizations of the histidine and aspartic acid residues and on the degree of mobility of the imidazole ring within the native protein at various pH's. These results lead to a modification of the generally held views of the role of the catalytic triad and charge transfer in hydrolytic catalysis by serine proteases.

State of Ionization of Histidine and Aspartic Acid Residues as a Function of pH in Serine Proteases. Knowledge of the microscopic ionization behavior of the residues of the catalytic triad is essential to a molecular understanding of catalysis by serine proteases. A great many studies (for example, 41-42) have shown that an ionization with an apparent pK_a of about 6.7 controls catalytic activity. Unfortunately, none of these studies uniquely defines the state of ionization of the residues of the triad as a function of pH. Thus, either of these situations could describe the available evidence:



(eq 1)



(eq 2)

This ionization (pK_a = 6.7) is commonly assigned to proton loss from the protonated imidazolium ring (eq 1) of the histidine residue in the catalytic triad (His 57 in chymotrypsin), an assignment which has seemed plausible as the pK_a of histidine side chains in many polypeptides is around 6.4 (43) and, in general, the pK_a of aspartic acid residues is around 4.5. However, in the conformation of the serine proteases present in crystals, the aspartic acid residue is buried within a hydrophobic region and is not exposed to solvent water (7). Further, in the crystalline enzyme, the histidine is only partially exposed to solvent as N^{π} is pointed toward aspartic acid and other residues within the hydrophobic interior of the protein. The environment experienced by the aspartic acid residue and, to a lesser extent, that of the histidine residue also, will be much less polar than water, a situation which would tend to raise the pK_a of aspartic acid above 4.5 and lower the pK_a of histidine below 6.4. Therefore, one

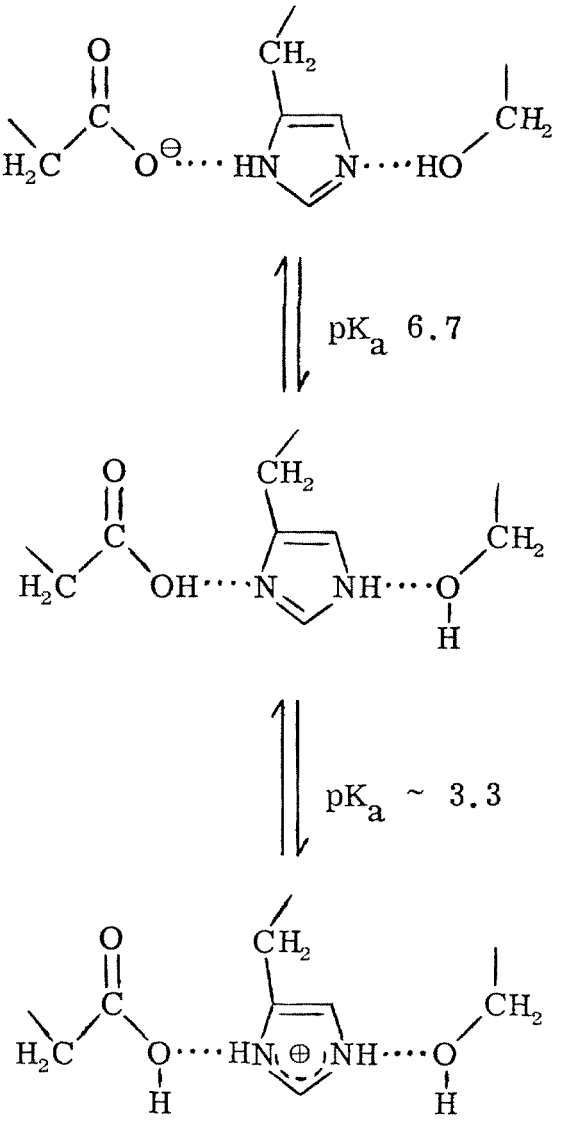
should seriously entertain the notion that the microscopic pK_a's of the aspartic acid and histidine residues are significantly perturbed from those expected in aqueous solution. As a result, their pK_a's may well be reversed, and the ionization that occurs with $pK_a = 6.7$ may actually reflect net loss of a proton from a neutral aspartic acid carboxyl group in the presence of a neutral imidazole ring rather than the more commonly accepted loss of a proton from an imidazolium cation in the presence of an aspartate carboxylate anion. Indeed, our interpretation of the nmr data supports the notion that the ionization at $pK_a = 6.7$ reflects net loss of a proton from aspartic acid.

Nmr Parameters. Two cmr parameters, chemical shift and coupling constant, of the C-2 carbon of an imidazole ring generally respond to the state of ionization of the ring. The data in Table I show that deprotonation of an imidazolium ring leads to downfield shifts of 1.4-5.6 ppm and changes in $^1J_{CH}$ from 220 Hz (cationic) to 207 Hz (neutral). These values agree with those reported for imidazole and its derivatives by Weigert and Roberts (44), Pugmire and Grant (45), and Reynolds et al. (46). The C-2 carbons of the eight histidine residues of denatured β-lytic protease on deprotonation exhibit normal changes in chemical shift (-134.1 to -136.7 ppm, a change of -2.6 ppm) and coupling constant (220 to 206 Hz) with an apparent pK_a of 6.4. The behavior of imidazole rings of histidine residues in a denatured protein, therefore, show no significant divergence from the behavior of imidazole rings in small molecules.

The situation for C-2 of the histidine residue of α-lytic protease is, in contrast, complex. The observed changes in chemical shift in the pH range 5-9 can be defined by a single ionization with $pK_a = 6.7$. However, the fact that $^1J_{CH}$ for the histidine C-2 has a value characteristic of neutral imidazole over this entire pH range suggests that the histidine itself does not titrate in this range. Rather, as revealed by the spectra taken at low pH, the histidine ionizes below pH 4. At pH 3.3, for example, three slowly exchanging species are evident from the cmr spectrum of the enzyme. Two of these signals, separated by 2.4 ppm, represent the neutral ($^1J_{CH} = 206$ Hz) and protonated ($^1J_{CH} = 220$ Hz) histidine at or near its normal position as a member of the catalytic triad.

Scheme I summarizes the ionization behavior of the aspartic acid and histidine residues of the catalytic triad. Although addition of the first proton ($pK_a = 6.7$) involves protonation of the aspartic acid, this group does not have to encounter solvent to acquire a proton as one is readily available from the neighboring N^{π} nitrogen of the histidine. The histidine can maintain its neutrality by simultaneously acquiring a proton from solvent at its exposed N^{τ} nitrogen. This process will be expected to be fast on the nmr time scale, and the changes in the C-2 resonance between pH 5 and 9 are consistent with such a rapid exchange process. Addition of a second proton to the catalytic triad must involve reorganization of the three residues owing to the inaccessibility of the aspartic acid and the N^{π} of the histidine to solvent. The histidine must

Scheme I


O
C
H2C
O⊖····
HN
N····HO
CH2
CH2
pKa 6.7
O
C
H2C
OH····
N
NH····O
H
CH2
CH2
pKa ~ 3.3
O
C
H2C
O····
H
HN⊕NH····O
H
CH2
CH2

first rotate out into solvent to expose N^{π} before it can acquire a proton, and this might reasonably be expected to be a relatively slow process (7). The multiplicity of peaks observed in the low pH spectra accords with this description.

In summary, only below pH 4 does the histidine exist in a protonated state. Around pH 5-6, the imidazole ring of the histidine is neutral which implies that the aspartic acid carboxyl group is likewise neutral. The proton added to the catalytic triad with pK_a = 6.7 does not produce an ion-pair system of carboxylate anion /imidazolium cation (eq 1), but rather a neutral system of carboxylic acid / imidazole (eq 2).

This conclusion should, in fact, occasion little surprise; analogous results can be obtained in model systems when they are in sufficiently nonaqueous environments. For example, equimolar solutions (1-2 M) of acetic acid and imidazole in pure dioxane show no protonation of the imidazole by the acetic acid. Such protonation (forming acetate anion and imidazolium cation) only becomes appreciable on addition of as little as 1-2 molar equivalents of water to the system.

Further, recent x-ray crystallographic results on trypsin (47) show that the interaction between the aspartate anion and neutral imidazole (in crystals of DIP-trypsin grown at pH 7.5) is unusual in that N^{π} of the imidazole ring points toward the center of the carboxylate anion. Unlike coordination in aqueous solution, this type of interaction seems more typical of that which one might expect in the gas phase or nonaqueous, nonpolar

solvents. This observation further supports the unusual character of the aspartate group in the catalytic triad.

Correlation with Other Data. Most techniques used to study ionization within the catalytic triad of serine proteases are inherently incapable of distinguishing between proton loss by an imidazolium cation as distinct from proton loss by a carboxylic acid group. Most kinetic data and other observations of macroscopic ionization behavior can only show that some group (or strongly interacting combination of groups) with a $pK_a = 6.7$ must be deprotonated to generate catalytically active enzyme.

For example, Fersht and Sperling (48) reported studies of the net release of protons upon denaturation of chymotrypsin in which most of the carboxyl groups (glutamic acid, aspartic acid, and terminal α-carboxyl residues) had been previously blocked. The authors concluded that denaturation of the enzyme at certain pH's led to uptake of one proton by the residues of the catalytic triad. They argued that Asp 102, present as the carboxylate anion in the undenatured, modified protein, was the residue responsible for uptake of this proton. The pK_a of Asp 102 was said to be depressed in the intact enzyme relative to its pK_a in denatured protein. Apart from the manifold uncertainties in accurately measuring quantitative proton uptake in such experiments, they cannot, in principle, define the microscopic ionization of groups involved in strongly interacting systems. (In the present case, for example, a system of Asp-His in which both groups are ionized will give the same result as a system of Asp-His in which

both groups are neutral.) Based on their assumption that Asp 102 is anionic and His 57 cationic in the pH 5-6.5 range, Fersht and Sperling argued that the resultant favorable electrostatic interaction must be responsible for a lowered pK_a of Asp 102 and a raised pK_a of His 57. The argument is, however, circular for it assumes that the pK_a of Asp 102 is lower than that of His 57 (so that the groups are in fact both ionized) to support, in turn, the abnormal pK_a's of these residues. If, in contrast, the pK_a of Asp 102 is not intrinsically lower than that of His 57, there will not be such an electrostatic interaction, and the pK_a's will be altered only as a result of the environment of the groups.

In fact, the hydrophobic nature of the environment is such that its more likely effect is to raise the pK_a of Asp 102 and lower that of His 57 so that pK_a Asp 102 > pK_a His 57. In this connection, in cysteine proteases such as papain, where the aspartic acid residue is replaced by a neutral asparagine, the active site histidine shows a pK_a around 4 (49). Further, at least six carboxylic acid side chains in the proteolytic enzyme pepsin have pK_a's higher than 6 (50). A particularly thorough kinetic study of chymotrypsin (39, 51) included both observations of the temperature and pH dependence of chymotryptic hydrolysis of N-acetyl-L-tryptophan ethyl ester. The results showed that the ΔH, ΔS, and "compensation" behavior of the residue with a $pK_a = 6.7$ were not those characteristic of ionization of an imidazole but did resemble those for ionization of a carboxylic acid. They suggested that the unusual environment of the histidine

accounted for their observations. We suggest that these values are normal and do indeed reflect ionization of the carboxylic acid group of Asp 102.

The very similar inhibition constants for binding neutral and anionic inhibitors in the pH 5-6.5 range also support the suggestion that the active site region is essentially electrostatically neutral in this pH range. For example, K_I values for neutral (N-trifluoroacetyl-D-tryptophanamide) and anionic (N-trifluoroacetyl-D-tryptophanate) inhibitors are virtually identical between pH 5 and 6.5 (52-54). Above pH 7, however, binding of the negatively charged inhibitor decreases drastically while that of the neutral inhibitor is virtually unchanged. This decreased binding of anionic inhibitors at high pH follows easily from the anticipated electrostatic repulsion between a negatively charged active site (because of the carboxylate anion of Asp 102) and a negatively charged inhibitor. However, below pH 6.5 one would have anticipated some favorable electrostatic interaction between the negatively charged inhibitor and the active site if His 57 were in fact protonated and, therefore, positively charged. Though the carboxylate anion of Asp 102 would formally neutralize this positive charge, the inhibitor would be sufficiently nearer the imidazolium ring that some electrostatic effect should be manifested. The absence of such electrostatic effect on binding, therefore, supports the suggestion that in the range pH 5-6.5 both Asp 102 and His 57 are electrically neutral.

Magnetic resonance observations allegedly of the N^{π} proton of the imidazolium ring of His 57 which forms a hydrogen bond to the carboxylate anion of Asp 102 and which exchanges only very slowly with solvent have been reported by Robillard and Shulman (55). They observed a broad (200 Hz) resonance at very low field (-18 to -15 ppm TMS) in the ^1H spectrum of chymotrypsinogen and δ-chymotrypsin whose chemical shift changed with an apparent pK_a of 7.2. The case of δ-chymotrypsin, especially, presents problems as, under the reported experimental conditions (2-4 mM enzyme, 14°), experience indicates that extensive autolysis, first to α- or γ-chymotrypsin and then to catalytically inactive products, would occur during the approximately 6 hr used to record each spectrum. We have attempted to duplicate these experiments with chymotrypsinogen, δ-chymotrypsin, and α-lytic protease (which is stable to autolysis) on three different Varian spectrometers (XL-100, HR-220, and HR-300) but have been unable to observe the low-field resonance. In any case, their result does not unambiguously distinguish between a proton attached to a carboxyl and one attached to an imidazole group.

They also reported that δ-chymotrypsin inhibited by alkylation of His 57 with tosyl-L-phenylalanine chloromethyl ketone exhibits a low-field proton resonance whose chemical shift is unchanged in the range pH 6-8.5. They argued that covalent attachment of the inhibitor raised the pK_a of the modified His 57 above 8.5 because deprotonation of the histidine would force the histidine to swing out toward solution and at the same time pull the phenyl

ring of the attached inhibitor out of its hydrophobic binding pocket. The resultant energy cost would prevent deprotonation of the modified His 57 and account for its markedly raised pK_a. However, in our view, if both groups (Asp 102 and modified His 57) were charged, the covalent attachment of the inhibitor to His 57 would, by completely excluding water from the active site, so lower the dielectric constant of this region of the protein molecule that the energetic disadvantage of two charged groups would more than offset the energetic advantage of filling the hydrophobic pocket. However, if above pH 5 Asp 102 and modified His 57 are actually neutral, the reduction in dielectric constant in the active site region could be expected to raise further the pK_a of Asp 102 so that it might not ionize appreciably even at pH 8.5, as observed.

One of the most interesting experiments which sheds light on the microscopic ionization behavior of the catalytic residues is the crystallographic work of Tulinsky and his collaborators (56, 57). They determined separately the structures of both crystallographically independent molecules in the dimeric state in which native α-chymotrypsin crystals exist. The two molecules are related to each other by a local twofold axis that interfaces the molecules (including their active sites) with one another to yield a dimeric unit. The x-ray crystallographic structure determined by Blow and his coworkers (7) was obtained by averaging the electron density distributions of the two independent molecules about the local twofold axis and by using this density to construct a model for the average molecule of the dimer. Tulinsky found that this

method led to several ambiguities in the structure along the dimer interface. Of particular note is the interaction he observed between the Tyr 146 α-carboxyl group (the B chain terminal residue) of one molecule and the active site His 57 of the other molecule. Both of these residues are crucial to formation of the α-chymotrypsin dimer in solution since either photooxidation of His 57 (58) or removal of Tyr 146 with carboxypeptidase (59) yields a protein with no tendency to associate into a structured, dimeric unit. Aune and Timasheff (60) investigated the pH dependence of the solution dimerization behavior and suggested that two identical pairs of ionic groups controlled the phenomenon. They described the pH dependence in terms of shifts of pK_a values for these groups during dimerization: one from pK_a 5.0 (monomer) to 6.2 (dimer) and the other from pK_a 3.6 (monomer) to 2.4 (dimer). They interpreted the interaction as being one between the imidazolium cation of His 57 (monomer pK_a = 5.0) and the carboxylate anion of Tyr 146 (monomer pK_a = 3.6). Tulinsky found, however, that at pH 4.2 the Tyr 146 carboxyl does not interact directly with the His 57 imidazole but that, instead, one of the carboxyl oxygens of Tyr 146 lies 3.0 Å from the carbonyl oxygen of His 57. The closeness of the latter two atoms implies that a hydrogen bond is formed between them, and this is possible only if the carboxyl group is neutral rather than ionized. Further, a reorientation of the carboxyl group occurs when the pH of the solution containing the crystals is raised above pH 5.4. Thus, Tulinsky suggested that the Tyr 146 carboxyl group in the dimer

possessed an elevated pK_a because it was buried within the interface between the molecules and thereby shielded from solvent. He failed to note, however, that the same dielectric effect should depress the pK_a of His 57. It seems very likely that the shifts observed by Aune and Timasheff (60) of pK_a 5.0 to 6.2 and pK_a 3.6 to 2.4 represent shifts for His 57 and Tyr 146, respectively. That is, the pK_a of His 57 (rather than Tyr 146) shifts from 3.6 (monomer) to 2.4 (dimer). This is entirely consistent with our interpretation of the cmr data for α-lytic protease which shows a pK_a of about 3.3 for His 56, and it represents important confirmatory evidence for our ionization scheme.

Mobility of Histidine 57 Side Chain. Dipolar interactions between a ^{13}C nucleus and directly bonded ^{1}H nuclei generally dominate relaxation of the carbon. For small molecules rapidly tumbling in solution, or for freely spinning methyl groups, spin rotation may contribute significantly to the relaxation process. When carbon is directly bonded to a nucleus with a spin greater than $\frac{1}{2}$ (such as nitrogen) scalar relaxation can also be important if the molecule has either a short or an extremely long correlation time. However, both spin rotation and scalar relaxation contribute only negligibly for ^{13}C nuclei having correlation times near the Larmor frequency. In these cases dipolar interactions dominate (61).

Doddrell et al. (39) have given a comprehensive theoretical treatment of T_1, T_2, and NOE values for ^{13}C nuclei in molecules

with long correlation times. In particular, they have dissected effects on the relaxation times and NOE of a ^{13}C nucleus directly bonded to a single ^{1}H into those that depend on overall molecular tumbling (characterized by a correlation time τ_R) and those that result from intramolecular reorientations (characterized by a correlation time τ_G).

In the absence of intramolecular reorientation, the molecular tumbling defines T_1, T_2, and NOE as

$$1/T_1 = (1/10)(\hbar\gamma_H\gamma_C)^2 r^{-6}[\chi_R]$$

$$1/T_2 = (1/20)(\hbar\gamma_H\gamma_C)^2 r^{-6}[\chi_R + 4\tau_R + 6\tau_R/(1+\omega^2{}_H\tau_R^2)]$$

$$NOE = 1 + 4\phi_R/\chi_R$$

where γ_H is the magnetogyric ratio for hydrogen, ω_H is the Larmor frequency for hydrogen, r is C-H bond distance (1.07 Å for the C-2 of imidazole),

$$\chi_R = \frac{\tau_R}{1+(\omega_H-\omega_C)^2\tau_R^2} + \frac{3\tau_R}{1+\omega_C^2\tau_R^2} + \frac{6\tau_R}{1+(\omega_H+\omega_C)^2\tau_R^2}, \text{ and}$$

$$\phi_R = \frac{6\tau_R}{1+(\omega_H+\omega_C)^2\tau_R^2} - \frac{\tau_R}{1+(\omega_H-\omega_C)^2\tau_R^2}.$$

The effects of internal motion are expressed by the following equations:

$$T_{1R}/T_1 = A + B(\chi_B/\chi_R) + C(\chi_C/\chi_R)$$

$$T_{2R}/T_2 = A + B(T_{2R}/T_{2B}) + C(T_{2R}/T_{2C})$$

$$NOE = \frac{1 + 4(A\phi_R + B\phi_B + C\phi_C)}{(A\chi_R + B\chi_B + C\chi_C)}$$

where

$$\tau_B^{-1} = \tau_R^{-1} + (6\tau_G)^{-1}$$

$$\tau_C^{-1} = \tau_R^{-1} + 2(3\tau_G)^{-1}$$

$$A = \tfrac{1}{4}(3\cos^2\theta - 1)^2$$

$$B = 3\sin^2\theta\cos^2\theta$$

$$C = \tfrac{3}{4}\sin^4\theta .$$

In the above equations, θ is the angle between the C-H bond in question and the axis of internal motion. Crystallographic models of serine proteases suggest that the principal motion available to the histidine is a swinging in and out from its partially buried position in the catalytic triad. This allowed motion is largely constrained such that it occurs within the plane containing the imidazole ring. The axis of the motion is therefore nearly perpendicular to the plane of the imidazole ring (though not passing through its center) and θ has a value close to $90°$.

Assumption of a value of 90° for θ allows calculation for a given value of τ_R of the dependence of T_1, T_2, and NOE on variations in τ_G. Globular proteins with a molecular weight of 20,000 have rotational correlation times, τ_R, between 1 and 2×10^{-8} sec in aqueous solution (62). If there is no independent, internal motion of the imidazole ring in α-lytic protease, the experimental results at pH 5.8 and 8.2 lead to a calculated $\tau_R = 1.7 \times 10^{-8}$ sec for the C-2 nucleus of the histidine. Table IV collects some calculated values of T_{1R}/T_1, T_{2R}/T_2, and NOE when τ_G is varied while τ_R is taken as 1.7×10^{-8} sec. These calculations show that even internal motion of the imidazole ring slower than a factor of 2 or 3 relative to the tumbling of the entire protein in solution would be clearly manifest in the observed relaxation parameters. We observe no such effects, and therefore conclude that, both at pH 5.8 and 8.2, the histidine side chain is locked within the catalytic triad of α-lytic protease and experiences no internal motion independent of the tumbling of the protein itself in solution.

At pH 3.3, two of the three observed resonances have line widths ($\sim$ 30 Hz) corresponding to a C-2 carbon with the same correlation time (1.7×10^{-8} sec) observed for the single resonance above pH 5. This suggests that these two signals arise from a carbon nucleus in an immobile histidine ring. The third signal ($\nu_{\frac{1}{2}} \leq 12$ Hz) represents a histidine ring with much greater mobility. The shorter correlation time, together with a chemical shift (-134.05 ppm) almost identical with that of the C-2 histidine

TABLE IV: Effect of τ_G on T_1, T_2, and NOE [a]

τ_G (sec)	T_{1R}/T_1	T_{2R}/T_2	NOE
$\gg 1 \times 10^{-7}$	1.00	1.00	1.18
1×10^{-7}	1.04	0.94	1.19
1×10^{-8}	1.33	0.72	1.24
1×10^{-9}	1.09	0.45	2.05
1×10^{-10}	0.39	0.28	1.86
1×10^{-11}	0.26	0.25	1.29
$\ll 1 \times 10^{-11}$	0.25	0.25	1.18

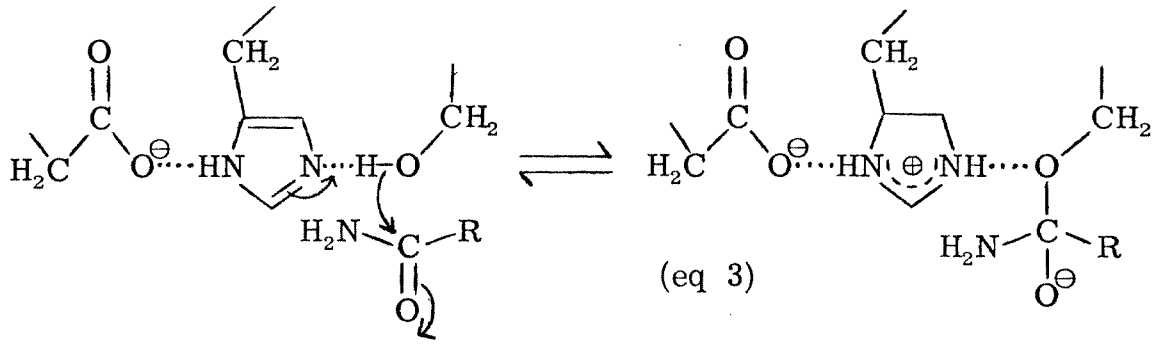
[a] Data calculated according to Doddrell et al. (39) for carbon bonded to a single hydrogen and having a $\tau_R = 1.7 \times 10^{-8}$ sec and only one degree of internal motion (rotation about an axis perpendicular to the C-H bond, see text).

carbons of denatured β-lytic protease, suggests that this signal arises from a histidine which is exposed to solvent and is relatively unrestrained in its movement.

Catalytic Mechanism of Serine Proteases. The precisely oriented array of three functional groups from three amino acid residues (a hydroxyl group of serine, an imidazole ring of histidine, and a carboxylate anion of aspartic acid) has been shown to be essential for the catalytic activity of serine proteases by both chemical and physical data. From the first discovery of the buried aspartate carboxylate and the abundant proposal of a "charge relay system" consisting of the hydrogen bonded network of carboxylate, imidazole, and hydroxyl, attempts have been made to account for catalysis by increased nucleophilicity of the serine hydroxyl by partial transfer of the negative charge from the carboxylate anion to the serine oxygen in the ground state (2). However, as there is no evidence for an abnormally low pK_a for the serine hydroxyl group, the degree of this charge transfer in the ground state should be minimal.

Both the formation of the isolable acyl-enzyme intermediate from substrate and enzyme and its subsequent hydrolysis by reaction with a molecule of water probably proceed by way of a tetrahedral intermediate. Considerable evidence for obligatory intervention of such a tetrahedral species in the formation of the acyl-enzyme intermediate has been presented (4, 5). Based on the commonly accepted relative pK_a values for the carboxylic acid

group and imidazole ring (pK_a His = 6.7, pK_a Asp = 3.5; pK_a His > pK_a Asp), the formation of this tetrahedral intermediate must involve some charge separation. Positive character will develop on the imidazole ring as negative character builds up on the carbonyl oxygen upon formation of the new carbon-oxygen bond between the carbonyl carbon of the substrate and the serine oxygen of the enzyme. At the same time, the proton of the serine hydroxyl will be transferred to N^{τ} of the imidazole ring without (given that pK_a His > pK_a Asp) concomitant transfer of the proton at N^{π} to the adjacent carboxylate anion. Accordingly, the imidazole ring will become positively charged (eq 3). Though some recent discussions of the formation of



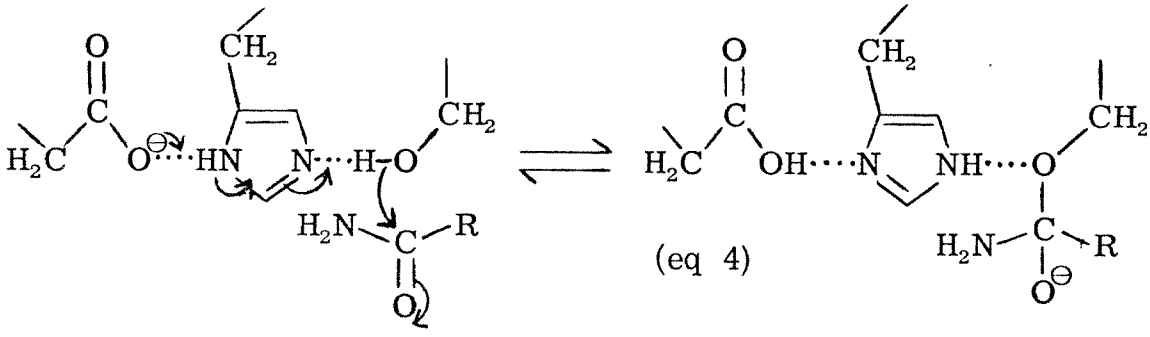
(eq 3)

the tetrahedral intermediate (7) have finessed the necessity for such charge separation by structures which show the relevant protons ambiguously bonded to both nitrogen and oxygen, its occurrence seems logically inescapable as long as the aspartic acid carboxyl is a significantly stronger acid (pK_a < 4) than the imidazolium ring of the histidine (pK_a = 6.7).

Such charge separation in the hydrophobic, nonpolar region of the active site should raise the energy required to form the tetrahedral intermediate and as a result seems a teleologically

unsatisfactory aspect of catalysis.

On the other hand, if, as the results of the present work show, the aspartic acid carboxyl is actually a weaker acid (pK_a = 6.7) than the imidazolium ring of the histidine (pK_a < 4), formation of the tetrahedral intermediate will be accompanied by proton transfer from the serine hydroxyl to N^{τ} of the histidine which will be concerted with transfer of the proton at N^{π} of the histidine to the carboxylate anion of aspartate. Charge separation will not be required. The negative charge on the carboxylate anion will be transferred fully to the carbonyl oxygen of the substrate and, from there, quite probably to other groups on the enzyme such as the backbone NH groups of Ser 195 and Gly 193 in chymotrypsin (7) by hydrogen bond formation to the oxyanion of the tetrahedral intermediate (eq 4).



(eq 4)

Thus, the carboxylate anion serves not just as a passive residue whose sole role is to keep the imidazole ring properly oriented, but rather as the ultimate base which accepts the proton taken from the serine hydroxyl upon formation of the tetrahedral intermediate. The imidazole ring serves a dual function. First, it insulates the carboxylic acid from water and thereby ensures it

a hydrophobic environment such that its pK_a is raised to 6.7. This makes the conjugate carboxylate anion unusually basic. Second, the imidazole ring, by virtue of being a bidentate acid-base, provides a relay for net transfer of a proton from the serine hydroxyl to the buried, basic carboxylate anion. The precise positioning and immobility of the imidazole ring demonstrated by the relaxation and NOE measurements are undoubtedly important in facilitating this proton transfer (63).

As the pK_a of the carboxylic acid group of 6.7 is so close to that of a normal imidazolium cation of histidine, one can ask what purpose is served in having a catalytic triad of Asp-His-Ser rather than a simpler diad of His-Ser. In the case of the His-Ser diad, the imidazole ring will be the ultimate acceptor of the proton which, for a nucleophilic addition to a carbonyl group, must inevitably lead to charge separation. The Asp-His-Ser triad, in contrast, can smoothly transfer a negative charge of the carboxylate anion through the imidazole ring and serine hydroxyl to the carbonyl oxygen of the substrate--no charge separation is required. Such factors as destabilization of the carboxylate anion of Asp 102 in the Michaelis complex (as evidenced by the unusually high pK_a of 6.7 we observe for Asp 102) in addition to stabilization of the tetrahedral intermediate by hydrogen bonding and the lack of charge separation during its formation, probably account in significant measure for the catalytic efficiency of the serine proteases.

REFERENCES

1. B. M. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, Nature (London), 214, 652 (1967).
2. D. M. Blow, J. J. Birktoft, and B. S. Hartley, Nature (London), 221, 337 (1969).
3. M. O. J. Olson, N. Nagabhushan, M. Dzwiniel, L. B. Smillie, and D. R. Whitaker, Nature (London), 228, 438 (1970).
4. A. R. Fersht and Y. Requena, J. Amer. Chem. Soc., 93, 7079 (1971).
5. A. R. Fersht, D. M. Blow, and J. Fastrez, Biochemistry, 12, 2035 (1973).
6. J. D. Robertus, J. Kraut, R. A. Alden, and J. J. Birktoft, Biochemistry, 11, 4293 (1973).
7. J. J. Birktoft and D. M. Blow, J. Mol. Biol., 68, 187 (1972).
8. M. L. Bender, G. E. Clement, F. J. Kezdy, and H. Heck, J. Amer. Chem. Soc., 86, 3680 (1964).
9. C. H. Johnson and J. R. Knowles, Biochem. J., 101, 56 (1966).
10. G. P. Hess, J. McConn, E. Ku, and G. McConkey, Phil. Trans. Roy. Soc. Lond. B, 257, 89 (1970).
11. W. H. Cruikshank and H. Kaplan, Biochem. J., 130, 1125 (1972).

12. D. H. Meadows, J. L. Markley, J. S. Cohen, and O. Jardetzky, Proc. Nat. Acad. Sci. U. S., 58, 1307 (1967).

13. D. H. Meadows, C. C. K. Roberts, and O. Jardetzky, J. Mol. Biol., 45, 491 (1969).

14. K. Wuthrich, R. G. Shulman, and T. Yamane, Proc. Nat. Acad. Sci. U. S., 61, 1199 (1968).

15. S. Ogawa and R. G. Shulman, Biochem. Biophys. Res. Commun., 42, 9 (1971).

16. M. A. Raftery, W. H. Huestis, and F. Millett, Cold Spring Harbor Symp. Quant. Biol., 36, 541 (1971).

17. W. H. Huestis and M. A. Raftery, Proc. Nat. Acad. Sci. U. S., 69 (1887).

18. W. Horsley, H. Sternlicht, and J. S. Cohen, J. Amer. Chem. Soc., 92, 680 (1970).

19. A. Allerhand, R. F. Childers, R. A. Goodman, E. Oldfield, and X. Ypern, Amer. Lab., 4, 19 (1972).

20. R. B. Moon and J. H. Richards, Proc. Nat. Acad. Sci. U. S., 69, 2193 (1972).

21. I. M. Chaiken, M. H. Freedman, J. R. Lyerla, and J. S. Cohen, J. Biol. Chem., 248, 884 (1973).

22. D. T. Browne, G. L. Kenyon, E. L. Packer, H. Sternlicht, and D. M. Wilson, J. Amer. Chem. Soc., 95, 1316 (1973).

23. M. W. Hunkapiller, S. H. Smallcombe, D. R. Whitaker, and J. H. Richards, Biochemistry, 12, 4732 (1973).

24. M. W. Hunkapiller, S. H. Smallcombe, D. R. Whitaker, and J. H. Richards, J. Biol. Chem., 248, 8306 (1973).

25. D. R. Whitaker, Methods Enzymol., 19, 599 (1970).

26. M. O. Dayhoff, Atlas Protein Sequence Structure, 5, D-101 (1972).

27. A. D. McLachlan and D. M. Shotton, Nature (London) New Biol., 229, 202 (1971).

28. H. Kaplan, V. Dugas, and D. R. Whitaker, Can. J. Biochem., 48, 649 (1970).

29. H. L. Oppenheimer, B. Labouesse, and G. P. Hess, J. Biol. Chem., 241, 2720 (1966).

30. M. Renard and A. R. Fersht, Biochemistry, 12, 4713 (1973).

31. D. R. Whitaker and C. Roy, Can. J. Biochem., 45, 911 (1967).

32. R. C. Thompson and E. R. Blout, Biochemistry, 12, 44 (1973).

33. H. Kaplan and D. R. Whitaker, Can. J. Biochem., 47, 305 (1969).

34. J. H. Ashley and R. Harrington, J. Chem. Soc., 2586 (1930).

35. H. Heath, A. Lawson, and C. Rimington, J. Chem. Soc., 2215 (1951).

36. L. Ornstein, Ann. N. Y. Acad. Sci., 121, 321 (1964).

37. R. Freeman and H. D. W. Hill, J. Chem. Phys., 54, 3367 (1971).

38. K. F. Kuhlmann, D. M. Grant, and R. K. Harris, J. Chem. Phys., 52, 3439 (1970).

39. D. Doddrell, V. Glushko, and A. Allerhand, J. Chem. Phys., 56, 3683 (1972).

40. J. A. Pople, W. J. Schneider, and K. J. Bernstein, "High-Resolution Nuclear Magnetic Resonance," New York, N. Y., McGraw-Hill, 1959, p. 122.

41. S. Rajender, R. Lumry, and M. Han, J. Phys. Chem., 75, 1375 (1971).

42. J. Fastrez and A. R. Fersht, Biochemistry, 12, 1067 (1973).

43. A. D. Buckingham, T. Schaeffer, and W. G. Sneider, J. Chem. Phys., 32, 1227 (1972).

44. F. J. Weigert and J. D. Roberts, J. Amer. Chem. Soc., 90, 3543 (1968).

45. R. J. Pugmire and D. M. Grant, J. Amer. Chem. Soc., 90, 4232 (1968).

46. W. F. Reynolds, I. R. Peat, M. H. Freedman, and J. R. Lyerla, J. Amer. Chem. Soc., 95, 328 (1973).

47. M. Krieger, L. M. Kay, and R. M. Stroud, J. Mol. Biol., in press.

48. A. R. Fersht and J. Sperling, J. Mol. Biol., 74, 137 (1973).

49. A. N. Glazer and E. L. Smith, Enzymes, Third Ed., 3, 501 (1971).

50. H. Edelhoch, J. Amer. Chem. Soc., 80, 6640 (1958).

51. R. Lumry and S. Rajender, J. Phys. Chem., 75, 1387 (1971).

52. K. L. Gammon, S. H. Smallcombe, and J. H. Richards, J. Amer. Chem. Soc., 94, 4573 (1972).

53. S. H. Smallcombe, K. L. Gammon, and J. H. Richards, J. Amer. Chem. Soc., 94, 4581 (1972).

54. K. L. Gammon, Ph. D. Thesis, California Institute of Technology, Pasadena, California (1973).

55. G. Robillard and R. G. Shulman, J. Mol. Biol, 71, 507 (1972).

56. A. Tulinsky, R. L. Vandlen, C. N. Morimoto, N. V. Mani, and L. H. Wright, Biochemistry, 12, 4185 (1973).

57. R. L. Vandlen and A. Tulinsky, Biochemistry, 12, 4193 (1973).

58. R. Egan, H. O. Michael, R. Schlueter, and B. J. Jandorf, Arch. Biochem. Biophys. Acta, 94, 535 (1957).

59. J. A. Gladner and H. Neurath, J. Biol. Chem., 206, 911 (1954).

60. K. C. Aune and S. N. Timasheff, Biochemistry, 10, 1609, (1971).

61. G. C. Levy and G. L. Nelson, "Carbon-13 Nuclear Magnetic Resonance for Organic Chemists," New York, N. Y., Wiley-Interscience, 1972, p.6.

62. J. W. Emsley, J. Feeny, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," New York, N. Y., Pergamon Press, 1965, p. 22.

63. J. H. Wang, Science, 161, 328 (1970).

PART II

Ionization Behavior of Enzymic and Inhibitor Groups in the Tetrahedral Adduct between α-Lytic Protease and a Peptide Aldehyde

INTRODUCTION

In order to provide a complete description of the processes involved in enzymic catalysis, one would like ideally to examine the structure of the enzyme and its substrate at each of the numerous stages of reaction. The most interesting and informative of these phases of the catalytic sequence, the transition states or high points in the free energy profile of the reaction, are, due to their transient nature, also the most difficult to observe (1, 2). Yet, since the enzyme functions by providing a reaction pathway in which the transition states are stabilized relative to those in the non-enzymic reactions, one must determine the peculiar characteristics of the transition states in the enzymic process before he understands the source of the power of these catalysts (see, for example, the statement by Linus Pauling quoted in reference 2).

Occasionally, one can infer characteristics of the transition states from the nature of intermediates whose finite lifetime is sufficiently long to allow their trapping or direct observation. This is most readily done, however, with poor substrates for which some step of the reaction is abnormally slow compared to that for good, specific substrates (3, 4). It is obvious, though, that great care must be exercised in interpreting the normal process based on analogy to the abnormal case. Therefore, the most reliable data obtainable may describe a phase of the reaction which is even one step further from the transition states than the meta-stable intermediates--the ground state of the enzyme.

The difficulties involved in characterizing even this "free enzyme" are hardly trivial, however, and in no case are they more apparent than in that of the serine proteases. These enzymes, partly because of their ready availability in purified form, have been the subject of numerous studies for many years. Particularly fruitful have been the crystallographic work of several groups (David Blow, M.R.C. at Cambridge; Bob Stroud and Richard Dickerson, Caltech; Joseph Kraut, U. C. San Diego; and Robert Huber, Max Planck Institute), which are excellent illustrations of the value of x-ray diffraction techniques for unraveling the structural secrets of proteins.

Blow et al. (5) were the first to observe the precisely arranged grouping of aspartic acid carboxyl, histidine imidazole, and serine hydroxyl now known to be an essential feature of the catalytic locus of these enzymes. Their hypothesis of a "charge relay system" in which the buried negative charge of the carboxylate was channeled through the imidazole ring to the serine hydroxyl to generate a highly reactive alkoxide nucleophile was an important contribution to mechanistic discussions. However, soon after their proposal was offered, it became apparent that it did not adequately explain the enzymes' catalytic power. The same alignment of these residues was discovered in chymotrypsinogen, the inactive precursor of chymotrypsin (6). Furthermore, the serine alkoxide anion would seem to be too strong and indiscriminate a nucleophile to be an integral part of an enzyme designed to function under physiological conditions. Therefore, the role of

the carboxylate was reduced to that of an anchor for the imidazole group (7). The apparent high reactivity of the hydroxyl group was ascribed to its precise orientation relative to the susceptible bond brought about by binding of part of the substrate in the enzymes' specificity pockets. The imidazole was thought to function as a general base/general acid to first remove the proton from the hydroxyl and then transfer it to the substrate leaving group.

Robertus et al. (8), however, observed another feature common to the catalytic locus of these enzymes which they interpreted as essential to catalysis--the "oxyanion hole." This structure consists of two hydrogen bond donors (the backbone NH groups of a glycine and serine residue in chymotrypsin). Its role is to stabilize the transient negative charge thought to develop on a substrate's carbonyl oxygen as the sp^2 hybridization of the carbonyl carbon changes to sp^3 upon attack of the serine hydroxyl group of the enzyme (9, 10). With the discovery of this oxyanion hole, it has become apparent once again that one must consider carefully the factors which might stabilize the transition states (or intermediates which closely resemble the transition states) when attempting to explain the catalytic mechanism of serine proteases. The nmr studies described in this section and in Part I of this thesis have allowed a reevaluation of these phenomena.

Part I of this thesis described our efforts to define the free enzyme ionization behavior of the crucial enzymic groups. The essence of the results of these studies is a reassignment of

the relative basicities of the carboxylate and imidazole groups. Our interpretation of the observed nmr parameters of the imidazole C-2 carbon of the catalytically essential histidine residue of α-lytic protease assigns a pK_a of 3.3 to the imidazolium group and a pK_a of 6.7 to the carboxyl group. The importance of this assignment becomes clear when one considers the development of ionic charge on the catalytic groups and substrate during the reaction process. If the imidazole were a stronger base than the carboxylate, then transfer of the hydroxyl proton to N^{τ} of the histidine ring would produce an imidazolium cation (in addition to the negative charge on what was the substrate carbonyl oxygen as the hydroxyl oxygen bonds to the carbonyl carbon). This separation of charge that would develop during the reaction hardly seems consistent with a process that must proceed with facility in the largely apolar environment of the catalytic locus. Since, however, the carboxylate is the stronger base, it can abstract a proton from N^{π} of the imidazole ring as the hydroxyl proton is transferred to N^{τ}. This concerted process can effectively minimize charge development during formation of the tetrahedral intermediate, and it includes an active rather than a passive role for the evolutionarily conserved carboxylate.

In view of the attractiveness of our interpretation of the microscopic ionization behavior of the catalytic groups in mechanistic terms, we sought a means to confirm our hypothesis. The ideal experiment would involve determination of the ionization state of the groups in the tetrahedral intermediate formed during

substrate hydrolysis. Unfortunately, direct observation of this state seems impossible with presently available techniques. An intriguing approach to the problem is available, however, through the use of peptide aldehydes to generate transition state analogs (11, 12). These compounds exhibit binding affinities 100-1000 times greater than those of the corresponding peptides whose C-terminal group is an amide, ester, or alcohol, and their unusually inhibitory power is thought to be due to reversible formation of a tetrahedral adduct (hemiacetal) with the serine hydroxyl. Many aldehydes are unusual among carbonyl compounds in preferring to exist as a tetrahedral addition complex, hydrate or hemiacetal, in aqueous or alcoholic media rather than as the free aldehyde. If, in fact, the peptide aldehydes bind as a tetrahedral adduct, then the complex may well resemble the tetrahedral intermediate formed during substrate hydrolysis. Consequently, an examination of the ionization behavior of the catalytic histidine residue when the enzyme is complexed with the aldehyde should provide insight into the catalytic process. For this reason, we undertook a study, using cmr spectroscopy, of the effect of binding of N-Ac-L-Ala-L-Pro-L-alaninal on the catalytic groups of α-lytic protease, and the results are reported below.

EXPERIMENTAL SECTION

Preparation of ^{13}C-enriched α-Lytic Protease. The procedure used for production and purification of ^{13}C-labeled α-lytic protease was essentially the same as that described in Part I of this thesis. Somewhat higher enrichment was obtained by adding the L-[2-^{13}C]-histidine in six 50-mg aliquots per liter of culture medium at 18-hr intervals during a 96-hr growth period.

N-Acetyl-L-alanyl-L-prolyl-L-alaninal. The procedure used for synthesis of this compound was (with slight modification) that described by Thompson (12).

(i) L-Proline Methyl Ester. L-Proline (25 g, 0.22 mol) was dissolved in 200 ml of absolute methanol and heated on a steam bath. Dry hydrogen chloride was bubbled through the suspension for 1 hr, and the solvent was removed by rotary evaporation. The oily residue was dissolved in 50 ml of ice-cold water, neutralized by addition of solid Na_2CO_3, and extracted with three 250-ml portions of chloroform. The combined chloroform solutions were dried over Na_2SO_4, filtered, and rotary evaporated at room temperature to remove the solvent. The residue was vacuum distilled to give a colorless liquid (bp 33°, 1 Torr). Yield: 18.3 g (65%).

(ii) N-Acetyl-L-alanyl-L-proline. L-Proline methyl ester (18.3 g, 0.13 mol) and N-acetyl-L-alanine (18.6 g, 0.13 mol) were dissolved in 300 ml of chloroform and cooled in an ice bath. Dicyclohexylcarbodiimide (32.1 g, 0.16 mol) was added, and the

mixture was stirred overnight at 4°. The solution was filtered, and the solvent was removed by rotary evaporation. The residue was extracted with 150 ml of warm water, and the solution was filtered and rotary evaporated to dryness. The residue was dissolved in 300 ml of ethanol and 33 ml of 5 N NaOH, and the solution was stirred for 2 hr. The pH was adjusted to pH 1 by addition of 6 N HCl, and the solvent was removed by rotary evaporation. The residue was extracted with 500 ml of chloroform, and the solution was dried over Na_2SO_4, filtered, and rotary evaporated to give a white solid which was crystallized from chloroform/n-hexane. Yield: 19.2 g (59%).

(iii) N-Acetyl-L-alanyl-L-prolyl-L-alaninol. N-Acetyl-L-alanyl-L-proline (8.5 g, 0.037 mol) and N-methylmorpholine (4.2 ml, 0.037 mol) were dissolved in 400 ml of acetonitrile. The solution was cooled to -20°, and isobutyl chloroformate (4.8 ml, 0.037 mol) was added with stirring. After 5 min, L-alaninol (5.5 ml, 0.074 mol) was added, and the mixture was stirred for 5 hr at room temperature. The solvent was removed by rotary evaporation, and the residue was dissolved in 100 ml of water. The solution was stirred, and Rexyn I-300 resin was added until the resin was no longer neutralized by the solution. The solution was filtered, and the solvent was removed by rotary evaporation to give an amorphous, white solid. Crystallization from ethyl acetate gave a colorless product (mp 174-176°), pure by thin layer chromatography on silica gel (90% chloroform/ 10% methanol). Yield: 7.5 g (71%).

(iv) N-Acetyl-L-alanyl-L-prolyl-L-alaninal. N-Acetyl-L-alanyl-L-prolyl-L-alaninol (7.5 g, 0.026 mol) and dicyclohexylcarbodiimide (16.1 g, 0.078 mol) were dissolved in 115 ml of ethanol-free chloroform and dimethylsulfoxide (11.4 ml, 0.156 mol). Eight 0.8-ml aliquots of dichloroacetic acid were added over a period of 2 hr. The mixture was then cooled to -20° for 2 hr, the dicyclohexylurea was filtered off, and the filtrate was extracted with three 250-ml portions of water. The combined aqueous solutions were rotary evaporated (40°) to give a gummy, yellow residue. This was extracted repeatedly with ether until it became a powdery, odorless solid. One gram of the solid was dissolved in a few ml of chloroform and chromatographed on a 1.5 x 100 cm column of silica gel (90% chloroform/10% methanol). A small amount (50 mg) of the product (R_f = 0.6) was eluted from the column. The silica gel was then washed with methanol, chloroform, and 90% chloroform/10% methanol. The remaining solid (5 g) was then chromatographed as before. Fractions containing the R_f = 0.6 material were pooled and rotary evaporated to remove solvent. The solid residue was dissolved in $CDCl_3$, filtered, and rotary evaporated to give 2.5 g (33%) of product. The nmr spectrum of this material in $CDCl_3$ showed a characteristic aldehyde proton resonance (9.6 ppm downfield from tetramethylsilane) which was removed by the addition of a drop of methanol. The product was stored as a 2 M aqueous solution at 0°.

Inhibition Studies. Inhibition of α-lytic protease-catalyzed hydrolysis of N-benzoyl-L-alanine methyl ester by N-Ac-L-Ala-L-Pro-L-alaninol and N-Ac-L-Ala-L-Pro-L-alaninal was measured with a pH stat (Radiometer Model TTT1c). Reactions were conducted in 10 ml of 0.10 M KCl at 34.0 ± 0.2°. The pH of the reaction mixture was maintained by addition of 0.0300 N KOH. Michaelis-Menten kinetics were observed, and K_I was evaluated from Dixon plots (13).

Nmr Spectra. Cmr spectra were recorded on a Varian XL-100-15 nmr spectrometer as described in Part I of this thesis. Directly bonded C-H coupling constants for the histidine C-2 carbon were determined by the difference spectra technique also described in Part I. Peak positions were determined both by using the Varian 16K Fourier transform program marking routine (which extrapolates to the highest point of the peak) and by measuring the center of the peak at half-maximum intensity. The two methods agreed to within ±1 Hz.

RESULTS

Kinetic Results. The reported specificity of α-lytic protease closely resembles that of porcine elastase (14), although kinetic studies with the bacterial enzyme have been limited to single amino acid derivatives. The affinity of both enzymes for such substrates is exceptionally weak even though their turnover (k_{cat}) rates are reasonably fast. Thompson and Blout (15) have shown, however, that, for elastase, several peptides containing a C-terminal alanine (amide or ester) with one or more proline or alanine residues attached to it exhibit both stronger binding affinities and higher k_{cat} values than compounds containing only an alanine derivative. Further, their studies suggest that inclusion of proline in the peptide tends to favor a single rather than multiple binding modes (16).

The results of our preliminary studies suggest that the specificity similarities between the two enzymes may be extended to include preference for extended chain length and proline in the position adjoining the C-terminal alanine. The data shown in Table I reveal that N-Ac-L-Ala-L-Pro-L-AlaOMe (k_{cat} = 384 sec^{-1}, K_M = 1.1 x 10^{-3} M) is a remarkably better substrate for α-lytic protease than the best of the substrates previously tested, N-benzoyl-L-alanine methyl ester (k_{cat} = 23 sec^{-1}, K_M = 0.035 M). K_M is less by a factor of 1/32 , and k_{cat} is greater by a factor of 17 .

TABLE I. Kinetic Parameters for α-Lytic Protease-catalyzed Hydrolysis of Peptide Esters and α-Lytic Protease Binding of Peptide Alcohols and Aldehydes [a]

Peptide	pH	K_I (M)	K_M (M)	k_{cat} (sec^{-1})
N-Ac-L-Ala-L-Pro-L-AlaOMe	8.0		1.1×10^{-3}	384
	5.0		8.0×10^{-3}	33
N-Ac-L-Ala-L-Pro-L-Alaninol	8.0	1.6×10^{-2}		
	5.0	1.4×10^{-1}		
N-Ac-L-Ala-L-Pro-L-Alaninal	8.0	5.6×10^{-5}		
	5.0	8.0×10^{-4}		

[a] K_I values are for inhibition of α-lytic protease-catalyzed hydrolysis of N-Benzoyl-L-AlaOMe at 34° in 0.2 M KCl.

The basic similarity between the two enzymes is also demonstrated by α-lytic protease's susceptibility to inhibition by peptide aldehydes (Table I). The much stronger binding of N-Ac-L-Ala-L-Pro-L-alaninal (K_I = 5.6 x 10^{-5} M) than of either the corresponding ester (for which the observed K_M = 1.1 x 10^{-3} M is considerably less than the true K_S since deacylation is rate-limiting for ester hydrolysis) or the corresponding alcohol (K_I = 0.016 M) lend further support to proposals that aldehydes do in fact possess such affinity for the serine proteases that they may reasonably be classed as transition state analogs. Additional arguments regarding this point are detailed by Thompson (12). It will, at any rate, be assumed in the following discussion that the α-lytic protease/aldehyde complex involves covalent bond formation between the aldehyde and the catalytic serine hydroxyl to produce a tetrahedral addition compound (hemiacetal), although unequivocable proof of this hypothesis requires confirmation by x-ray diffraction techniques.

Magnetic Resonance Parameters. Figure 1 shows the low-field region of the proton noise-decoupled cmr spectrum of α-lytic protease (enriched with ^{13}C at C-2 of the histidine residue) saturated (greater than 98% of the enzyme in solution is complexed) with N-Ac-L-Ala-L-Pro-L-alaninal at pH 4.60, 6.25, and 8.60. Figures 2 and 3 show proton coupled cmr spectra (pH 4.60 and 8.60, respectively) from which values of $^1J_{CH}$ can be calculated. We assign the resonance whose chemical shift changes from

FIGURE 1. ^{13}C-enriched, Proton Noise Decoupled Cmr Spectra of α-Lytic Protease/Peptide Aldehyde Complex. Enzyme, 4 mM; N-Ac-L-Ala-L-Pro-L-alaninal, 50 mM; 0.2 M KCl; 34°. Spectra were recorded using a 5000 Hz sweepwidth, 0.15 sec acquisition time, and 90° pulse. pH 4.60 spectrum, 190,000 transients; pH 6.25 spectrum, 80,000 transients; pH 8.60 spectrum, 90,000 transients.

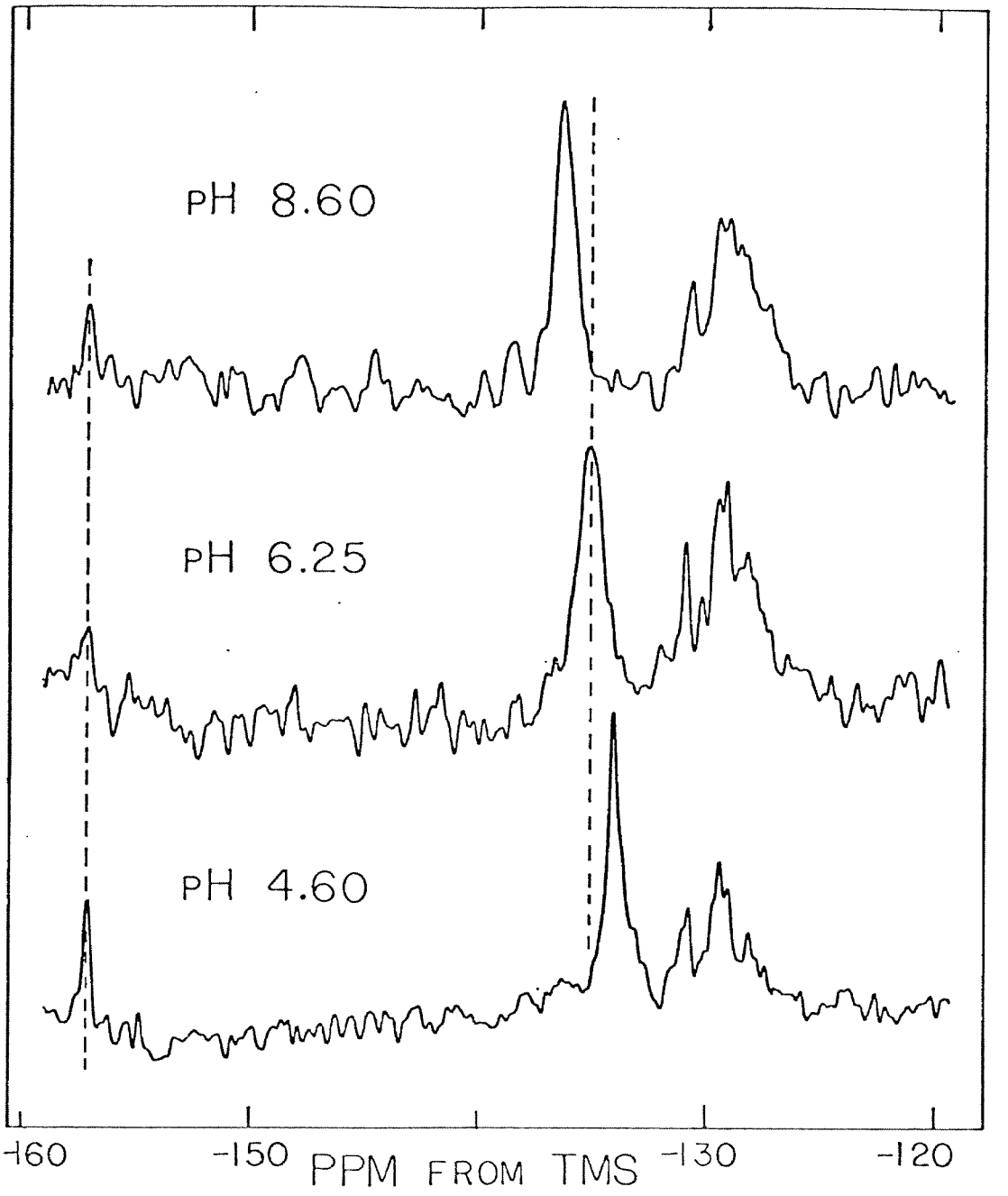

pH 8.60
pH 6.25
pH 4.60
-160
-150
PPM FROM TMS
-130
-120

FIGURE 2. Measurement of $^{1}J_{CH}$ for Histidine C-2 in α-Lytic Protease/Peptide Aldehyde Complex at pH 4.60. (a) Proton coupled cmr spectra of 4 mM α-lytic protease and 50 mM N-Ac-L-Ala-L-Pro-L-alaninal. (——) ^{13}C-enriched enzyme (His 57 C-2). (---) ^{13}C-natural abundance enzyme. (b) Difference spectrum obtained by computer subtraction of the natural abundance spectrum (---) from ^{13}C-enriched spectrum (——) of (a).

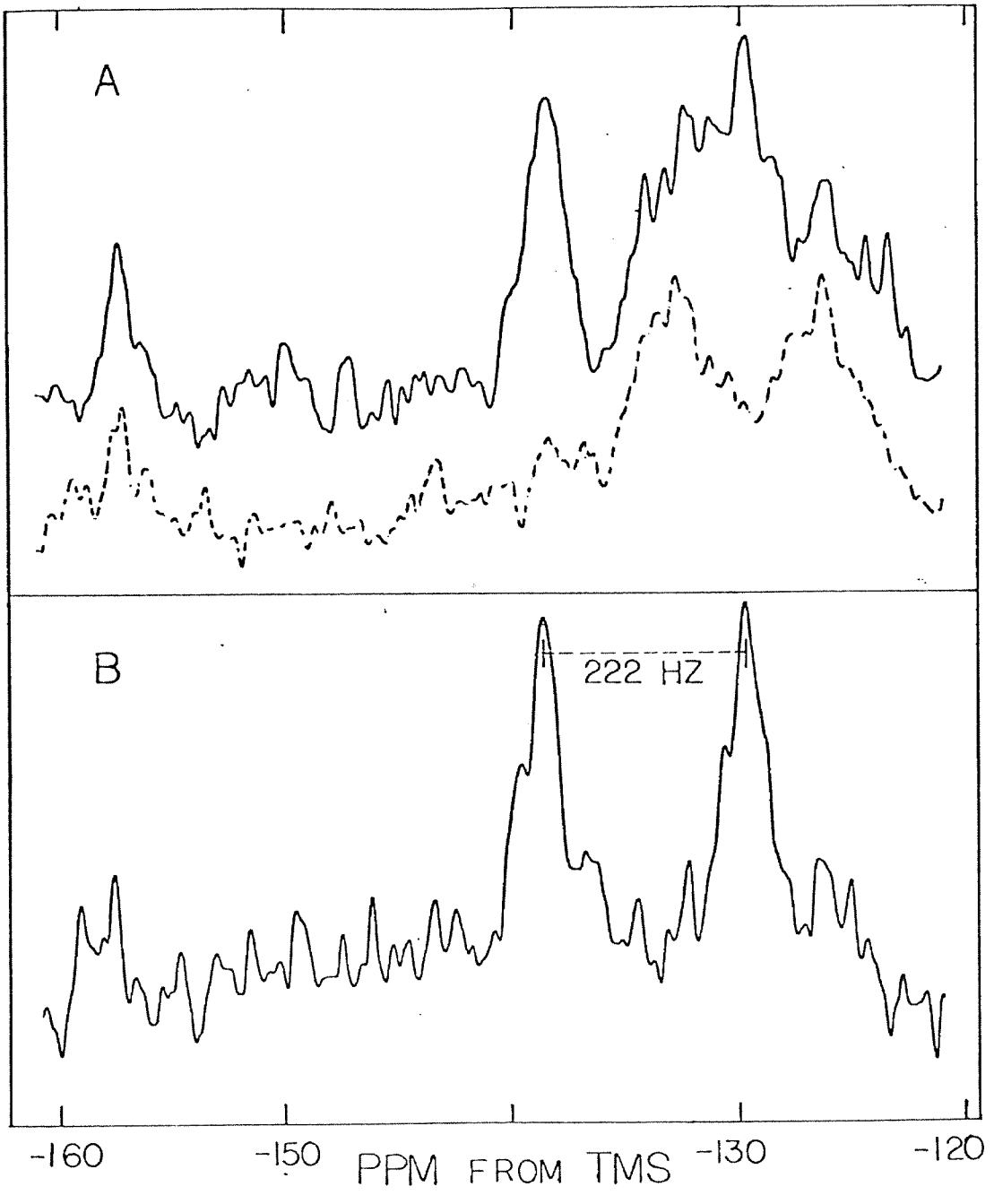
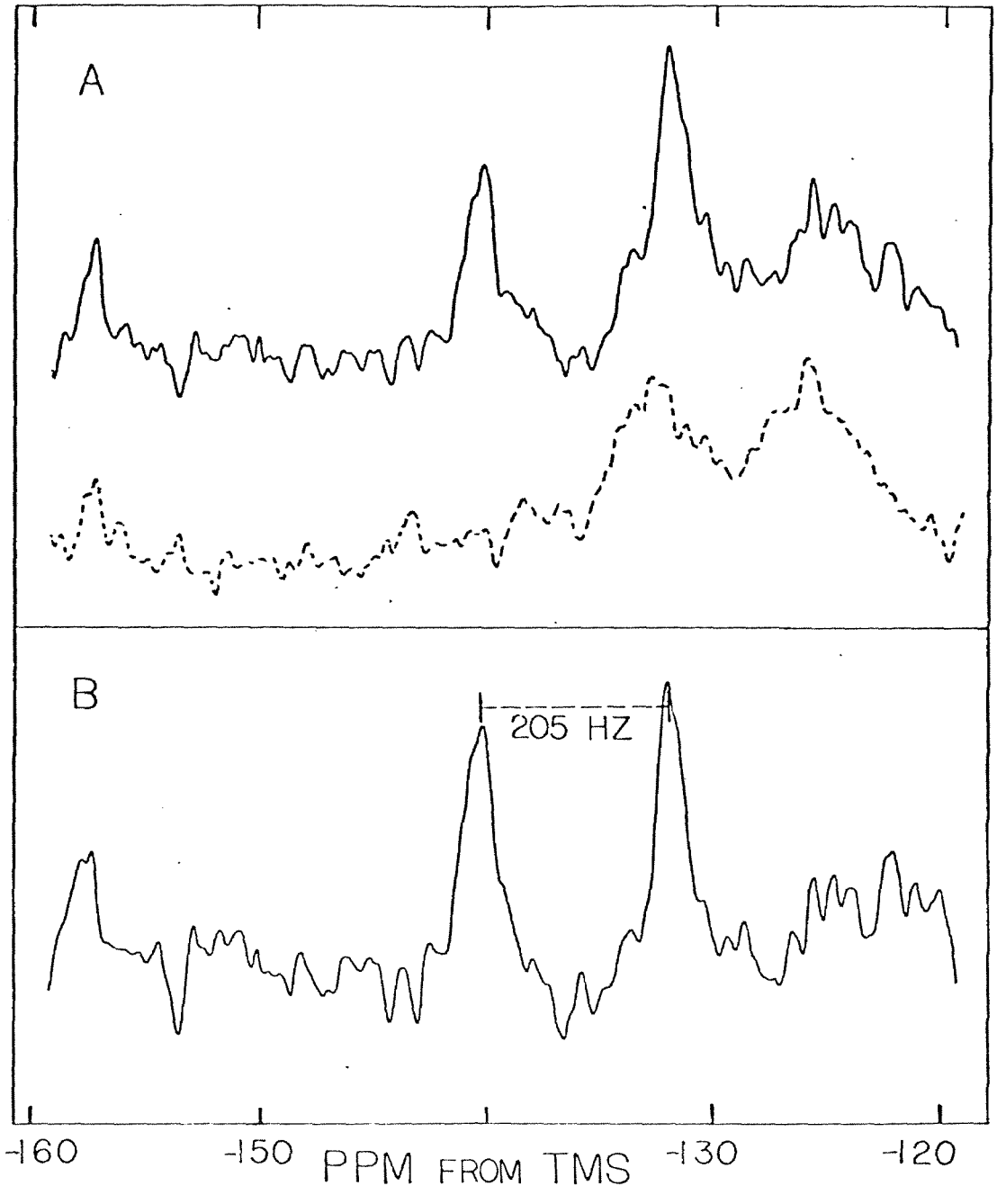

A
B
222 HZ
-160
-150
PPM FROM TMS
-130
-120

FIGURE 3. Measurement of $^{1}J_{CH}$ for Histidine C-2 in α-Lytic Protease/Peptide Aldehyde Complex at pH 8.60. (a) Proton coupled cmr spectra of 4 mM α-lytic protease and 50 mM N-Ac-L-Ala-L-Pro-L-alaninal. (——) ^{13}C-enriched enzyme (His 57 C-2). (---) ^{13}C-natural abundance enzyme. (b) Difference spectrum obtained by computer subtraction of the natural abundance spectrum (---) from ^{13}C-enriched spectrum (——) of (a).


A
B
205 HZ
-160
-150
PPM FROM TMS
-130
-120

-134.11 to -136.33 ppm and whose $^1J_{CH}$ changes from 222 to 205 Hz between pH 4.6 and 8.5 to the histidine C-2 carbon. It is important to point out that the chemical shift is pH-independent from pH 2-5 and from pH 7.5-9. The observed change in chemical shift (-2.22 ppm) occurs over a narrow pH range centered at pH 6.25 (Figure 4). Furthermore, we do not observe the multiplicity of resonances for the histidine C-2 in spectra of aldehyde-complexed enzyme at pH $<$ 4 as we do in spectra of the native enzyme at low pH (Figure 5, Part I of this thesis, p.37). The δ and $^1J_{CH}$ values for the C-2 nucleus in the pH-independent regions are listed in Table II along with comparable values for C-2 in the native enzyme.

Titration Results. The change in chemical shift as a function of pH ostensibly resembles a titration curve with a midpoint at pH 6.25. Analysis of the data in terms of an ionization equilibrium between two species is unsatisfactory, however, since it predicts a significantly flatter sigmoid curve than is actually observed. A mathematical treatment of such ionization behavior has been presented by Huestis (17). She noted that curves with a sharp slope can be obtained if two or more strongly interacting groups ionize in the same range. In order to analyze the data, one can consider the case of two interacting, ionizable groups with the microscopic ionization constants defined as below:

FIGURE 4. Chemical Shift of Histidine C-2 Resonance of α-Lytic Protease/Peptide Aldehyde Complex as a Function of pH.
(o) observed chemical shift; (---) theoretical curve calculated for single group ionization using pK_a = 6.25 and Δ = 56 Hz;
(——) theoretical curve calculated for two interacting ionizations (see text) using $pK_{A1} = pK_{B1} = 7.0$, $pK_{A2} = pK_{B2} = 5.5$, and Δ = 56 Hz.

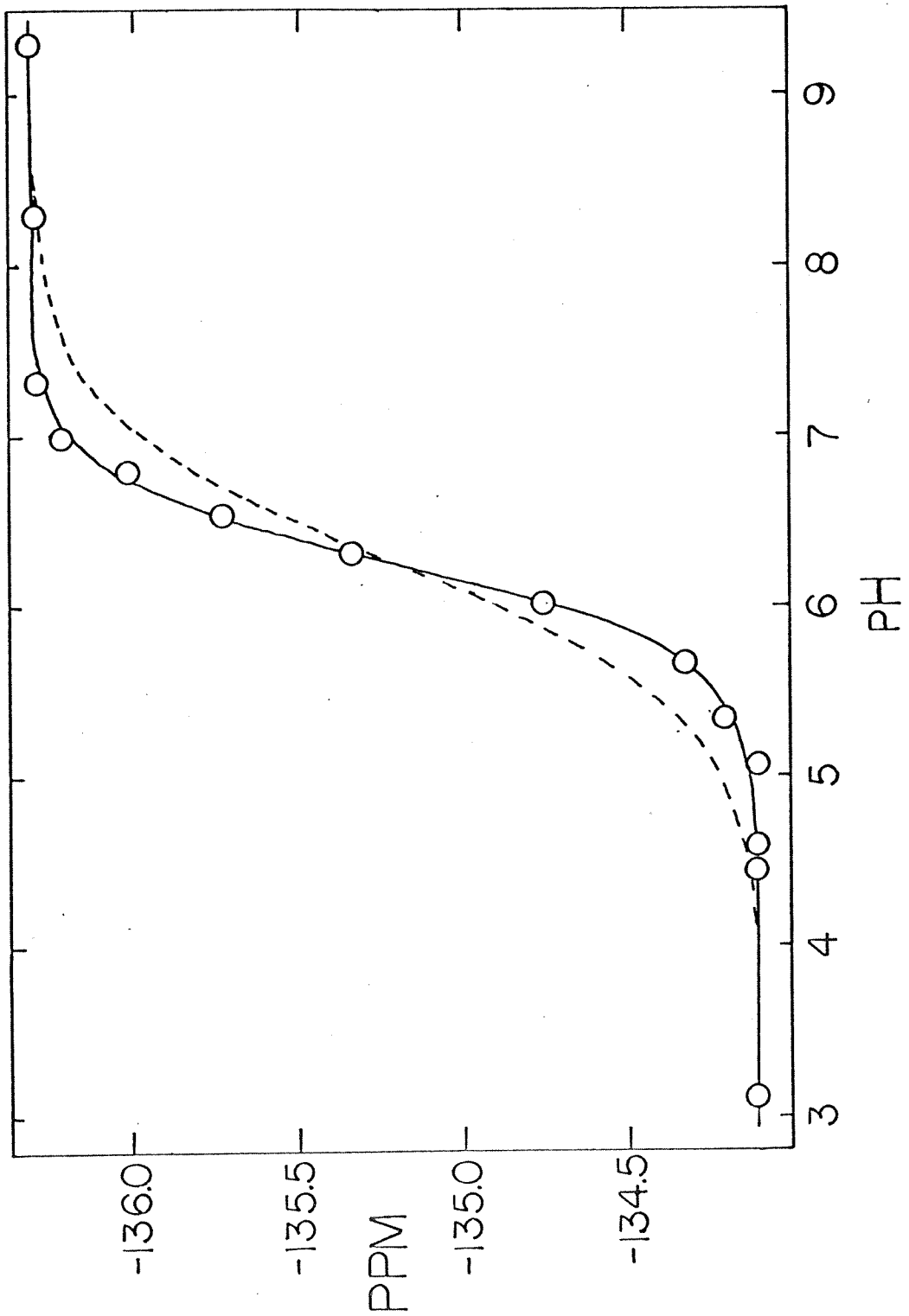

-136.0
-135.5
-135.0
-134.5
PPM
3
4
5
6
7
8
9
PH

TABLE II: Chemical Shift and Coupling Constant Values for C-2 Carbon in Histidine Residue of α-Lytic Protease, α-Lytic Protease/ Peptide Aldehyde Complex, and Denatured β-Lytic Protease

Enzyme	pH	Chemical Shift (ppm ± 0.08 from TMS)	$^1J_{CH}$ (Hz ± 3)
α-lytic protease/aldehyde [a]	8.2	-136.33	205
α-lytic protease/aldehyde [a]	4.2	-134.11	222
α-lytic protease [b]	8.7	-137.26	205
α-lytic protease [b]	4.7	-134.79	205
α-lytic protease [b]	3.3	-134.81	206
		-134.05	222
		-132.46	220
β-lytic protease (denatured) [c]	8.4	-136.67	206
β-lytic protease (denatured) [c]	4.4	-134.09	220

[a] Enzyme, 4 mM; N-Ac-L-Ala-L-Pro-L-alaninal, 50 mM; 0.2 M KCl; 34°.

[b] 5-6 mM enzyme in 0.2 M KCl.

[c] 1-2 mM enzyme in 0.2 M KCl.

$$HA\sim\sim BH \overset{K_{A1}}{\rightleftharpoons} {}^{\ominus}A\sim\sim BH + H^{\oplus}$$

$$HA\sim\sim B^{\ominus} \overset{K_{A2}}{\rightleftharpoons} {}^{\ominus}A\sim\sim B^{\ominus} + H^{\oplus}$$

$$HA\sim\sim BH \overset{K_{B1}}{\rightleftharpoons} HA\sim\sim B^{\ominus} + H^{\oplus}$$

$$K_{A1}K_{B2} = K_{A2}K_{B1}$$

$${}^{\ominus}A\sim\sim BH \overset{K_{B2}}{\rightleftharpoons} {}^{\ominus}A\sim\sim B^{\ominus} + H^{\oplus}$$

The condition necessary for a sharp slope is that ionization of one acidic group depresses the pK_a of the other--that is, $pK_{A1} > pK_{A2}$. The opposite condition in which ionization of one group elevates the pK_a of the other (as is observed for dicarboxylic acids such as maleic acid) actually produces a titration curve with a less steep slope at the midpoint of the titration. In the present case, the observed data can be fitted quite satisfactorily by a theoretical curve calculated using $pK_{A1} = pK_{B1} = 7.0$ and $pK_{A2} = pK_{B2} = 5.5$ (although it must be pointed out that equally good fits can be obtained by considering more than two ionizable groups whose interactions are not quite so strong). Therefore, in interpreting the observed titration data, one must consider situations which could give rise to the seemingly unusual pK_a shifts.

DISCUSSION

Aldehydes as Transition State Analogs. An essential aspect of transition state theory as it applies to enzymic reactions is that in the transition state the substrate is bound much more tightly to the enzyme than it is in the Michaelis-Menten complex (2). Numerous studies (9,10) have suggested that a tetrahedral addition compound is an obligatory intermediate along the pathway of hydrolysis of specific amide and ester substrates catalyzed by serine proteases, and it is likely that this tetrahedral intermediate closely resembles the true transition state for the reaction. In this regard, Robertus et al. (8) have suggested that three strong hydrogen bonds may be formed between the enzyme and substrate in the tetrahedral intermediate which are either weak or nonexistent in the more stable acyl enzyme intermediate and the Michaelis-Menten complex. Two of these hydrogen bonds are formed between the substrate oxyanion (ground state carbonyl oxygen) and two hydrogen bond donors, the backbone NH groups of Gly 193 and Ser 195 in chymotrypsin. The other is formed between the substrate α-amido (NH) group and the backbone carbonyl of Ser 214. All three result from rearrangements necessitated by the change in hybridization ($sp^2 \rightarrow sp^3$) of the substrate carbonyl carbon. Their existence, as well as that of the tetrahedral intermediate itself, has been confirmed by the x-ray diffraction studies of Ruhlmann et al. (18) who determined the crystal structure of the trypsin/pancreatic trypsin inhibitor

complex. The remarkable stability of this protein/protein interaction ($K_{diss} = 10^{-14}$ M) apparently arises partly because of covalent bond formation between the active site serine (Ser 195) and the carbonyl group of an inhibitor lysine (Lys 15I) residue. The crystal structure clearly displays covalent bonding between the Lys 15I carbonyl carbon and both the amido nitrogen of Ala 16I and O^{γ} of Ser 195. The structure is apparently trapped by steric blocking (caused by bulky side chain of Cys 14I) of movement of the His 57 side chain to a position where it can transfer its N^{τ} proton to the Ala 16I amido nitrogen. The three hydrogen bonds predicted by Robertus et al. (8) are present in the crystal structure, and the ability of the "oxyanion hole" to stabilize an otherwise unstable structure is obvious.

Since the source of the stability, and hence tighter binding, of the tetrahedral intermediate is apparent, one can expect that compounds which can mimic the essential structure of the intermediate should act as potent, reversible inhibitors of enzymic action. Thompson (12) recognized that aldehydes, which readily form tetrahedral addition complexes with alcohols, should serve as transition state (or at least high-energy intermediate) analogs providing they otherwise satisfy the specificity requirements of the enzyme. Thus, he demonstrated that with elastase two peptide aldehydes, N-Ac-L-Ala-L-Pro-L-alaninal and N-Ac-L-Pro-L-Ala-L-Pro-L-alaninal, exhibit K_I values approximately 1/1000 that of the K_S values for the corresponding peptide amides (which are excellent substrates) and K_I values for the corresponding peptide

alcohols (which are only moderately effective inhibitors). We have demonstrated that one of these peptide aldehydes is also an effective inhibitor for α-lytic protease, whose specificity closely resembles that of elastase. Shimizu et al. (19) and Ito et al. (11) have also shown that appropriate peptide aldehydes strongly inhibit both chymotrypsin and trypsin. Thus, the inhibitor potency of these compounds seems to be a general phenomenon for serine proteases, and the most likely explanation for it is their ability to form a stable, covalent tetrahedral complex with the enzymic Ser 195 hydroxyl group (12). The detailed nature of this covalent structure, in particular the distribution of charge on the various atoms, has not been established, however, and it is to this question that our present nmr investigations are directed.

Ionization Behavior of Enzymic and Inhibitor Groups in α-Lytic Protease/Peptide Aldehyde Complex. The tetrahedral complex contains three potentially ionizable groups in the pH range of catalytic activity (Asp 102 carboxyl, His 57 imidazole, and hemicacetal hydroxyl), and determination of the actual ionization behavior of these groups may provide insight into the mechanism by which serine proteases catalyze hydrolysis of ester and amide bonds. The ^{13}C-enriched C-2 carbon of the His 57 imidazole serves as a valuable probe for monitoring this ionization behavior since several nmr parameters (chemical shifts, C-H coupling constants, and relaxation times) respond to ionization of the imidazole and neighboring groups. The $^{1}J_{CH}$ value for the

histidine C-2, as shown in Part I of this thesis, is the most reliable of these parameters as as indicator of the state of ionization of the imidazole itself. We have measured $^1J_{CH}$ for C-2 (His 57) in the aldehyde complex in the pH ranges 4-5.5 and 7-8.5. The results clearly show that in the former, pH 4-5.5, the histidine is cationic ($^1J_{CH}$ = 222 Hz), while in the latter, pH 7-8.5, it is neutral ($^1J_{CH}$ = 205 Hz). This implies that in the aldehyde complex the histidine has an apparent pK_a in the range of pH 5.5-7, and this observation is consistent with the change in chemical shift also seen in this pH range.

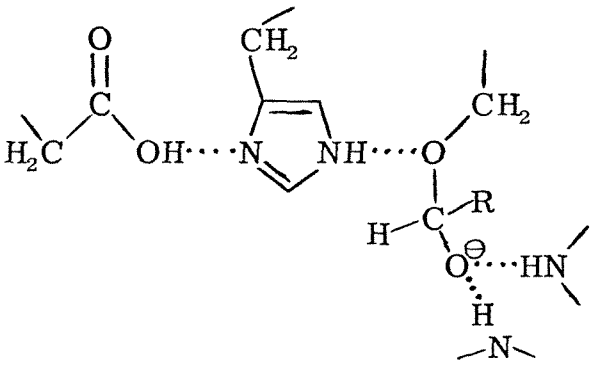
As might be expected at this point, however, the observed ionization behavior is vastly more complex than simple titration of the imidazole group. Although the observed pH dependence of the C-2 chemical shift appears at first glance to represent an ordinary titration curve, mathematical analysis suggests that the empirical curve represents ionization of at least two strongly interacting groups. A simple, one-group ionization would produce a curve with a much less steep slope at the midpoint of the titration than is actually observed. Such steep titration curves arise when two (or more) acidic groups interact in such a way that ionization of one depresses the pK_a of the other. The result of this cooperative ionization is a destabilization of the two singly ionized species relative to the doubly ionized or nonionized species. In other words, ionization of one group tends to force ionization of the other. Since one of the acidic groups is the histidine imidazolium cation, we may be able to determine the other group if we

can show that its ionization would produce the observed perturbation on the pK_a of the histidine.

In attempting to answer this question, we are aided by observation of a pH dependent change in another nmr parameter for the C-2 carbon, its relaxation behavior. The spectra in Figure 1 show that the resonance observed at pH 4.6 is significantly sharper than that observed at the higher pH values. The narrow line width, together with an observed NOE of 1.6, suggest considerable side chain mobility in this pH range (see Part I of this thesis, p. 52). In fact, the chemical shift (-134.11 ppm) and line width (10 Hz) are almost identical to those for a histidine imidazolium group on a random coil polypeptide (denatured β-lytic protease, Table II). It seems likely, then that the histidine imidazolium group in the aldehyde-complexed enzyme exists not as a tightly anchored part of the catalytic triad but as a relatively freely moving group that has been ejected into a solution-like environment. Ionization of the histidine residue involves not only a change in charge on the ring but also a change in the position and mobility of the entire side chain.

With these ideas in mind, we can consider the source of the ionization which exhibits cooperativity with that of the histidine. First, we will consider what we feel to be the most likely group, the hemiacetal hydroxyl. If the aldehyde complex actually mimics the true tetrahedral intermediate, then in the pH range of maximum catalytic activity (pH $>$ 7) the hemiacetal should contain an oxyanion stabilized by hydrogen bonds to the two backbone NH groups in the

oxyanion hole. Further, based on our knowledge of the relative pK_a's of Asp 102 and His 57 in the free enzyme (pK_a Asp > pK_a His), we can predict that both the aspartic acid carboxyl and the histidine imidazole in the complex would be neutral. The system should be stabilized by the same hydrogen bonding network shown in the crystal structure of the trypsin/pancreatic trypsin inhibitor complex (18). Thus, the imidazole would be held in place by hydrogen bonds between N^{π} and Asp 102 O^{δ} and between N^{τ} and Ser 195 O^{γ}. It seems reasonable that addition of a proton

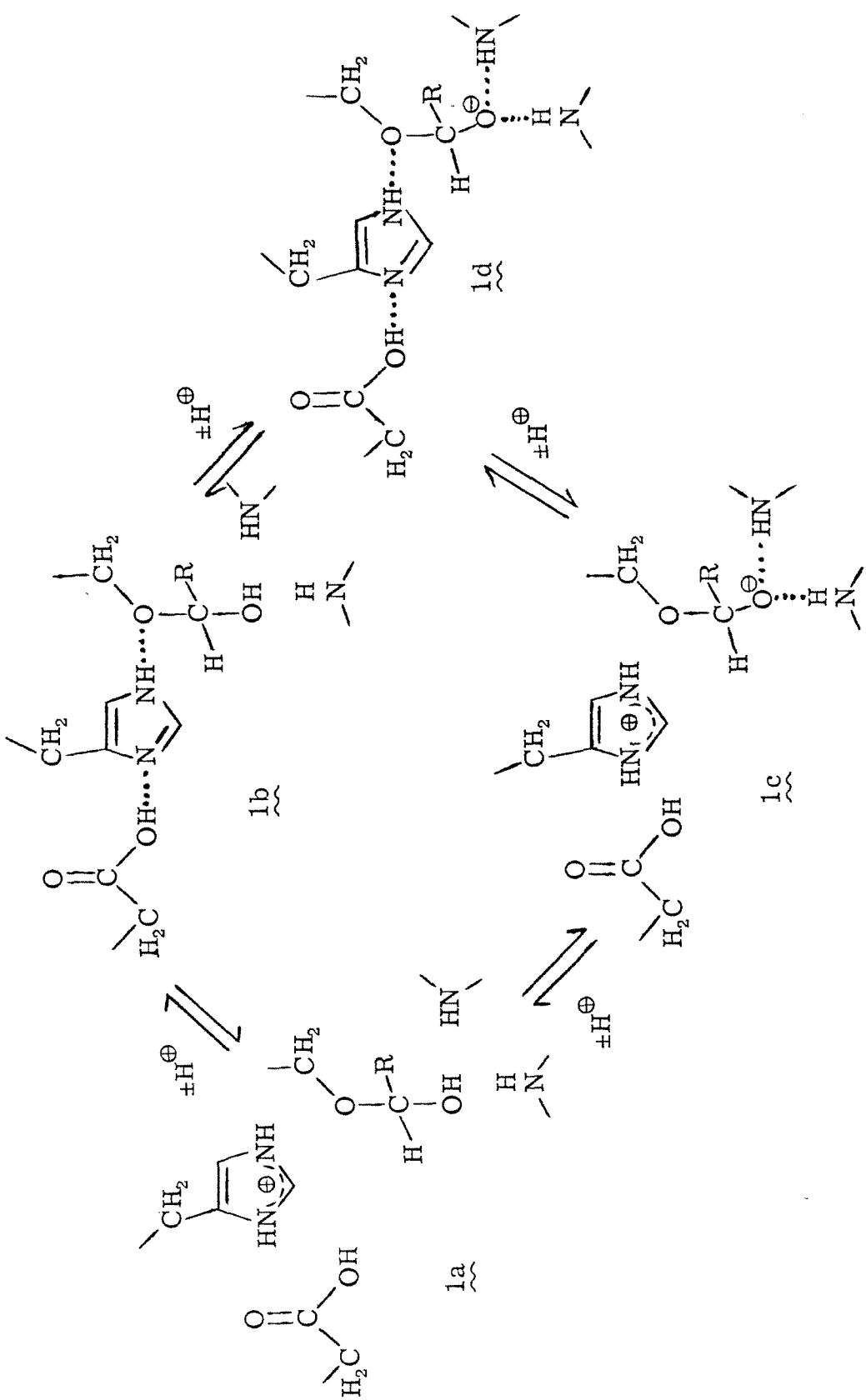


to this system, either at N^{π} or at the oxyanion, should lessen the stability of the system. The steric strain caused by the protonation could be relieved either by expulsion of the aldehyde from the enzyme or by a rotation of the histidine out towards solution and away from Ser 195 and Asp 102. Since the aldehydes are still potent inhibitors below pH 6 (though K_I is larger by a factor of 14 than that at pH 8), the complex itself does not seem to disperse. This suggests, then, that the strain caused by the addition of a proton is relieved by displacement of the histidine side chain into solution. Such a movement is entirely consistent with the observed nmr parameters, as described above, and has

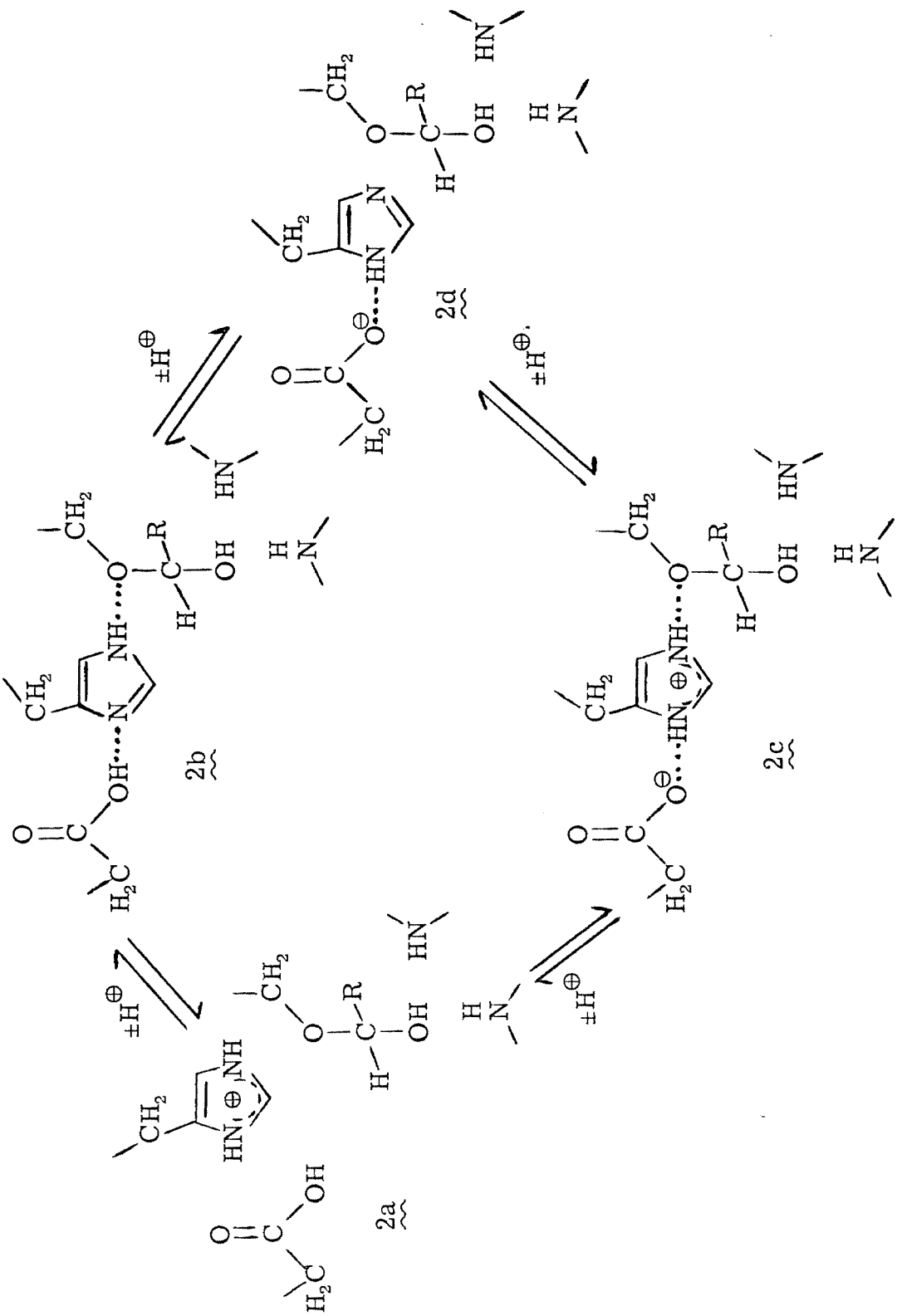
been observed in x-ray diffraction studies of other inhibited forms of serine proteases, including tosyl-Ser 195 elastase (20), phenylmethanesulfonyl-Ser 221 subtilisin (21), and silver (I)-Asp 102 trypsin (22). Moreover, since both the histidine and the oxyanion would then be more fully exposed to solvent, both would tend to become protonated. In other words, addition of a proton to either group triggers a rearrangement which promotes protonation of the other. The system has two stable configurations, and the equilibrium between the two involves the net, cooperative loss or gain of two protons (Scheme I). We cannot accurately describe the processes involved in this equilibrium in terms of several microscopic ionization constants because we can observe separately only two of the species (1a and 1d) shown in Scheme I. However, if our interpretation of the system is correct, it is remarkable testimony to the intricate structural design that the enzymes have evolved in order to stabilize a high-energy intermediate along their reaction pathway.

Before accepting the above description, however, we must also consider the possibility that the source of the cooperative ionization may be the aspartic acid carboxyl. If Asp 102 were the source of the second ionization, then the hemiacetal hydroxyl would be neutral throughout the observed pH range, and the ionizations could be depicted as in Scheme II. However, we do not believe such a scheme adequately predicts our experimental results. In particular, one might expect reasonable stability for at least one of the intermediate species (2b and 2c) since

Scheme I



Scheme II



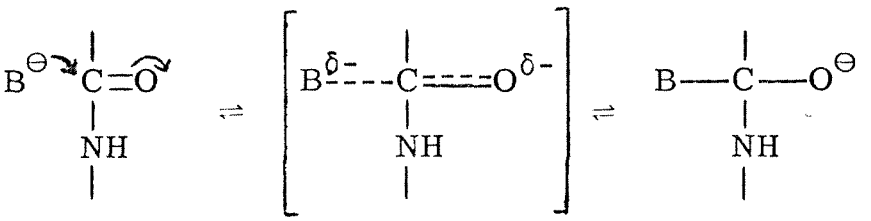
hydrogen bonding possibilities for each are greater than for the other two species (2a and 2d). This is not consistent with the observed cooperativity of the two ionizations. There is no apparent reason why ionization of either Asp 102 or His 57 should promote ionization of the other. Comparison of 2d with 1d also suggests that 1d has the greater potential for hydrogen bonding. At least one and possibly three hydrogen bonds may be formed in 1d which are absent in 2d. They could easily explain the greater stability of the hemiacetal oxyanion 1d relative to that of the carboxylate anion 2d. In other words, they may be responsible for the apparently low pK_a of the hemiacetal and the abnormally high pK_a of Asp 102. It must be emphasized, however, that one cannot logically assign pK_a's for either Asp 102 or the hemiacetal as separate entities. Their apparent ionization behavior must be viewed in the context of the entire complex of which they are part. One seeming energetically unfavorable structure (the negative charge on the hemiacetal rather than on the carboxyl group) can be compensated for by other favorable interactions (the hydrogen bonding network, for example) in the complex. It must be remembered that one strong hydrogen bond (3.2 kcal) can compensate for a shift in pK_a of as much as 2.5 pK_a units. Thus, it is not unreasonable to conclude that the apparent pK_a of Asp 102 in the complex has been raised considerably above its pK_a of 6.7 in the free enzyme. If our interpretation of the cmr data is correct, we have not been able to observe the Asp 102 ionization in the complex up to pH 9 (cmr studies above

this pH are prevented by instability of the aldehyde in basic, pH > 10, aqueous solution). Although we cannot yet say with certainty that His 57 and the hemiacetal, rather than His 57 and Asp 102, are the groups involved in the cooperative ionization around pH 6.25, we feel that their ionizations are more likely to produce the experimental results we have obtained. (One final point that should be noted is that the observed chemical shift [-136.33 ppm] for C-2 of His 57 [neutral] in the aldehyde complex at pH 7.5-9 is intermediate between the δ values for His 57 in the free enzyme at pH 4-5.5 [neutral His 57, neutral Asp 102, δ = -134.79 ppm] and at pH 8-9.5 [neutral His 57, anionic Asp 102, δ = -137.26 ppm]. Therefore, the chemical shift value does not provide as much assistance in deciding whether Asp 102 is ionized in the aldehyde complex as we might have hoped. However, the observed value is at the very least not inconsistent with our interpretation.)

The Catalytic Mechanism of Serine Proteases--An Overview. In detailing the factors responsible for the catalytic efficacy of serine proteases, we take particular note of the structural features which seem to have been tailored to stabilize what otherwise would be high-energy transition states and intermediates along the reaction coordinate between substrate and product. Indeed, one of the most striking characteristics of these proteins is their system of discontinuous environments for the various catalytic groups and substrate moieties. The ordered multi-phased network creates a

pathway for the enzymic reaction which avoids the high-energy states that would otherwise plague the reaction (hydrolysis of peptide bonds) in a physiological milieu. Its catalytic power is attested to by its appearance in two otherwise unrelated groups of proteins, the chymotrypsin family and the subtilisin family, in which almost identical catalytic loci have developed through a process of convergent evolution (8).

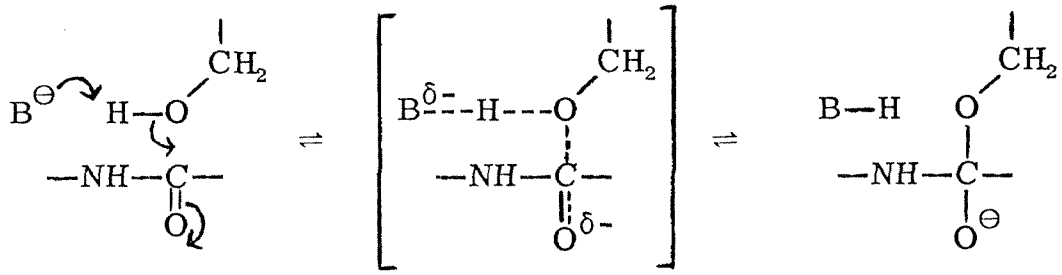
It appears that a prerequisite for enzymic action is the removal of the reacting groups, both substrate and enzyme, from homogeneous solvation by the external medium (which is essentially water). Otherwise, for the enzymic groups to be effective as catalysts they would need to be so reactive that they would be indiscriminate and hence unstable. The reactants in this largely hydrophobic environment must therefore be tailored to produce transition states compatible with the low dielectric constant immediately surrounding them. Since the most obvious requirement is that the transition states should involve a minimum of charge development, we might suppose that for hydrolysis of a neutral species such as an amide bond the attacking nucleophile should be negatively charged. However, amino acid groups which



could produce anionic species under physiological conditions have serious drawbacks as nucleophilic catalysts. One of these, the

cysteine sulfhydryl, is too unstable to provide the basis for a very efficient catalytic system. The other, the aspartic (or glutamic) acid carboxyl, produces an anionic species (carboxylate anion) which is generally so stable that it is not an effective nucleophile at the slightly alkaline pH where serine proteases function. Thus, the enzymes have been forced to utilize an intricate, but elegant, scheme which promotes a weak, neutral nucleophile (a serine hydroxyl group) into a potent catalyst by providing a facile pathway to a well-stabilized transition state.

The essential feature of this process is that an anionic species (carboxylate anion), by acting as a general base, can assist the nucleophilic attack through concerted removal of the proton from the hydroxyl group during bond formation between the hydroxyl oxygen and substrate carbonyl carbon. The efficiency of

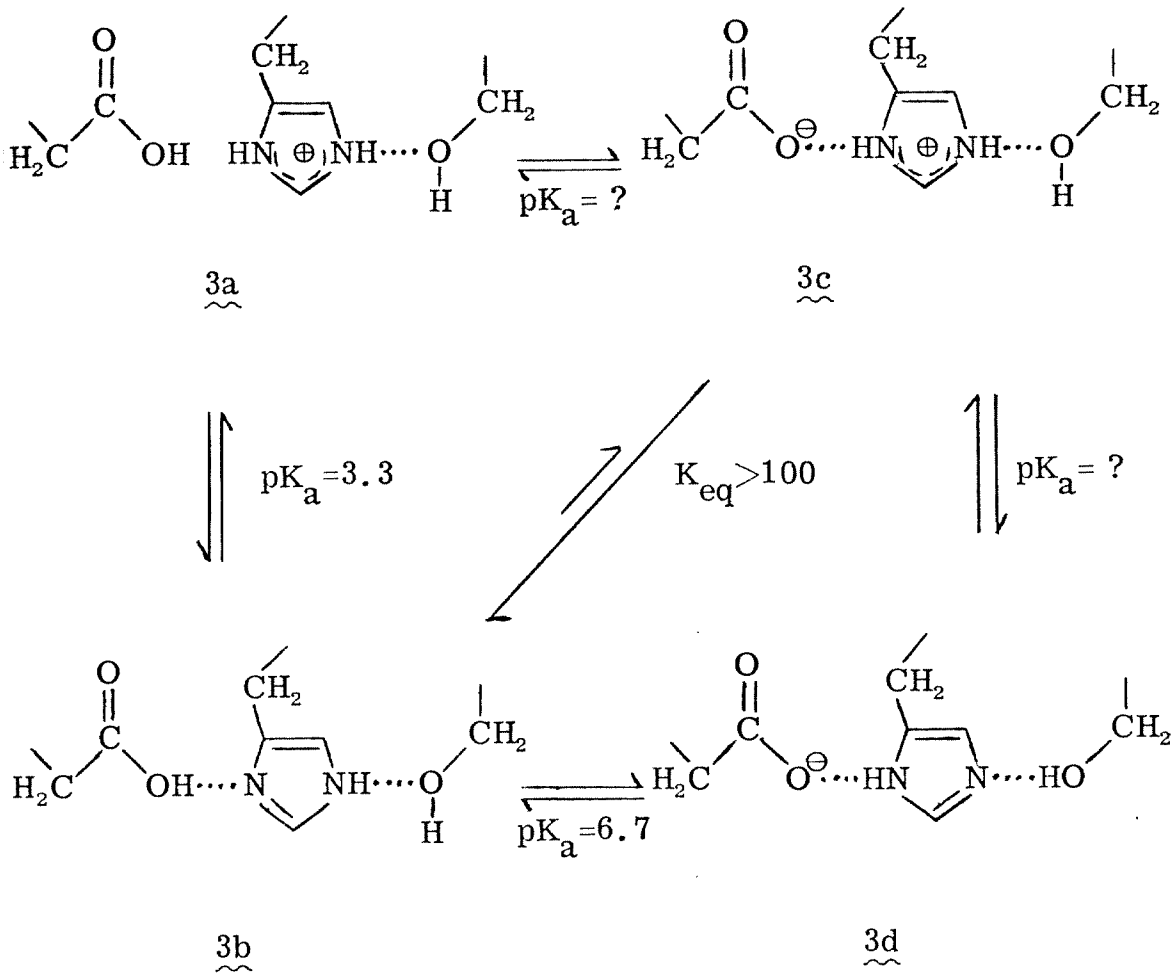


the proton abstraction will be related to the basicity of the carboxylate, and this characteristic can be manipulated by controlling the microenvironment of the base. Hence, if the local dielectric around the carboxylate is low, then the basicity of the group will be greater than if it were exposed fully to the aqueous solvent. However, if the carboxylate is shielded from solvent, it must also be removed from contact with the hydroxyl group

which must have adequate access to substrate and, hence, solvent. Consequently, the carboxylate/solvent barrier must be capable both of insulating the carboxylate and of relaying the proton from the hydroxyl to the buried negative charge. The histidine imidazole fulfills this role admirably if, and this point is crucial, it is a weaker base than the carboxylate. Since the basicity of the imidazole is also a function of the local environment, the enzyme can (and in the case of the serine proteases apparently does) meet this requirement.

The importance of the enzymes' ability to provide distinct, discontinuous microscopic environments for the two neighboring basic groups lies in this need to regulate their relative basicities. In the serine proteases, the local environments serve to raise the effective pK_a of the carboxyl group above that of the imidazolium cation. This is achieved partly by surrounding the two groups with hydrophobic amino acid residues and partly by alignment of a series of hydrogen bond donors and acceptors which can stabilize certain species (carboxyl/imidazole) relative to others (carboxylate/imidazolium) (4). We must remember that in discussing pK_a's of acidic groups which interact strongly with other groups we cannot dissociate the acidic group from the interacting complex of which it is a part. Thus, we cannot assign microscopic pK_a's for several possible equilibria because we can never detect the presence of some of the species(for example, 3c in Scheme III). Scheme III is even much simpler than the actual situation since (at the very least) we should also consider conformational and

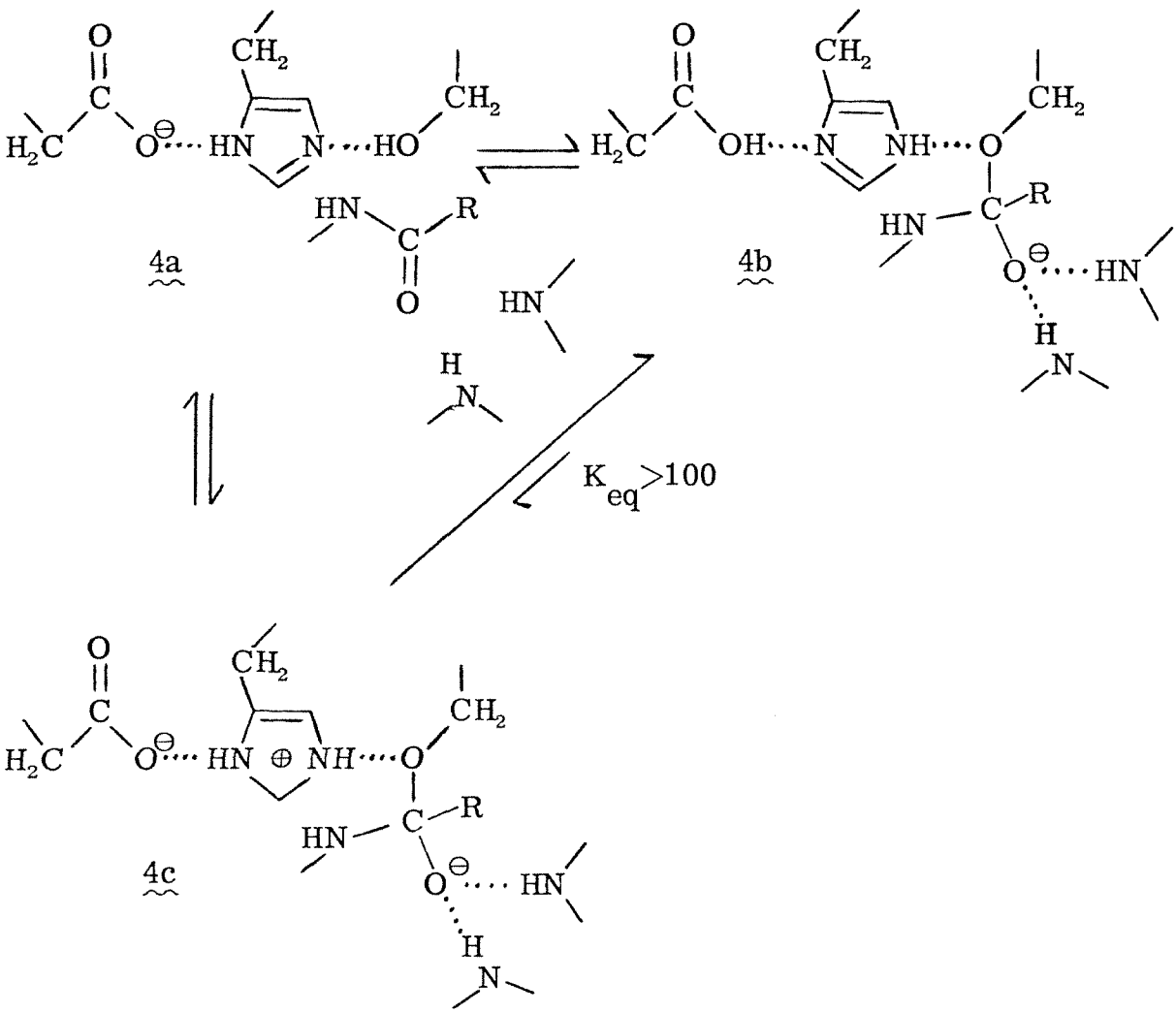
Scheme III



ionization equilibria for the various species in which the imidazole ring is displaced from its buried position toward solvent where interaction with the other groups is minimized.

We can, however, define the relative stabilities of several species in the free enzyme, and from these comparisons we can predict the contribution of analogous species to the structure of the transition states and intermediates involved in catalysis. Therefore, since species 3b is more stable than 3c, we can predict that the intermediate 4b is more stable than 4c (Scheme IV). Although we do not yet have direct evidence which confirms this prediction, the apparent relative stabilities of related species in which the amide is replaced by an aldehyde (see preceding section of this discussion) support our hypothesis. The aldehyde/enzyme complex in its "active" state (that is, the complex at pH $>$ 7) certainly contains a neutral imidazole rather than an imidazolium cation, and it very likely contains a neutral carboxyl and hemiacetal oxyanion. If this latter hypothesis proves correct, then the enzyme has designed a structure which accommodates and stabilizes a negative charge on the tetrahedral adduct at the expense of neutralizing the buried carboxylate. This is not really too surprising since the microenvironment of the tetrahedral oxyanion is complementary to a negative charge. It consists of two hydrogen bond donors (oxyanion acceptors), Gly 193 and Ser 195 backbone NH groups, which can diffuse the charge. Moreover, the entire complex is stabilized by a network of hydrogen bonds, including those between Asp 102 O^{δ} and His 57

Scheme IV



N^{π}, His 57 N^{τ} and Ser 195 O^{γ}, and Ser 214 carbonyl and substrate α-amido.

While it may prove difficult to assign meaningful pK_a's to the various ionizable species in the tetrahedral complex (whether the complex includes aldehyde or amide), the serine proteases almost certainly achieve catalysis by stabilizing this intermediate and the transition states leading to and from it. (The observation that the primary nitrogen isotope effect for chymotrypsin-catalyzed amide hydrolysis ($k_{14}{}_N/k_{15}{}_N = 1.010$) is very small is confirmatory evidence for the existence of the tetrahedral intermediate and the high degree of resemblance between it and the transition states.) Thus, the enzymes have coupled a negatively charged base (carboxylate) through an insulating relay (imidazole) with a weak, neutral nucleophile (hydroxyl) to produce a catalytic system which functions not by generating a highly reactive, highly unstable nucleophile but by promoting a pathway for a series of concerted proton migrations which effectively minimize charge development during the transition state. Stabilization of the transition state by formation of additional hydrogen bonds between enzymic and substrate moieties complements this bond-making/bond-breaking process so that the real difference in the free energy of the ground state and that of the transition state, the key to catalysis, is remarkably small.

REFERENCES

1. W. P. Jencks, Cold Spring Harbor Symp. Quant. Biol., 36, 1 (1971).
2. G. E. Lienhard, I. I. Secemski, K. A. Koehler, and R. N. Lindquist, Cold Spring Harbor Symp. Quant. Biol., 36, 45 (1971).
3. S. A. Bernhard and S.-J. Lau, Cold Spring Harbor Symp. Quant. Biol., 36, 75 (1971).
4. J. J. Birktoft and D. M. Blow, J. Mol. Biol., 68, 187 (1972).
5. D. M. Blow, J. J. Birktoft, and B. S. Hartley, Nature, 221, 337 (1969).
6. S. T. Freer, J. Kraut, J. D. Robertus, H. T. Wright, and N. H. Xuong, Biochemistry, 9, 1997 (1970).
7. A. R. Fersht and J. Sperling, J. Mol. Biol., 74, 137 (1973).
8. J. D. Robertus, J. Kraut, R. A. Alden, and J. J. Birktoft, Biochemistry, 11, 4293 (1972).
9. M. Caplow, J. Amer. Chem. Soc., 91, 3639 (1969).
10. A. R. Fersht and Y. Requena, J. Amer. Chem. Soc., 93, 7079 (1971).
11. A. Ito, K. Tokawa, and B. Shimizu, Biochem. Biophys. Res. Commun., 49, 343 (1972).
12. R. C. Thompson, Biochemistry, 12, 47 (1973).
13. M. Dixon, Biochem. J., 55, 170 (1953).

14. D. R. Whitaker, Methods Enzym., 19, 599 (1970).

15. R. C. Thompson and E. R. Blout, Biochemistry, 12, 57 (1973).

16. R. C. Thompson and E. R. Blout, Biochemistry, 12, 51 (1973).

17. W. H. Huestis, Ph. D. Thesis, California Institute of Technology, Pasadena, California, 1972, p. 48.

18. A. Ruhlmann, D. Kukla, P. Schwager, K. Bartels, and R. Huber, J. Mol. Biol., 77, 417 (1973).

19. B. Shimizu, A. Saito, A. Ito, K. Tokawa, R. Maeda, and H. Umezawa, J. Antibiotics, 25, 515 (1972).

20. D. M. Shotton, N. J. White, and H. C. Watson, Cold Spring Harbor Symp. Quant. Biol., 36, 91 (1971).

21. J. Drenth, W. G. J. Hol, J. N. Jansonius, and R. Koekoek, Cold Spring Harbor Symp. Quant. Biol., 36, 107 (1971).

22. R. Stroud, personal communication.

23. M. H. O'Leary and M. D. Kluetz, J. Amer. Chem. Soc., 92, 6089 (1970).

PART III

Kinetics of Pepsin-Catalyzed Hydrolysis of N-Trifluoroacetyl Amino Acids

INTRODUCTION

Porcine pepsin is an endopeptidase with maximal activity at acidic pH. It catalyzes the hydrolysis of a wide range of small synthetic peptides and displays a preference for substrates with hydrophobic substituents on the amino acid residues on both sides of the sensitive bond (1-2). Pepsin does not hydrolyze simple amides or esters of amino acids, nor does it normally cleave N-acetyl or N-carbobenzoxy groups from amino acids (3). Its only reported carboxyl esterase activity is toward the depsipeptide methyl N-carbobenzoxy-L-histidyl-L-p-nitrophenylalanyl-L-β-phenyllactate, in which the ester bond between the p-nitrophenylalanyl and β-phenyllactate residues is cleaved (4). Hydrolysis of sulfite esters can also be catalyzed by pepsin (5). Furthermore, pepsin can catalyze transpeptidation reactions of both the amino (6-7) and acyl (8) transfer type and exchange of ^{18}O between $H_2{}^{18}O$ and the carboxyl group of N-acetyl amino acids (9). All of these catalytic activities (hydrolysis, transpeptidation, and ^{18}O exchange) show similar specificity and probably occur at a single catalytic locus on the enzyme (3).

Knowles (3) and Fruton (2) have proposed a mechanism for pepsin catalysis which involves two enzymic carboxyl groups. They suggest that one of the groups, with an apparent pK_a in the free enzyme of 4.7, protonates the carbonyl oxygen of the sensitive peptide bond while the other, with an apparent pK_a of 1.1, as the anion, nucleophilically attacks the carbonyl carbon of the

protonated amide. The resulting tetrahedral intermediate then undergoes a reversible four-center exchange reaction to liberate the free acid product and generate an amino enzyme intermediate. Subsequent reaction with water liberates the free amine product and regenerates active enzyme. Though attractively simple, this mechanism suffers from several inconsistencies: (i) the as yet unsuccessful isolation of the proposed covalent amino enzyme, (ii) the absence of a kinetic isotope effect upon hydrolysis of dipeptide substrates such as N-Ac-L-Phe-L-TyrOMe (10) [Knowles (3) postulates proton transfer in the rate limiting steps], (iii) indications that more than two carboxyl groups may play important roles at pepsin's active site (11-12), and (iv) the mechanism's inability to allow for transpeptidation of the acyl transfer type.

Clement (13) has proposed a mechanism in which an enzymic carboxylate group acts as a nucleophile toward the carbonyl group of the substrate amide bond to yield a tetrahedral intermediate. Concurrent with breakdown of this intermediate is an attack by the liberated amine anion on an enzymic carboxyl group to yield both amine (amide) and acyl (anhydride) covalent intermediates. Subsequent hydrolysis of the amide and anhydride bonds would lead to formation of products and regeneration of active enzyme. Although this mechanism is in greater agreement than that of Knowles with the experimental observations of pepsin's activity, it is not satisfactorily consistent with the failure to isolate the covalent amino enzyme intermediate and the observation that pepsin's catalytic mechanism seems to involve a significant

electrophilic component (3).

To date, no generally accepted mechanism of pepsin action has been formulated, and progress toward understanding the catalytic process has been hampered by the narrow substrate specificity of the enzyme which permits study of only a very limited class of substrates. The following paper is a report on the activity of pepsin in catalyzing the hydrolysis of some N-TFA-L-amino acids* [an activity serendipituously observed during attempts to study binding of these substances to pepsin by magnetic resonance techniques (14)] and conclusions, based on this catalytic activity, about the mechanism of pepsin action (15).

---

*The abbreviation used is: N-TFA, N-trifluoroacetyl.

EXPERIMENTAL SECTION

Materials. Crystalline porcine pepsin (1:60,000; lot 70C-9280) and pepsinogen (Grade 1; lot 117B-1130) were obtained from Sigma and used without further purification. Standard hemoglobin assay for pepsin (described below) showed an activity of 4100 ± 200 units/mg pepsin. Human gastric juice was supplied by the University of Southern California Medical Center. Human pepsin and gastricsin were prepared by chromatography on Amberlite CG-50 ion exchange resin as described by Richmond et al. (16). N-TFA amino acids were synthesized according to the procedure of Fones (1952). N-TFA-L-Phe had a melting point of 119.5-120.5°. Fones reported mp 119.4-120.6°. Diazoacetylglycine ethyl ester, prepared by diazotizing Gly-GlyOEt hydrochloride according to the procedure of Kozlov et al. (18), had a melting point of 107-108° (lit. mp 107-108°). L-Tryptophan ethyl ester hydrochloride, prepared by treating L-tryptophan with HCl in ethanol, had a melting point of 224-225° [lit. mp 225-226°; (19)]. 2,4'-Dibromoacetophenone was obtained from Sigma. Deuterium oxide (99.7%) was obtained from Columbia Organic Chemicals.

Determination of Rate of Hydrolysis of N-TFA Amino Acids by Pepsin. A weighed sample of the N-TFA amino acid was dissolved in 2.50 ml of 0.040 M sodium citrate buffer (pH 4.5), 0.50 ml of a freshly prepared 5.0 mM pepsin solution was added, and the pH was adjusted to the desired value by addition of 0.20 M

citric acid, 0.20 M sodium citrate, or 0.20 M citric acid-HCl buffer (pH 1.0). The solution was then made up to 5.0 ml by addition of 0.20 M sodium citrate buffer of the desired pH and placed in a water bath maintained at 34 ± 1°. In order to measure the per cent hydrolysis, the solution was transferred to a 12 mm O.D. nmr tube and inserted into a Varian XL-100-15 nmr spectrometer with probe temperature at 34 ±1°. The hydrogen resonance of the solvent (water or deuterium oxide) was used for field-frequency stabilization, and the fluorine spectrum of trifluoroacetate anion and the N-TFA amino acid was time-averaged (four scans) using a Fabritek Model C-1062 time-averaging computer. Digital integration of the fluorine spectrum was also performed with the computer. The per cent hydrolysis for each time point was calculated from the relative areas of the trifluoroacetyl and trifluoroacetate fluorine peaks. The hydrolysis of any given sample was followed until 10-15% hydrolysis had been obtained, and v_o was calculated by fitting the data for time points taken at intervals during the 2-15% hydrolysis range to a straight line with the aid of a least squares computer program.

All pH measurements were made on a Radiometer Model 26 pH meter zeroed on pH 4.01 and 7.00 standard buffers. Values of pD were calculated by adding 0.4 to the observed pH meter reading for D_2O solutions (20). Measurements at the start and end of hydrolysis runs showed essentially no change in pH during the reaction (± 0.02 pH units).

Proteolytic Activity. The procedure of Anson and Mirsky (21) was used. The hydrolysis of acid-denatured bovine hemoglobin at pH 1.8, 37°, was followed by measuring the 280 nm absorbence of trichloroacetic acid-soluble hydrolysis products in a Beckman DU spectrophotometer. Activity is defined as (1000 x ΔO.D./10 min) units/mg enzyme. Hemoglobin assays with pepsin incubated at 34° for 24 hours in 0.10 M sodium citrate buffer (pH 2.0 or 5.4) showed 3900 ± 300 units/mg. This rules out significant loss of activity (< 5%) during the kinetic experiments.

Preparation of Pepsin Inactivated by Diazoacetylglycine Ethyl Ester. The procedure of Lundblad and Stein (12) was used. Five milliliters of a 0.1 M ethanolic solution of cupric acetate was mixed with 5 ml of a 0.06 M ethanolic solution of diazoacetylglycine ethyl ester and allowed to stand for 10 min at room temperature. This solution was then added with gentle stirring to a solution of 500 mg of pepsin in 90 ml of 0.1 M sodium acetate buffer (pH 5.4). The solution was allowed to stand at room temperature for 15 min, dialyzed against three liters of distilled water for two hr at 4°, and lyophilized. The dried material was dissolved in a minimum amount of 0.1 M sodium acetate buffer (pH 5.4), and the inactivated enzyme was freed from excess reagents by passage through a column of Sephadex G-25 (3 x 60 cm) equilibrated with the same buffer. The eluted protein fraction was dialyzed against distilled water at 4° and lyophilized. Hemoglobin assay showed no pepsin activity.

Preparation of Pepsin Inactivated by 2,4'-Dibromoacetophenone. The procedure of Erlanger et al. (22) was used. Five hundred milligrams of pepsin was dissolved in 90 ml of 0.1 M sodium citrate buffer (pH 3.0), and 10 ml of a 0.03 M ethanolic solution of 2,4'-dibromoacetophenone was added with gentle stirring. The solution was allowed to stand at room temperature for 24 hr, dialyzed against three liters of distilled water for two hr at 4°, and lyophilized. The inactivated enzyme was then freed from excess reagents on Sephadex G-25 as above. Hemoglobin assay showed that only 22% of the initial pepsin activity remained.

Determination of pK_a of N-TFA-L-Phe. A 0.01 M solution of N-TFA-L-Phe was made up in 0.2 M citric acid-HCl buffer (pH 1.15), and trifluoroacetamide (to 0.01 M) was added as an internal reference. The fluorine nmr spectrum of the solution at this and higher pH's was observed, the pH being changed by addition of 0.2 M sodium citrate buffer (pH 7.0) containing 0.01 M N-TFA-L-Phe and 0.01 M trifluoroacetamide) to the original sample. The separation, δ, in Hz between the fluorine peaks of the two compounds was measured as a function of pH over the range of pH 1.15-5.00. δ decreased from 48.6 to 43.2 Hz over this range and showed a smooth titration curve with a pK_a of 2.85.

Determination of K_M and k_{cat}. The initial velocity, v_o, of the pepsin-catalyzed hydrolysis of N-TFA-L-Phe at each pH and pD studied was measured at substrate concentrations, $[S_o]$, ranging from 7.5-40.0 mM. The Michaelis constant, K_M, and the catalytic constant, k_{cat}, were calculated from a plot of $[S_o]/v_o$

versus $[S_o]$. The intercept on the abscissa is equal to $-K_M$, and the slope is equal to $1/(k_{cat}[E_o])$ where $[E_o]$ is the molar concentration of enzyme.

Kinetic Analysis. Examination of the pH dependence of the observed kinetic parameters for hydrolysis of N-TFA-L-Phe suggested the occurrence of significant nonproductive binding which would greatly complicate a complete kinetic analysis. Indeed, such nonproductive binding might be expected in light of studies showing that pepsin has two binding sites with high affinity for the side chains of hydrophobic amino acids (2). Thus, good pepsin substrates contain two hydrophobic amino acid residues, both with L configurations, which can fill both binding pockets simultaneously. However, N-TFA amino acids having only one hydrophobic side chain might be able to bind in either pocket, although binding in only one of these would lead to catalytic activity.

Hein and Niemann (23) have developed equations describing the effect of nonproductive binding on the observed kinetic parameters, K_M and k_{cat}, which they show are related to the true values for the productive reaction by equations 1 and 2 where K_{ES^*} is the dissociation constant for the active complex, K_{N_j} is the dissociation constant for a nonproductive complex, and k_{cat^*} is the actual value for the catalytic constant.

$$\frac{1}{K_M} = \frac{1}{K_{ES^*}} + \frac{1}{K_{N_1}} + \frac{1}{K_{N_2}} + \ldots + \frac{1}{K_{N_j}} \qquad (1)$$

$$k_{cat} = \frac{k_{cat}^*}{K_{ES^*}(1/K_{ES^*} + 1/K_{N_1} + 1/K_{N_2} + \ldots + 1/K_{N_j})} \qquad (2)$$

If K_{ES^*} is greater than K_{N_j}, the observed K_M will reflect the value of K_{N_j} more than that of K_{ES^*}, and the observed k_{cat} will be significantly less than k_{cat}^*. Moreover, since each dissociation constant can have a different pH dependence, an analysis of the ionizations affecting either k_{cat} or K_M can easily become impossible if several binding modes contribute significantly to observed binding. Equation 3, however, shows that k_{cat}/K_M depends not on nonproductive binding but only upon those ionizations that affect the active reaction.

$$k_{cat}/K_M = \left[\frac{k_{cat}^*}{K_{ES^*}(1/K_{ES^*} + 1/K_{N_1} + 1/K_{N_2} + \ldots + 1/K_{N_j})}\right](1/K_{ES^*} + 1/K_{N_1} + \ldots + 1/K_{N_j}) =$$

$$k_{cat}^*/K_{ES^*} \qquad (3)$$

Dixon and Webb (24) have developed equations for the dependence of k_{cat}^* on pH in terms of $\tilde{k}_{cat}^*$ (the pH-independent velocity constant for breakdown of the active complex) and the appropriate pH function (f) which determines the concentration of this complex at any pH (equation 4).

$$k_{cat}^* = \tilde{k}_{cat}^*/f_{ES^*} \qquad (4)$$

Similarly, K_{ES^*} is represented by equation 5.

$$K_{ES^*} = \tilde{K}_{ES^*}(f_{E^*}f_{S^*}/f_{ES^*}) \tag{5}$$

Equations 4 and 5 show that k_{cat}/K_M is only dependent upon ionizations on the free enzyme and substrate that affect the productive reaction (equation 6).

$$k_{cat}/K_M = k_{cat}^*/K_{ES^*} = (\tilde{k}_{cat}^*/\tilde{K}_{ES^*})(1/f_{E^*}f_{S^*}) \tag{6}$$

For a system involving an enzyme active only when a critical ionizable group is undissociated and a substrate active only when it is ionized, k_{cat}/K_M may be represented by equation 7 where K_E and K_S are defined by $EH \underset{K_E}{\rightleftharpoons} E + H$ and $SH \underset{K_S}{\rightleftharpoons} S + H$, respectively.

$$k_{cat}/K_M = (\tilde{k}_{cat}^*/K_{ES^*})\left[\frac{1}{(1 + K_E/[H^+])(1 + [H^+]/K_S)}\right] \tag{7}$$

In order to determine K_E and K_S, a computer program based upon equation 7 was written the outputs of which were a tabulation of values for k_{cat}/K_M as a function of pH for a set of assumed values of K_E and K_S. The input consisted of trial values for each quantity needed to define k_{cat}/K_M and was varied until the best fit was achieved as determined by visual comparison of the curves generated from these trial values of $\tilde{k}_{cat}^*/\tilde{K}_{ES^*}$, K_E, and K_S and the experimental pH profile.

RESULTS

A typical fluorine nmr spectrum of a hydrolysis experiment is shown in Figure 1. Digital integration by a Fabritek computer showed an accuracy of better than 2% in determining concentrations of trifluoroacetate and N-TFA amino acids. This was demonstrated by taking spectra of several mixtures of known concentrations of trifluoroacetic acid and N-TFA-L-Phe.

Table I lists data for the per cent hydrolysis of several N-TFA amino acids by porcine pepsin, and Table II lists data for the per cent hydrolysis of N-TFA-L-Phe by several enzyme preparations. Under similar conditions, these substrates are stable to nonenzymic hydrolysis.

A plot of per cent hydrolysis versus time for a typical hydrolysis experiment is shown in Figure 2; v_o was calculated by a least squares fit. Hydrolysis was, however, essentially linear even after 35-40% reaction. In practice, therefore, most determinations of v_o were made from averages of several spectra taken in the range of 10-15% hydrolysis.

Figure 3 shows a plot of $[S_o]/v_o$ versus $[S_o]$ for a kinetic experiment done at pH 4.05. The range of substrate concentration used to evaluate the kinetic constants was limited somewhat by the maximum solubility of the substrate and the need to keep $[E_o] \ll [S_o]$. These factors restricted the kinetic studies to a 4-6 fold range of substrate concentration (7.5×10^{-3} to 4.0×10^{-2} M). However, as the K_M values fell within this range, they could be

FIGURE 1. Fluorine Nmr Spectrum of N-TFA-L-Phe Hydrolysis Experiment. $[E_o]$, 5.0×10^{-4} M; $[S_o]$, 4.0×10^{-2} M; 0.10 M sodium citrate buffer, pH 4.05; temp., 34°; incubation time, 550 min. The peak at right (upfield) is N-TFA-L-Phe fluorine. The smaller peak is trifluoroacetate fluorine. Per cent hydrolysis is 9.6%.

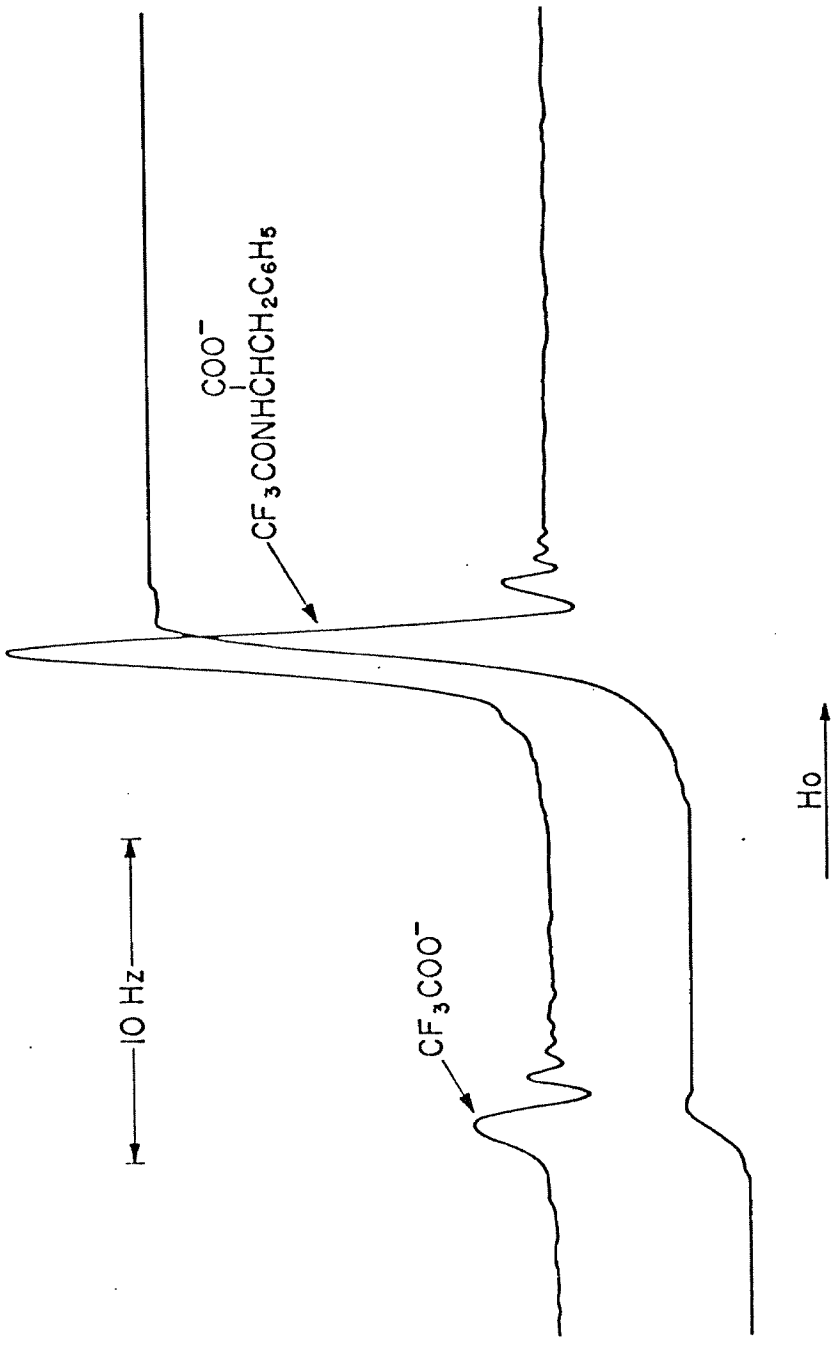

COO⁻
CF3CONHCHCH2C6H5
CF3COO⁻
10 Hz
Ho

TABLE I: Hydrolysis of Several N-TFA Amino Acids by Pepsin[a]

	Time (hr)	Per Cent Hydrolysis
N-TFA-L-Phe	12	58
	48	98
N-TFA-D-Phe	48	0
N-TFA-D, L-Phe	12	23
	48	49
N-TFA-L-p-F-Phe	12	59
N-TFA-D, L-p-F-Phe	12	30
	48	50
N-TFA-D, L-o-F-Phe	12	19
N-TFA-L-Trp	12	37
N-TFA-D-Trp	48	0
N-TFA-D, L-Trp	12	17
N-TFA-D, L-Tyr	12	16
N-TFA-L-phenylglycine	12	0
N-TFA-Gly	12	0

[a] $[E_o]$, 5.0×10^{-4} M, based on molecular weight of 34,200 (28); $[S_o]$, 5.0×10^{-3} M; temp., 34°; buffer, 0.10 M sodium citrate, pH 3.0.

TABLE II: Hydrolysis of N-TFA-L-Phe by Several Enzymes [a]

Enzyme	pH	Time (hr)	Per Cent Hydrolysis
Pepsin (Commercial, 2x Crystallized)	3.5	12	55
Pepsin (from pepsinogen activated according to ref. 28)	3.5	12	50
Pepsin (diazoacetylglycine ethyl ester - treated)	3.5	12	0
Pepsin (2,4'-dibromoacetophenone-treated)	3.5	12	10
Human Pepsin	3.5	12	53
Human Gastricsin	3.5	12	13
None	1.0	24	0
None	5.4	24	0

[a] $[E_o]$, 5.0×10^{-4} M; $[S_o]$, 0.01 M; temp., 34°; buffer, 0.10 M sodium citrate (pH 3.5 and 5.4), 0.10 M citric acid-HCl (pH 1.0).

FIGURE 2. Hydrolysis of N-TFA-L-Phe by Pepsin. $[E_o]$, 5.0×10^{-4} M; $[S_o]$, 7.5×10^{-3} M; 0.10 M sodium citrate buffer, pH 3.60; temp., 34°. v_o was calculated from a least squares fit.

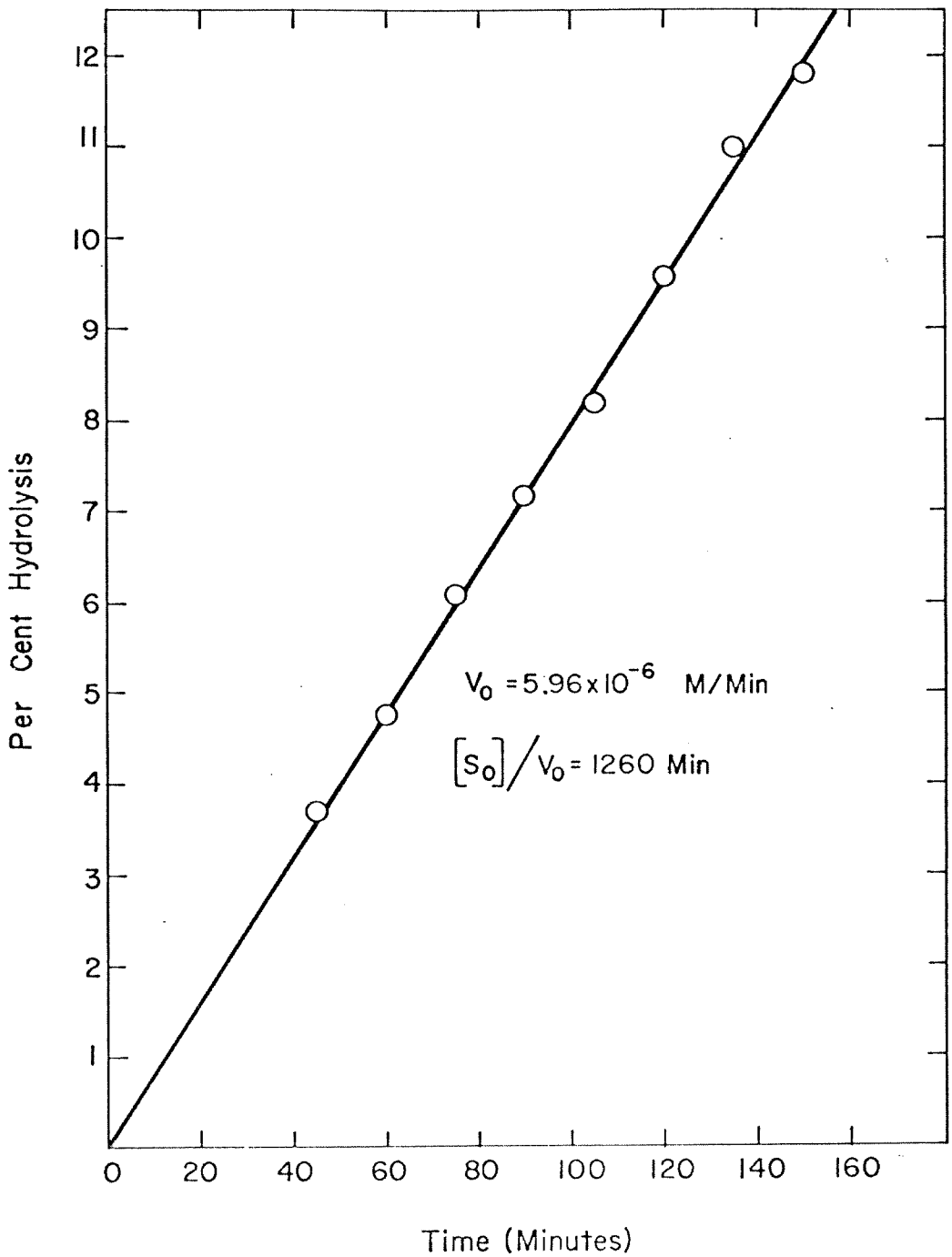
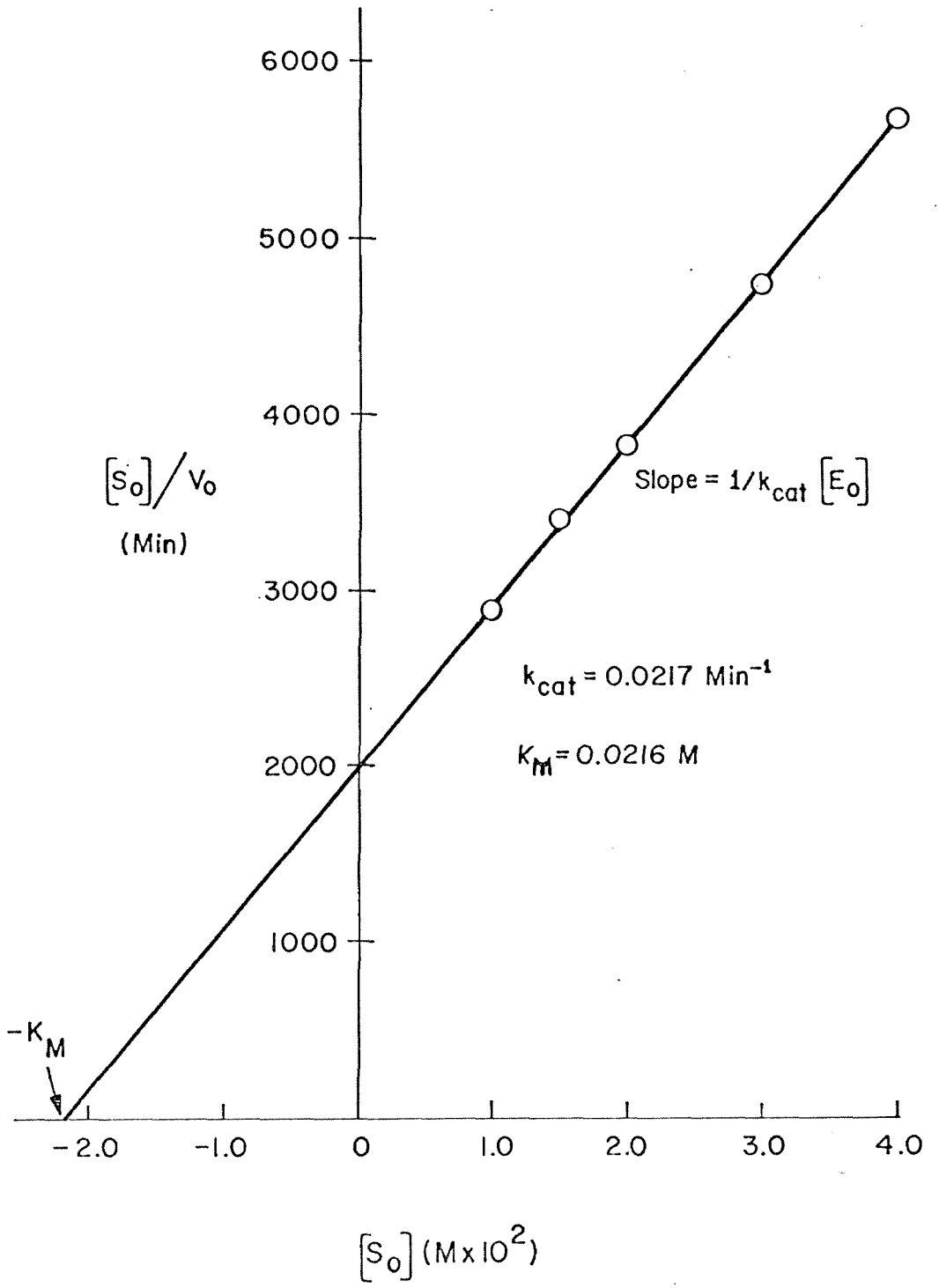

Per Cent Hydrolysis
Time (Minutes)
$V_0 = 5.96 \times 10^{-6}$ M/Min
$[S_0]/V_0 = 1260$ Min
0
20
40
60
80
100
120
140
160
1
2
3
4
5
6
7
8
9
10
11
12

FIGURE 3. Plot of $[S_o]/v_o$ versus $[S_o]$ for the Hydrolysis of N-TFA-L-Phe by Pepsin at pH 4.05. $[E_o]$, 5.0×10^{-4} M; 0.10 M sodium citrate buffer; temp., 34°. The straight line was determined by a least squares fit. k_{cat} and K_M were calculated as described in the text.


6000
5000
4000
3000
2000
1000
[S0]/V0
(Min)
Slope = 1/kcat [E0]
kcat = 0.0217 Min-1
KM = 0.0216 M
-KM
-2.0
-1.0
0
1.0
2.0
3.0
4.0
[S0] (M x 10^2)

determined accurately from the $[S_o]/v_o$ versus $[S_o]$ plots.

Kinetic data for hydrolysis of N-TFA-L-Phe are shown for the pH range 1.7-5.4 in Table III and for the pD range 3.4-4.3 in Table IV. The errors quoted are probable errors from the plots of $[S_o]/v_o$ versus $[S_o]$ and are not accuracy estimates. However, duplicate experiments performed at pH 4.05 suggested that the data have a precision of ±4-8%. Plots of the kinetic parameters from Tables III and IV versus pH and pD are shown in Figure 4 (for k_{cat}), Figure 5 (for K_M), and Figure 6 (for k_{cat}/K_M).

The computer fit of the data for k_{cat}/K_M to equation 7 gave a value for pK_E of 3.7 and a value for pK_S of 2.8. Figure 5 shows the theoretical curve obtained using these values for K_E and K_S and compares it with the experimental dependence of k_{cat}/K_M on pH.

Calculation of the deuterium isotope effect on k_{cat}/K_M was made from the formula $k_{H_2O}/k_{D_2O} = k_{max\ H_2O}/k_{max\ D_2O}$ where k_{max} was the apparent maximum value of the curves for k_{cat}/K_M versus pH and pD. (Such a comparison depends on the independence of K_M on H_2O and D_2O as solvent; within experimental error, D_2O caused a decrease in k_{cat} without affecting K_M.) The value of the isotope effect (k_{H_2O}/k_{D_2O}) so determined was 2.8±0.3.

TABLE III: Kinetic Parameters for the Hydrolysis of N-TFA-L-Phe by Pepsin [a]

pH	$[S_o]$ (mM)	k_{cat} ($min^{-1} \times 10^3$)	K_M (mM)	k_{cat}/K_M ($min^{-1}\ M^{-1}$)
1.70[b]	10.0-40.0	2.4 ± 0.2	6.6 ± 0.8	0.36 ± 0.02
2.25[b]	10.0-40.0	6.0 ± 0.6	5.9 ± 1.2	1.01 ± 0.10
2.80[b]	10.0-40.0	14.3 ± 0.8	8.0 ± 0.8	1.79 ± 0.10
3.05[c]	10.0-40.0	26.8 ± 1.0	14.0 ± 0.8	1.91 ± 0.06
3.35[c]	7.5-30.0	34.1 ± 3.5	16.6 ± 3.4	2.06 ± 0.21
3.60[c]	7.5-30.0	31.7 ± 1.0	15.5 ± 0.8	2.05 ± 0.06
4.05[c]	10.0-40.0	21.7 ± 0.4	21.6 ± 0.8	1.01 ± 0.02
4.50[c]	7.5-30.0	15.8 ± 1.0	28.6 ± 4.0	0.55 ± 0.04
4.65[c]	7.5-30.0	13.1 ± 0.6	29.0 ± 3.2	0.45 ± 0.02
5.40[c]	10.0-40.0	5.5 ± 1.0	81.8 ±15.0	0.07 ± 0.02

[a] $[E_o]$, 5.0×10^{-4} M; temp., 34±1°; [b] 0.10 M citric acid-HCl buffer. [c] 0.10 M sodium citrate buffer.

TABLE IV: Kinetic Parameters for the Hydrolysis of N-TFA-L-Phe by Pepsin in D_2O [a]

pH	$[S_o]$ (mM)	k_{cat} (min^{-1} x 10^3)	K_M (mM)	k_{cat}/K_M (min^{-1} M^{-1})
3.45	10.0-40.0	8.1 ± 1.0	14.1 ± 1.6	0.57 ± 0.06
3.65	10.0-40.0	10.7 ± 1.8	14.1 ± 2.4	0.76 ± 0.12
3.85	10.0-40.0	10.8 ± 0.6	14.3 ± 0.8	0.76 ± 0.04
4.05	10.0-40.0	12.5 ± 1.2	18.7 ± 1.8	0.67 ± 0.12
4.35	10.0-40.0	6.7 ± 0.4	21.0 ± 1.0	0.32 ± 0.02

[a] $[E_o]$, 5.0×10^{-4} M; temp., 34 ± 1°; buffer, 0.10 M sodium citrate in D_2O.

FIGURE 4. Plot of k_{cat} versus pH for the Hydrolysis of N-TFA-L-Phe by Pepsin at 34°. The circles are data for experiments in H_2O; the squares are data for experiments in D_2O.

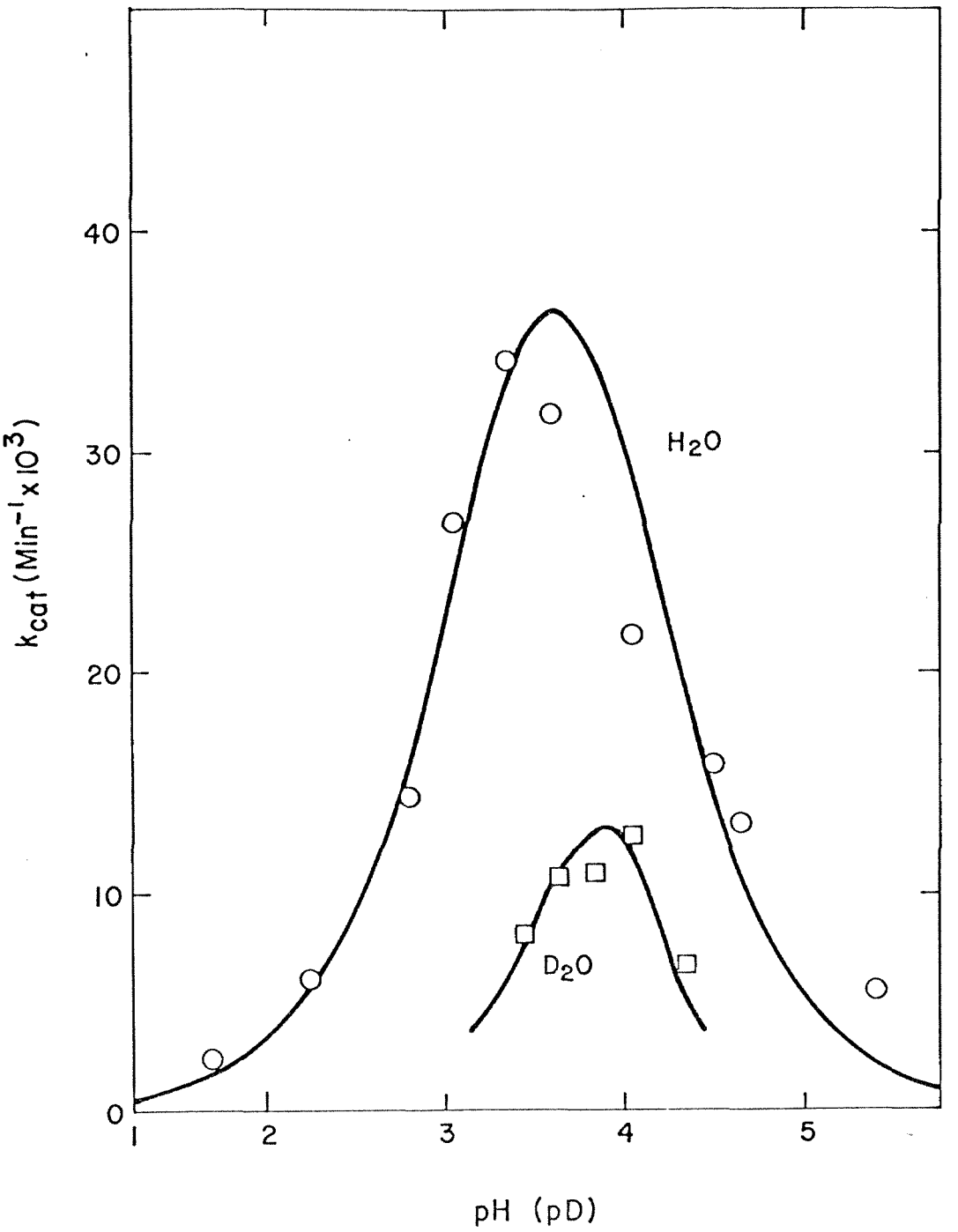

40
30
20
10
0
1
2
3
4
5
k_{cat} ($Min^{-1} \times 10^3$)
pH (pD)
H_2O
D_2O

FIGURE 5. Plot of K_M versus pH for the hydrolysis of N-TFA-L-Phe by Pepsin at 34°. The circles are data for experiments in H_2O; the squares are data for experiments in D_2O.

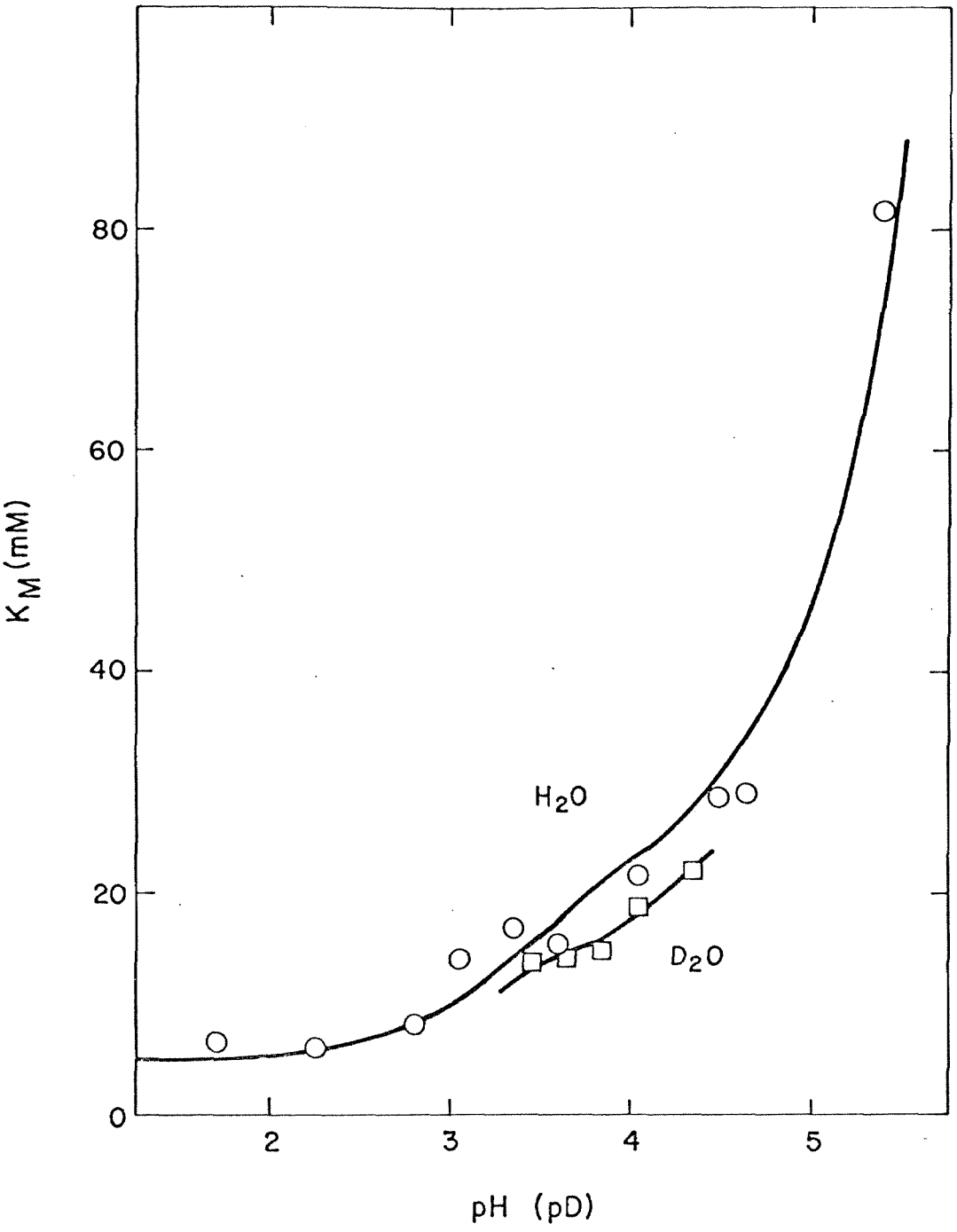
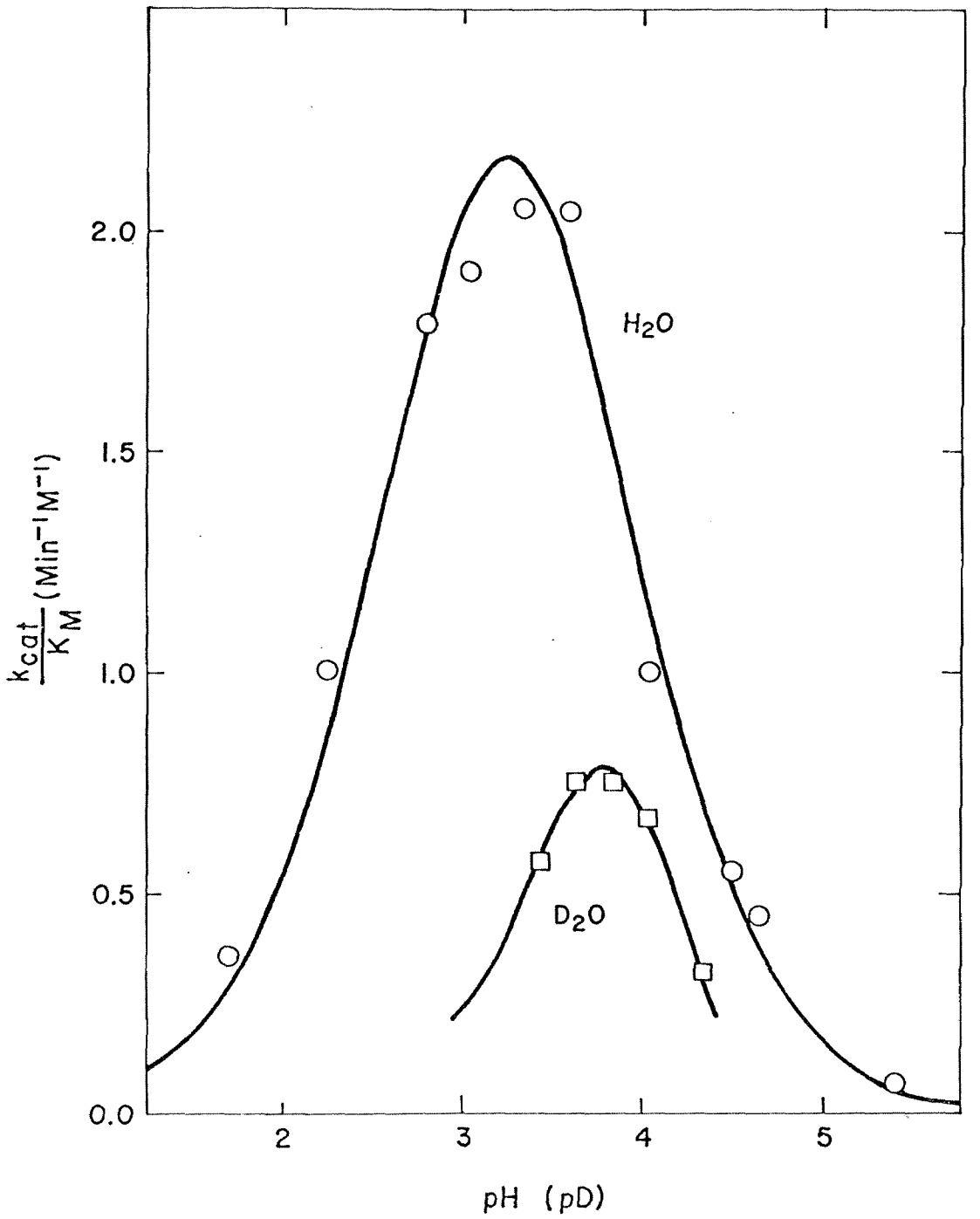

80
60
40
20
0
K_M (mM)
2
3
4
5
pH (pD)
H_2O
D_2O

FIGURE 6. Plot of k_{cat}/K_M versus pH for the Hydrolysis of N-TFA-L-Phe by Pepsin at 34°. The circles are data for experiments in H_2O; the squares are data for experiments in D_2O. The solid line for the H_2O data is the theoretical line calculated from equation 7 for $pK_S = 2.8$ and $pK_E = 3.7$.


2.0
1.5
1.0
0.5
0.0
2
3
4
5
pH (pD)
$\frac{k_{cat}}{K_M}$ ($Min^{-1}M^{-1}$)
H_2O
D_2O

DISCUSSION

Analytical Method. The nmr technique described above has been found to be a convenient and accurate method for studying the kinetics of the pepsin-catalyzed hydrolysis of N-TFA amino acids. Commonly used assay procedures for studying pepsin kinetics involve spectrophotometric measurements of ninhydrin reactive products released during hydrolysis of peptides (25) or differences in the ultraviolet absorption of substrates and products (4). The former often suffer from the need to correct for "enzyme blank" caused by ninhydrin-positive material in pepsin preparations, and the latter suffers from the small change in absorbence observed upon hydrolysis of most substrates.

In our procedure, the only fluorine nmr signals came from the substrate and one of the hydrolysis products (trifluoroacetate), and the signals were far enough apart (10-18 Hz depending upon pH) so that they could be integrated without overlap.

Pepsin kinetic studies have also suffered from the low solubility of pepsin substrates in aqueous solution, and failure to obtain substrate concentrations greater than K_M without adding organic solvents to the reaction solution has often prevented accurate separate determinations of k_{cat} and K_M. N-TFA amino acids are soluble to concentrations well above K_M, and such determinations were easily possible in our experiments. The chief limitation of the nmr technique involved the relative slowness of the hydrolysis of N-TFA amino acids (even at the pH optimum

for the reaction) which necessitated use of fairly high enzyme concentrations (5×10^{-4} M).

Specificity. The specificity of pepsin in hydrolysis of N-TFA amino acids parallels its specificity in hydrolysis of peptides. Only N-TFA amino acids with the L configuration are cleaved, and aromatic amino acids are preferred. Pepsin's failure to hydrolyze N-TFA-Gly and N-TFA-L-phenylglycine is consistent with its known specificity detailed by Fruton (2). He found that, although a hydrophobic side chain is normally required of a pepsin substrate, branching at the β-carbon of an amino acid involved in a peptide bond prevents hydrolysis of that bond (i.e., a β-methylene group is essential for catalytic activity).

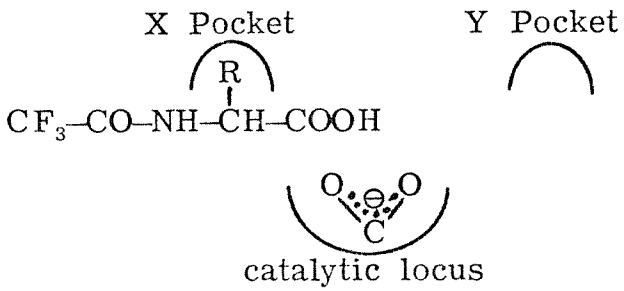
Human and porcine pepsin, known to possess almost identical catalytic specificity and activity (26), hydrolyzed N-TFA-L-Phe at approximately the same rate. Human gastricsin, which possesses a very similar, but not identical, catalytic specificity (27), also hydrolyzes this substrate, but at a markedly slower rate. A highly purified porcine pepsin sample obtained by activation of commercial pepsinogen according to the method of Rajagopalan et al. (28) showed the same activity toward N-TFA-L-Phe as did commercial 2x crystallized pepsin.

The only products detected during hydrolysis of N-TFA-L-Phe by pepsin were trifluoroacetic acid and phenylalanine. No transpeptidation producing N-TFA-L-Phe-L-Phe was observed. There was no appearance of a fluorine resonance for N-TFA-L-Phe-L-Phe

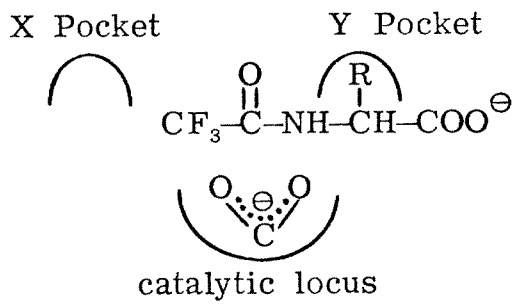
(which comes 15-20 Hz upfield of the fluorine resonance for N-TFA-L-Phe), and the combined area of the peaks for N-TFA-L-Phe and trifluoroacetic acid was constant.

Porcine pepsin previously treated with diazoacetylglycine ethyl ester and thereby inactivated toward hemoglobin substrate was also inactive toward N-TFA-L-Phe. Porcine pepsin previously treated with 2,4'-dibromoacetophenone and possessing only 22% of its initial activity in hemoglobin assay retained only 18% of activity toward N-TFA-L-Phe. Accordingly, hydrolysis of N-TFA-amino acids seems to be a true pepsin-catalyzed reaction involving the same catalytic site as that used in pepsin's other catalytic activities.

pH Dependence and Mode of Substrate Binding. The pH dependence of the kinetic parameters for the hydrolysis of N-TFA amino acids suggests that, although the undissociated substrate binds more tightly to the enzyme than does the ionized substrate, only ionized substrate is hydrolyzed appreciably. One possible explanation for this observation is that neutral substrate binds in an unproductive mode with its side chain "R" filling the hydrophobic pocket normally occupied by the "X" side chain of a dipeptide substrate such as CH_3-CO-NH-CHX-CO-NH-CHY-COOH.



For anionic substrate, binding will be expected to decrease due to electrostatic repulsion between the negatively charged substrate and the enzymic carboxylate anion at the catalytic locus. This would be particularly important if the side chain were to bind in the "X" pocket as this would position the carboxylate group of the substrate near the carboxylate anion on the enzyme. Accordingly, the substrate, when bound, will be more likely to have its side chain in the "Y"pocket which will minimize the electrostatic repulsion of the two carboxylate anions and position the sensitive amide bond near the catalytic locus.



In fact, this type of binding accounts for the observed pH dependence of hydrolysis at low pH. The substrate has a pK_a of 2.85 and the kinetic measurements show that the hydrolysis is dependent upon an ionization of pK_a 2.8 for the free substrate or enzyme (presumably the substrate).

We find that the basic side of the curve of k_{cat}/K_M versus pH depends on ionization of a group on the free enzyme with an apparent pK_a of 3.7 which does not correspond to that of either of the catalytic carboxyl groups (pK_a's 1.1 and 4.7) implicated in pepsin-catalyzed hydrolysis of peptide substrates (29). However,

esterification of the β-carboxyl group of an aspartyl residue of pepsin by 2,4'-dibromoacetophenone is known to partially inactivate pepsin by interfering with substrate binding without affecting k_{cat} (22). Further, Hollands and Fruton (11) have shown that a non-catalytic carboxyl group with an apparent pK_a of 3.8 strongly influences both binding and hydrolysis of a series of cationic substrates. Thus, this acidic group (of pK_a ~ 3.7) is apparently located sufficiently near the site occupied by the free carboxyl group of a bound substrate (as, for example, N-Ac-L-Phe-L-Phe) to interfere significantly with binding when both it and the substrate are negatively charged (29). Accordingly, ionization of this group on the enzyme (pK_a 3.7) decreases binding of N-TFA amino acids (whose carboxyl groups, with pK_a's of 2.8, are already ionized) in the "Y" pocket which is the productive mode. This decrease in productive binding as the enzyme group of pK_a 3.7 is ionized will lead, in turn, to the observed decrease in hydrolytic activity in this pH region.

An alternative explanation for the observed pH dependence of binding and catalysis involves formation of a favorable interaction between the negative carboxylate (but not the neutral carboxyl) of the substrate and some group on the enzyme which is positioned near the substrate carboxylate when the substrate binds in the "Y" pocket. Thus, binding in the productive mode ("Y" pocket) would not depend on a decrease in the strength of binding to the nonproductive mode ("X" pocket) when the substrate ionizes, but rather on an increase in affinity for the "Y" pocket. Decrease in

binding in the productive mode resulting from ionization of the enzymic group (pK_a 3.7) would result from a disruption of the favorable substrate carboxylate-enzyme interaction. The relatively small change in overall binding (Figure 5) associated with the substrate ionization (pK_a 2.8) and the observation that the "X" pocket is the predominant binding mode at all values of pH (a conclusion based upon the mode of inhibition by L-tryptophan ethyl ester of N-TFA-L-Phe hydrolysis, see below) support this alternative scheme. Its kinetic manifestations are actually indistinguishable from the former scheme, however.

Finally, the dependence of K_M on pH (Figure 5) suggests that ionization of a group on the free enzyme with an apparent pK_a around 4.8 decreases binding drastically. This ionization corresponds closely to that of one of the catalytic carboxyl groups, and it might be expected to prevent binding since it would create significant negative charge in both the preferred "X" site and the productive "Y" site. Ionizations of the catalytic carboxyl groups with pK_a's 1.1 and 4.7 are not manifest in the plot of k_{cat}/K_M versus pH as their states of ionization are effectively unchanged over the pH range where activity against N-TFA amino acids is observed.

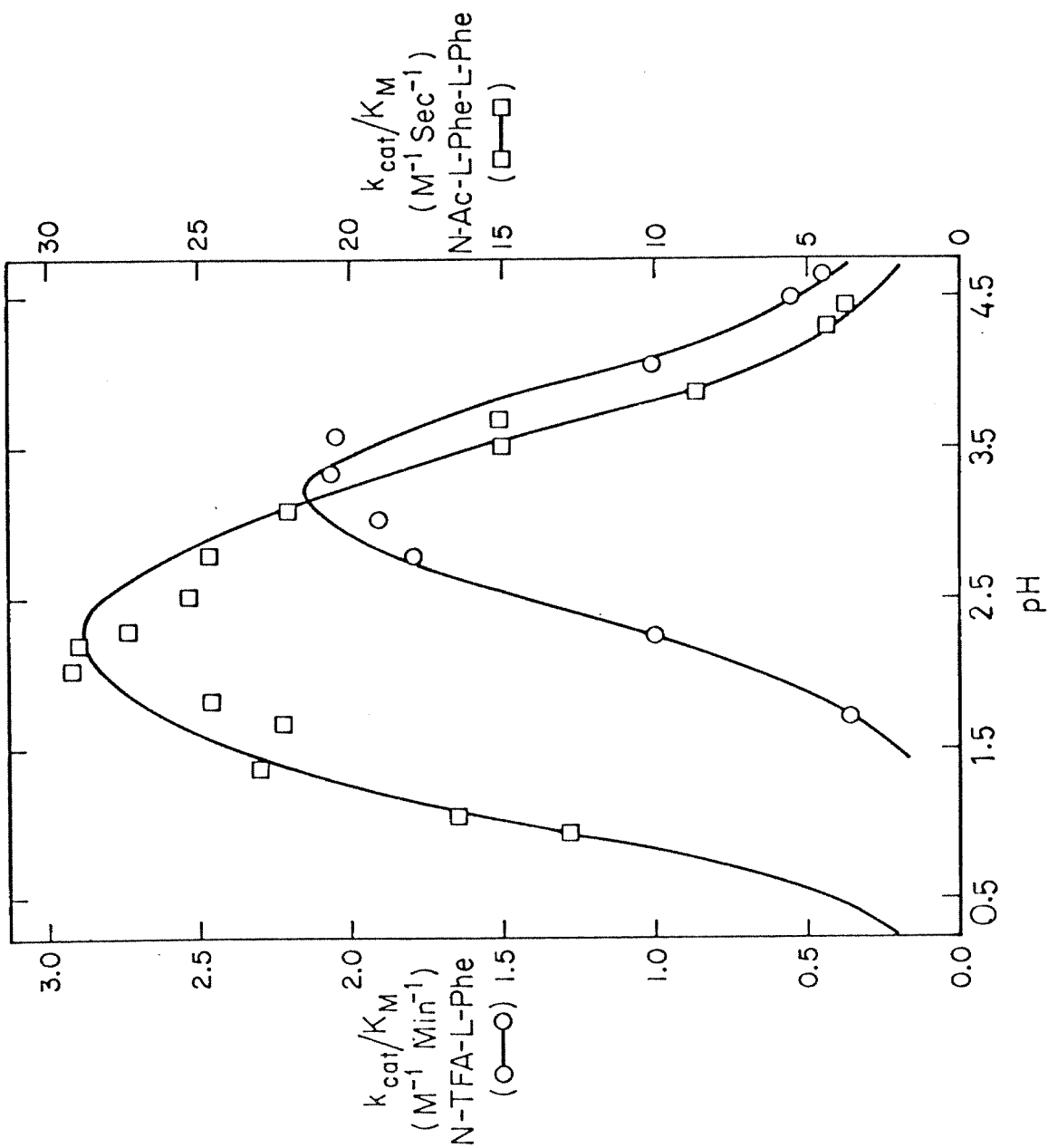
Why are N-TFA amino acid derivatives hydrolyzed by pepsin when N-acetyl derivatives are not? Failure to observe hydrolysis of N-acetyl amino acids probably results from the small fraction that would be bound in a productive mode. With pK_a's generally around 3.5-3.7, they ionize at essentially the same pH as the

enzymic carboxyl group with pK_a of 3.7. In contrast, for the N-TFA derivatives of amino acids which have a significantly lower pK_a than the corresponding acetyl amino acids, there should exist an appreciable pH range over which the N-TFA amino acid is ionized while the enzymic carboxyl group of pK_a 3.7 is still neutral. Over this pH range, the N-TFA amino acid will bind to pepsin in a productive mode, with the aromatic residue in the "Y" pocket and the trifluoroacetamido bond in the catalytic locus. Even so, the favorable pH range will be small, and the catalytic rate, further decreased by the presence of appreciable nonproductive binding, might be considerably slower than that for most good pepsin substrates. The observed rates are, in fact, slower by a factor of 100-1000.

These effects of pH on binding and catalysis of normal acid substrates (N-Ac-L-Phe-L-Phe) and N-TFA amino acids (N-TFA-L-Phe) are shown graphically in Figure 7. Whereas a normal acid substrate binds productively in the low pH range, an N-TFA amino acid derivative does not begin to bind in a productive manner until its carboxyl group is ionized (pK_a 2.8). Above this pH, its activity as a substrate increases until titration of the carboxylic acid group on the enzyme with pK_a of 3.7 leads to decreased binding both for normal acid substrates and N-TFA amino acids. Thus, in the higher pH region both curves fall off with ionization of this enzymic carboxyl group (pK_a 3.7).

Essentially, the scheme just discussed proposes that N-TFA-L-Phe usually binds to the enzyme in the "X" pocket in a

FIGURE 7. Comparison of pH Dependence of k_{cat}/K_M for Pepsin-catalyzed Hydrolysis of N-TFA-L-Phe and N-Ac-L-Phe-L-Phe. Data for N-TFA-L-Phe are from Table I. Data for N-Ac-L-Phe-L-Phe are from Cornish-Bowden and Knowles (29).


k_{cat}/K_M (M^{-1} Min^{-1}) N-TFA-L-Phe (○—○)
k_{cat}/K_M (M^{-1} Sec^{-1}) N-Ac-L-Phe-L-Phe (□—□)
pH
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
5
10
15
20
25
30
0.5
1.5
2.5
3.5
4.5

nonproductive mode; only when bound in the "Y" pocket does hydrolysis occur. We sought further substantiation of this proposal by studying the inhibition of the pepsin-catalyzed hydrolysis of N-TFA-L-Phe by L-tryptophan ethyl ester which has been shown (30) to behave as a linear competitive inhibitor (K_I = 6 mM at pH 4.0) for the pepsin-catalyzed hydrolysis of N-CBZ-L-His-L-Phe(NO_2)-L-PheOMe and to bind preferentially in the "Y" pocket (2). Thus, L-tryptophan ethyl ester should, according to our scheme, compete only with the productive binding of N-TFA-L-Phe.

The kinetic manifestations of such competition between L-tryptophan ethyl ester and hydrolysis of N-TFA-L-Phe can be described by the following equations:

$$E + S \underset{}{\overset{K_{ES*}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}^*} E + P$$

$$E + S \overset{K_N}{\rightleftharpoons} ES_N$$

$$E + I \overset{K_I}{\rightleftharpoons} EI$$

$$EI + S \overset{K_N}{\rightleftharpoons} ES_NI \; ; \; ES + I \overset{K_N}{\rightleftharpoons} ES_NI$$

Solution of these equations leads to the following relations between the molecular and observed parameters (k_{cat} and K_M):

$$k_{cat} = \frac{k_{cat}^*}{(1 + K_{ES*}/K_N + K_{ES*}[I]/K_NK_I)}$$

$$K_M = \frac{K_{ES*}(1 + [I]/K_I)}{(1 + K_{ES*}/K_N + K_{ES*}[I]/K_N K_I)}$$

Under conditions where $K_{ES*} > K_N$ (i.e., most binding occurs in a nonproductive fashion as in the present case) and $[I]/K_I > 1$ (a condition which can be, and in the present case was, imposed on the system by keeping $[I] > K_I$), these equations simplify to:

$$k_{cat} \cong \frac{k_{cat}^*}{(K_{ES*}/K_N)(1 + [I]/K_I)}$$

$$K_M \cong K_N$$

Thus, such an inhibitor under these conditions, though competing with the productive binding of substrate, will cause no observable change in K_M, but will cause a decrease in the observed k_{cat} which will be a function of $[I]$; the inhibitor will manifest the symptoms of noncompetitive inhibition (decrease in k_{cat}, no change in K_M).

A study of the pepsin-catalyzed hydrolysis of N-TFA-L-Phe at pH 3.60 in the presence of 12.0 mM L-TrpOEt showed that k_{cat} was reduced to 11.3×10^{-3} min^{-1} (compared to 31.7×10^{-3} min^{-1} in the absence of inhibitor) and that K_M was 15.1 mM in the presence of inhibitor (essentially unchanged from its value of 15.5 mM without inhibitor). Moreover, the value of K_I of 6.6 mM based on these results and calculated from the relationship

$$k_{cat}/K_M = k_{cat}^*/K_{ES*}(1 + [I]/K_I) \qquad \text{(note: } K_M \cong K_N\text{)}$$

agrees well with the value of 6 mM reported by Inouye and Fruton (30). The central point of this particular experiment is, however, the support it lends to the general scheme proposed in this paper for the interaction of N-TFA-L-Phe.

Isotope Effect. Replacement of water by deuterium oxide reduces the rate of pepsin-catalyzed hydrolysis of N-TFA-L-Phe by a factor of three. The effect is apparently entirely on k_{cat}; K_M within experimental error remains unchanged. An isotope effect of this type manifested in k_{cat} suggests that proton transfer occurs in the rate determining catalytic step. The magnitude of this effect and its relation to the nature of the trifluoroacetyl group has significance in regard to the mechanism of pepsin-catalyzed reactions and will be discussed after some preliminary comments on the probable nature of the catalytic groups and mechanism.

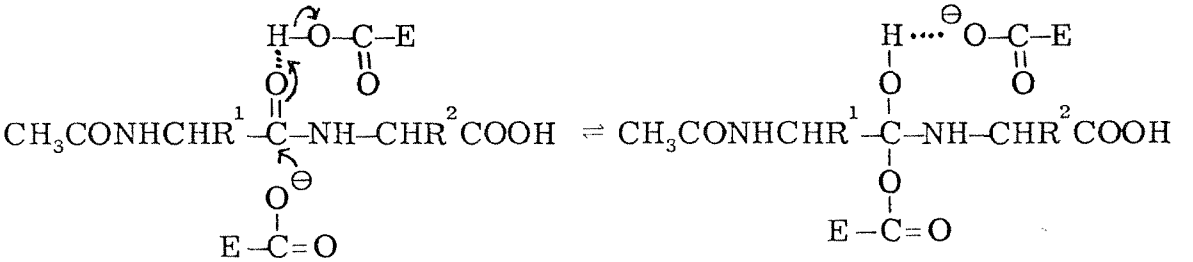
Mechanism. An important question concerns the identity and nature of the carboxylic acid groups (or carboxylate anions) involved in pepsin-catalyzed hydrolysis. Kinetic studies of the hydrolysis of substrates such as N-Ac-L-Phe-L-PheNH_2 which do not ionize in the range of pepsin activity have shown that at least two carboxyl groups at pepsin's active center function in catalysis (29). One of these, with an apparent pK_a of 1.1, must be present in the ionized form. The other, with an apparent pK_a of 1.1, must be present in the ionized form. The other, with an apparent pK_a of 4.7, must be present in the undissociated form. Modification studies involving active site-directed, irreversible, epoxide inhibitors have shown that

esterification of a single carboxyl group with a pK_a below 2 (presumably the same one implicated in kinetic studies) destroys pepsin's catalytic activity (31). Modification studies involving active site-directed, irreversible, diazocarbonyl inhibitors have shown that esterification of a different carboxyl group, one with a much higher pK_a, also destroys catalytic activity (32). Lundblad and Stein (12) have argued that the Cu(II) catalyzed reaction of diazocarbonyl compounds with carboxylic acids invariably involves reaction with an undissociated carboxyl group. Thus, the generally accepted notion that the catalytic carboxyl group with pK_a 4.7 is the group blocked by diazocarbonyl inhibitors seems unlikely as the pH dependence of this inactivation indicates that the group with pK_a 4.7 must be ionized for inactivation to occur. In fact, Lundblad and Stein (12) proposed a reaction in which the positively charged, copper-complexed inhibitor is attracted to the negatively charged active site in which the carboxyl group with pK_a 4.7 is ionized. In this way, the inhibitor is positioned for reaction with a third, still undissociated, catalytic carboxyl group with a pK_a around 6. This conclusion, that several carboxyl groups may function in catalysis, is also supported by the work of Fruton (2) who found that the pH optimum for pepsin hydrolysis may vary from 2.0-4.5 depending upon the substrate.

Though the exact function of these various carboxyl groups is presently unknown, an electrophilic and a nucleophilic component seem to play a role in pepsin catalysis. The presence of an electrophilic component in the catalytic mechanism is suggested,

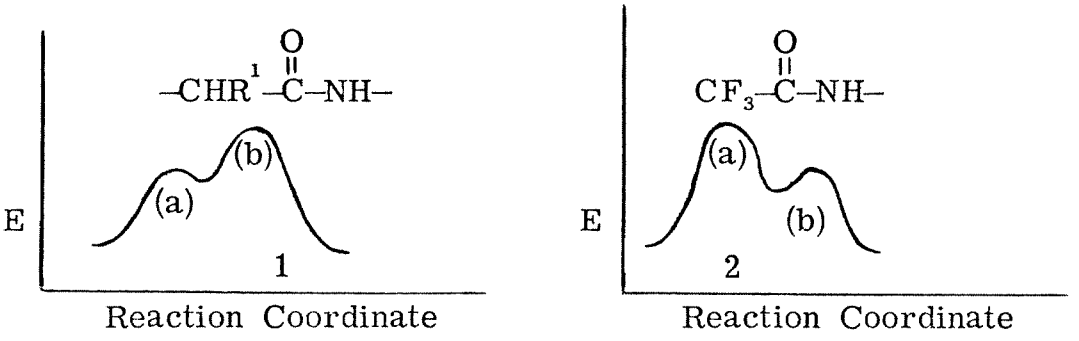
for example, by the relative rates of the hydrolysis of depsipeptide (ester) and peptide (amide) substrates ($k_{depsipeptide}/k_{amide} \cong 2$) (4). Also, the observed isotope effect ($k_{H_2O}/k_{D_2O} = 2$) for hydrolysis of Gly-Gly-Gly-L-Phe(NO_2)-L-PheOMe at the Phe(NO_2)-Phe bond reveals that the appropriate amino acid residues in the peptide substrates can cause the electrophilic component to manifest itself in the solvent isotope effect (33).

On the other hand, the absence of a significant deuterium isotope effect with peptides such as N-Ac-L-Phe-L-TyrOMe ($k_{H_2O}/k_{D_2O} = 1.05 \pm 0.30$) or sulfite esters such as methyl phenyl sulfite ($k_{H_2O}/k_{D_2O} = 1.1 \pm 0.1$) suggests that the electrophilic component is not the rate-limiting step in hydrolysis of these pepsin substrates (5,10) and implies, in turn, that a nucleophilic component occurs in pepsin's mechanism and can become the rate-limiting step. Accordingly, the initial hydrolytic step may be a concerted attack by two enzymic carboxyl groups, one in the ionized form acting as a nucleophile and the other in the undissociated form acting as a proton or hydrogen bond donor (3).



The influence on the deuterium isotope effect observed in this work by replacing the $CH_3CONHCHR^1$-group with a CF_3-group supports

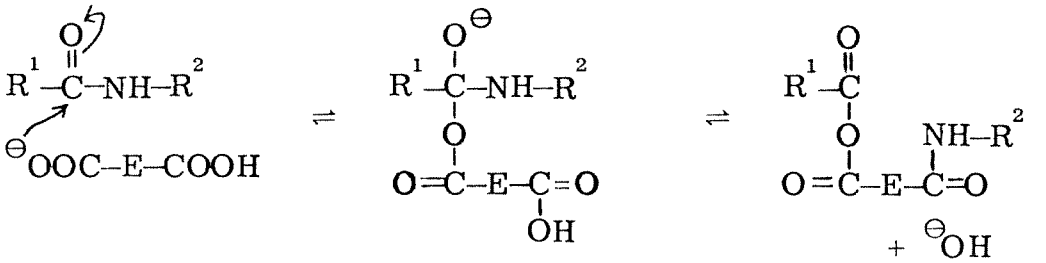
such a mechanism. The electron-withdrawing nature of the trifluoromethyl group will decrease electron density at the carbonyl group at which reaction occurs. This will decrease the ability of the oxygen atom of this carbonyl group to function as a hydrogen bond or proton acceptor, but at the same time it will increase the susceptibility of the carbon atom to nucleophilic attack. This effect may be represented by the following energy profiles in which the first transition state (a) represents proton transfer (the electrophilic component of the mechanism) and the second (b) represents nucleophilic attack.



The isotope effect of 3 associated with hydrolysis of N-TFA amino acids by pepsin suggests that the energy profile for this reaction may resemble diagram 2 in which proton transfer is rate-limiting for the overall reaction. The isotope effect results from the freezing out of an oxygen-hydrogen stretching vibration in the transition state (34). The absence of an isotope effect in the hydrolysis of N-Ac-L-Phe-L-TyrOMe (10) suggests that this reaction may have an energy profile like diagram 1. The zero-point energy of the O-H and O-D bonds in the ground state of the reactant is lost in the initial transition state involving proton

transfer, but it is regained in new O-H and O-D bonds on the substrate before the rate-determining step (nucleophilic attack) occurs.

This variation in the deuterium isotope effect of pepsin-catalyzed reactions with the nature of the substrate supports a mechanism of pepsin action involving concerted electrophilic-nucleophilic attack. (Incidentally, the order along the reaction coordinate of electrophilic or nucleophilic attack is not essential to this argument.) It is not quite so consistent with the essentially nucleophilic mechanism supported by Clement (13).



Modifications such as addition of a proton donor to facilitate removal of OH from the carboxyl group could make this mechanism consistent with the observed isotope effects. However, if, for hydrolysis of N-TFA-L-Phe, the second step is rate limiting and requires an obligatory protonation of the leaving OH, one might expect to see a buildup of the tetrahedral intermediate. No such intermediate was observed in the nmr experiments, although it is possible that its fluorine chemical shift was outside the observed chemical shift range.

In either of the above mechanisms, the attack of the carboxylate anion of pepsin on the carbonyl group of the substrate

yields a tetrahedral intermediate whose formation probably represents the slow step in the overall reaction for most peptide substrates and whose breakdown leads to eventual release of products. Attempts to isolate any stable intermediate have been inconclusive (13). One might expect such results if formation of the tetrahedral intermediate is very slow compared to subsequent steps. In this connection, Knowles (3) has shown that the rate-limiting step is that which immediately follows formation of the Michaelis complex (i) by failure to observe any burst release of either of the two products of hydrolysis, (ii) by the nonequivalence of k_{cat} for a series of substrates in which either the acyl or amino moiety is the same, and (iii) by the apparent equivalence of K_M and the enzyme-substrate dissociation constant (i.e., $k_2 \ll k_1$ in the normal Michaelis-Menten kinetic scheme).

The nature of pepsin-catalyzed transpeptidation indicates that the release of products may indeed be random. In contrast to the serine proteases which catalyze transpeptidation by an acyl transfer mechanism, pepsin catalyzes transpeptidation through an amino transfer reaction (6-7). In this latter reaction, the acyl moiety of the substrate is first released by the enzyme while the amino moiety remains bound to the enzyme long enough for a new acyl moiety to bind and undergo reaction (the reverse of the initial hydrolysis step) leading to formation of a new peptide bond. This type of transpeptidation is a characteristic of the hydrolysis of peptide substrates of the type N-Ac-L-Phe-L-Tyr with an ionizable carboxyl group. Somewhat surprisingly, the reaction is readily

detectable only above pH 4 even though the pH optimum for substrate hydrolysis is normally around pH 2. Moreover, Takahashi and Hofmann (8) have shown that pepsin can, with certain substrates, also catalyze transpeptidation by acyl transfer. Finally, Silver and Stoddard (35) have shown that with peptide substrates containing blocked amino and carboxyl end groups, such as N-Ac-L-Phe-L-TyrNH_2, no transpeptidation is observed.

Thus, the order of product release during substrate hydrolysis appears to depend upon the nature of the substrate. Furthermore, Ginodman and Lutsenko (36) have shown that the order of product release depends upon the pH at which the reaction is carried out. After studying the dependence of the rates of the exchange of substrate and products (N-Ac-L-Phe-L-TyrOEt, N-Ac-L-Phe, and L-TyrOEt) on the concentration of the components in a state of equilibrium, they concluded that at pH 4.7 N-Ac-L-Phe is released first, while at pH 3.3 there is a random release of the two products.

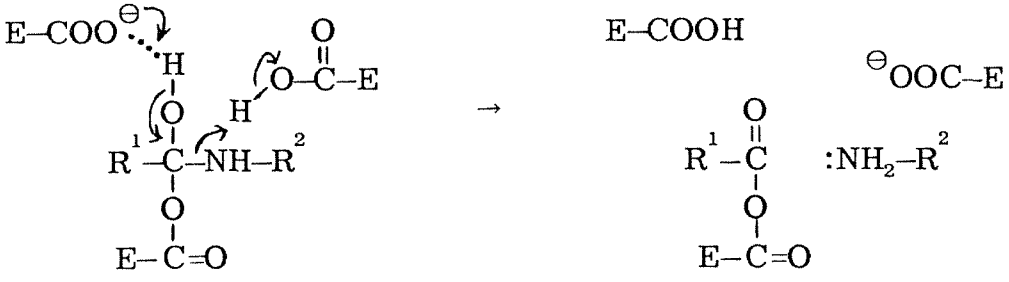
The transpeptidation experiments suggest the existence of two distinct covalent intermediates (or at least their kinetic eqivalents). They do not, however, conclusively prove that either the acyl enzyme or amino enzyme detected by transpeptidation involves a covalent bond between an enzymic group and one of the product moieties. One might have expected that the covalent amino enzyme intermediate (presumably involving an amide bond between one of the reactive carboxyl groups of the enzyme and the amido nitrogen of the substrate) would be stable enough to allow its isolation.

The failure of numerous attempts at such an isolation and the failure of hydrolysis schemes based on an obligatory covalent amino enzyme intermediate to explain the kinetics of the transpeptidation reactions have led Silver and Stoddard (35) to suggest that the amino enzyme is not a covalent intermediate.

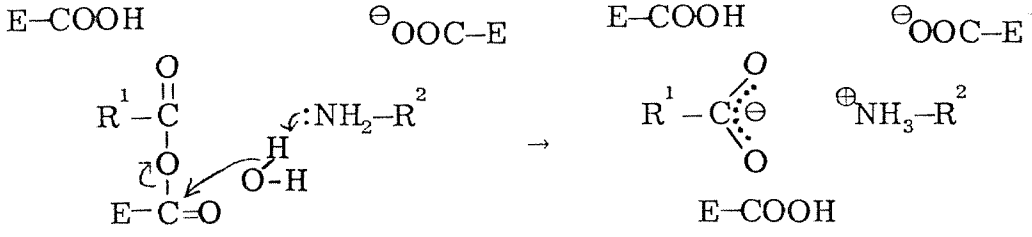
A reasonable mechanistic scheme must account for several other observations concerning pepsin's catalytic activity. First, it must account for the results of ^{18}O tracer experiments on the source of the oxygen atom added to the acyl moiety during hydrolysis. Stein and Fahrney (37) have shown that an oxygen atom is transferred from an enzymic carboxyl group to the sulfite group of the substrate during hydrolysis of methyl phenyl sulfite. Second, the scheme must account for the observation by Kitson and Knowles (38-39) that the acyl product leaves as the anion during hydrolysis and enters as the anion during transpeptidation of the amino transfer type. This conclusion was based on the dependence on pH of the nature of the inhibition by N-Ac-L-Phe of the pepsin-catalyzed hydrolysis of N-Ac-L-Phe-L-Phe-Gly. At pH 2.1, when it is largely undissociated, N-Ac-L-Phe behaves as a linear noncompetitive inhibitor and binds both to free enzyme and to the enzyme-amino moiety complex in a nonproductive manner such that transpeptidation is not possible. At pH 4.3, however, when it is largely ionized, N-Ac-L-Phe approximates a linear competitive inhibitor, binding both to the free enzyme and to the complex in a productive manner so that transpeptidation is possible. That it is the anion of N-Ac-L-Phe which functions as the

transpeptidation acceptor explains the failure of N-Ac-L-PheOMe and N-Ac-L-PheSMe to serve as acceptors since the latter two compounds will not form the ionized species necessary to attack the enzyme-amino moiety complex.

The above observations are incorporated into the following scheme for the mechanism of pepsin catalysis. The tetrahedral intermediate resulting from the initial concerted nucleophilic-electrophilic attack on the substrate is decomposed to give a bound (but noncovalent) amine product and a covalent acyl (anhydride) enzyme in a reaction catalyzed by a third carboxyl group (one of the two with high pK_a's implicated by kinetic and inactivation studies):



After the reaction above, if the amine then diffuses away and is replaced by a new amine, the reaction could proceed in reverse to yield a new peptide. If a water molecule is properly oriented, however, hydrolysis of the anhydride could be very rapid.



The liberated acid anion might well leave and be replaced by a new acid anion before the protonated amine (held in place by electrostatic interaction with the negatively charged enzymic carboxylate group) departs. A reversal of the hydrolysis step could then result in transpeptidation by amino transfer.

This mechanism is consistent with available information concerning pepsin activity, but it is still a very tentative proposal. Much additional study, involving imaginative and innovative techniques, must be completed before this enzyme and its catalytic mechanism will be understood.

CONCLUSION

The main point from this work is that pepsin catalysis of the hydrolysis of N-TFA-L-Phe leads to insight into the dramatic effect of pH on binding into the two aromatic pockets of pepsin. The observed kinetic isotope effect supports a mechanism for formation of a tetrahedral intermediate involving two carboxyl groups (one as a neutral carboxylic acid, the other as a carboxylate anion) which mount a concerted electrophilic-nucleophilic attack on the substrate leading to a tetrahedral intermediate whose subsequent breakdown may require the intervention of a third carboxylic acid group. Moreover, the stronger binding of the amino moiety than of the carboxylate moiety of the substrate may be caused by secondary forces and may not require covalent attachment of the amino fragment to the enzyme.

REFERENCES

1. J. Tang, Nature, 199, 1094 (1963).
2. J. S. Fruton, Adv. Enzym., 33, 401 (1970).
3. J. R. Knowles, Phil. Trans. Roy. Soc. Lond. B, 257, 135 (1970).
4. K. Inouye and J. S. Fruton, Biochemistry, 6, 1765 (1967).
5. T. W. Reid and D. Fahrney, J. Amer. Chem. Soc., 89, 3941 (1967).
6. H. Neumann, Y. Levin, A. Berger, and E. Katchalski, Biochem. J., 73, 33 (1959).
7. J. S. Fruton, S. Fujii, and M. H. Knappenberger, Proc. Nat. Acad. Sci. U. S., 47, 759 (1961).
8. M. Takahashi and T. Hofmann, Proc. Bioch. Soc., 127, 35P (1972).
9. L. V. Kozlov, L. M. Ginodman, B. M. Zolotarev, and V. N. Orekhovich, Dokl. Akad. Nauk S. S. S. R., 146, 943 (1962).
10. G. E. Clement and S. L. Snyder, J. Amer. Chem. Soc., 88, 5338 (1966).
11. T. R. Hollands and J. S. Fruton, Biochemistry, 7, 2045 (1968).
12. R. L. Lundblad and W. H. Stein, J. Biol. Chem., 244, 154 (1969).
13. G. E. Clement, Progr. Bioorg. Chem., 2, 177 (1973).

14. K. L. Gammon, S. H. Smallcombe, and J. H. Richards, J. Amer. Chem. Soc., 94, 4573 (1972).

15. M. W. Hunkapiller and J. H. Richards, Biochemistry, 11, 2829 (1972).

16. V. Richmond, J. Tang, S. Wolf, R. Trucco, and R. Caputto, Biochim. Biophys. Acta, 29, 453 (1958).

17. W. S. Fones, J. Org. Chem., 17, 1661 (1952).

18. L. V. Kozlov, L. M. Ginodman, and V. N. Orekhovich, Biokhimiya, 32, 1011 (1967).

19. J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Wiley, New York, 1961, p. 932.

20. R. P. Bell, "The Proton in Chemistry," Cornell University Press, Ithaca, N. Y., 1959, p. 188.

21. M. L. Anson and A. E. Mirsky, J. Gen. Physiol., 16, 59 (1932).

22. B. F. Erlanger, S. M. Vratsanos, N. Wassermann, and A. G. Cooper, Biochem. Biophys. Res. Commun., 28, 203 (1967).

23. G. E. Hein and C. Niemann, J. Amer. Chem. Soc., 84, 4495 (1962).

24. M. Dixon and E. C. Webb, "Enzymes," Academic Press, New York, 1960, p. 129.

25. K. Inouye, I. Voynick, G. R. Delpierre, and J. S. Fruton, Biochemistry, 5, 2473 (1966).

26. J. N. Mills and J. Tang, J. Biol. Chem., 242, 3093 (1967).

27. J. A. Hartsuck and J. Tang, J. Biol. Chem., 247, 2575 (1972).

28. T. G. Rajagopalan, S. Moore, and W. H. Stein, J. Biol. Chem., 241, 4940 (1966).

29. A. J. Cornish-Bowden and J. R. Knowles, Biochem. J., 113, 353 (1969).

30. K. Inouye and J. S. Fruton, Biochemistry, 7, 1611 (1968).

31. J. Tang, J. Biol. Chem., 246, 4510 (1971).

32. R. S. Bayliss, J. R. Knowles, and G. B. Wybrandt, Biochem. J., 113, 377 (1969).

33. T. R. Hollands and J. S. Fruton, Proc. Nat. Acad. Sci. U. S., 62, 1116 (1969).

34. W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, 1969, p. 246.

35. M. S. Silver and M. Stoddard, Biochemistry, 11, 191 (1972).

36. L. M. Ginodman and N. G. Lutsenko, Biokhimiya, 37, 81 (1972).

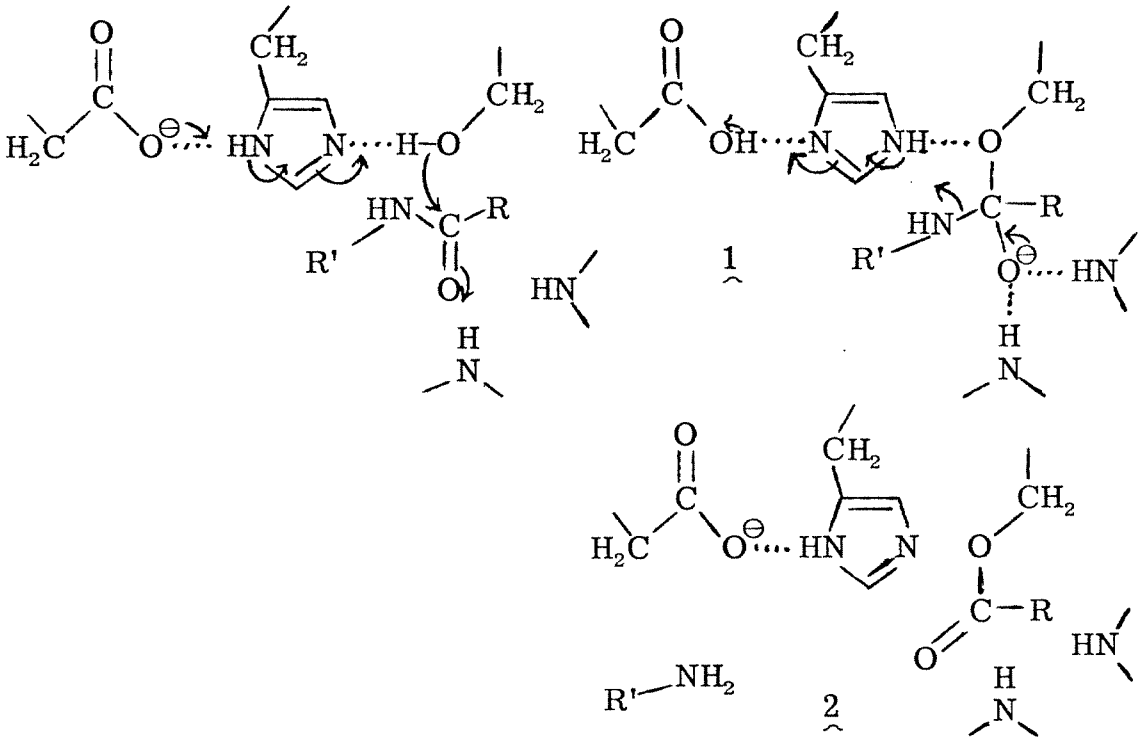
37. T. P. Stein and D. Fahrney, Chem. Commun., 555 (1968).

38. T. M. Kitson and J. R. Knowles, Biochem. J., 122, 241 (1971).

39. T. M. Kitson and J. R. Knowles, Biochem. J., 122, 249 (1971).

PROPOSITION I

Considerable experimental evidence (1, 2) suggests that much of the catalytic power of serine proteases arises from structural features of the enzymes which stabilize transition states resembling a tetrahedral intermediate (adduct between serine hydroxyl and substrate carbonyl) which contains a formal negative charge (eq 1).



Formation of this intermediate (1) is assisted by net transfer of the hydroxyl proton to a buried carboxylate anion via an intervening imidazole group. Breakdown of the adduct involves transfer of the proton (actually on N^{τ} of the imidazole) to the substrate amido nitrogen to liberate the amine product and produce the acyl intermediate (2). Replacement of the amine by a water molecule is then followed by a reversal of the reaction sequence to liberate the

acid product and regenerate active enzyme.

Available evidence (3) suggests that, for specific amide substrates, the rate-determining step for the entire reaction is breakdown to the tetrahedral intermediate $\underset{\wedge}{1}$ to the free amine and acyl enzyme $\underset{\wedge}{2}$. Accumulation of $\underset{\wedge}{1}$ is, nevertheless, too small for direct spectrophotometric observation. Determination of the actual charge distribution of $\underset{\wedge}{1}$ is, however, so crucial to understanding the catalytic process that it is highly desirable to have some way to characterize the adduct. Magnetic resonance studies (4) of the tetrahedral adduct (hemiacetal) formed between a bacterial serine protease, α-lytic protease, and a peptide aldehyde (using a ^{13}C label at C-2 of the histidine imidazole as the probe) indicate that the structure shown above for $\underset{\wedge}{1}$ (rather than one containing carboxylate/imidazolium) is correct). A direct characterization of the actual reaction intermediate is still needed, though, since the aldehyde lacks an essential feature (the potential leaving group) of this structure, and an experimental approach using carbon magnetic resonance spectroscopy is proposed below.

Several naturally-occuring proteins strongly inhibit the catalytic activity of trypsin (5). All seem to contain a reactive peptide bond (whose carbonyl is contributed by an arginine or lysine residue) which can be attacked by the enzyme. Secondary binding forces, however, tend to maintain the complex between enzyme and inhibitor, and in some cases the dissociation constants are as low as 10^{-14} M (6). Ruhlmann et al. (7) have shown by x-ray diffraction techniques that the complex between pancreatic trypsin inhibitor and trypsin

actually contains a covalent bond between the active site serine O^{γ} and the inhibitor lysine carbonyl carbon. Further, this bond is maintained in the complex without breaking of the bond between lysine and the adjoining alanine residue. Thus, the stable structure of the crystalline complex is a covalent, tetrahedral adduct which should be an excellent model for the desired tetrahedral intermediate (since, after all, the reactive inhibitor peptide bond is a specific substrate). Breakdown of the adduct to liberate the alanine amino group is apparently inhibited by steric constraints on movement of the histidine imidazole to allow transfer of its N^{τ} proton to the amido nitrogen of the alanine. The hydrogen bonding structure linking aspartic acid O^{δ} with histidine N^{π} and serine O^{γ} with histidine N^{τ} is not perturbed, however, and neither is that between the trypsin backbone NH groups (Gly 193 and Ser 195) and the proposed oxyanion (inhibitor lysine carbonyl oxygen).

A study of the charge distribution on the various groups of the trypsin/inhibitor complex as a function of pH can be made by specifically enriching the lysine carbonyl carbon with ^{13}C and observing this label with carbon magnetic resonance spectroscopy. This carbon, which undergoes a change in hybridization (sp^2 to sp^3) during formation of the complex, should be a sensitive probe for the study since it is directly bonded to one ionizable group and is located near the histidine and aspartic acid. Although it might seem difficult to devise a method of achieving this enrichment, Sealock and Laskowski (8) have developed an elegant procedure by which the reactive arginyl residue in soybean trypsin inhibitor

(Kunitz) is enzymatically replaced by a lysine residue. Desarginine inhibitor is obtained by first treating the virgin inhibitor with catalytic (2 mole %) amounts of trypsin at pH 3.7 (to establish equilibrium between virgin and modified inhibitor in which the reactive arginine-isoleucine bond is cleaved) and then treating the modified inhibitor with carboxypeptidase B at pH 7.6 (to liberate free arginine from the newly-formed carboxy-terminus). Addition of free lysine to a solution containing equimolar amounts of desarginine inhibitor, trypsin, and carboxypeptidase B at pH 6.7 results in covalent attachment of the lysine at the position previously occupied by the arginine since the enzymatic reactions are reversible and the equilibrium lies heavily ($K = 10^6\ M^{-2}$) in favor of the modified inhibitor which complexes strongly with the trypsin (whereas the desarginine inhibitor does not). The latter complex can be

$$\text{desarginine inhibitor} + \text{lysine} \underset{\text{carboxypeptidase B}}{\rightleftharpoons} \text{modified inhibitor} \quad (K = 10^{-2}M^{-1})$$

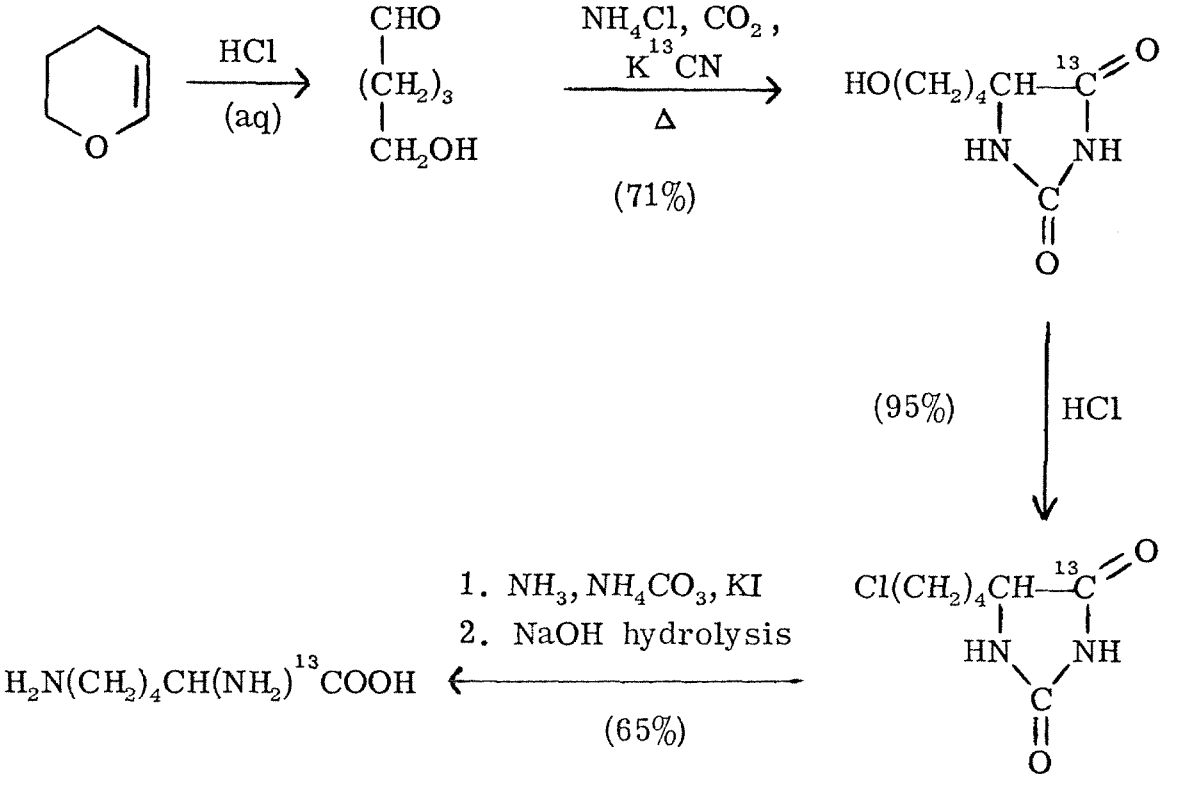
$$\text{native inhibitor} + \text{trypsin} \rightleftharpoons \text{complex} \quad (K = 10^{8}M^{-1})$$

$$\text{desarginine inhibitor} + \text{lysine} + \text{trypsin} \underset{\text{carboxypeptidase B}}{\rightleftharpoons} \text{complex} \quad (K = 10^{6}M^{-2})$$

dissociated in 6 M guanidine hydrochloride at pH 7 to yield denatured trypsin and native inhibitor which is purified by gel filtration.

Thus, if one uses L-[1-^{13}C]lysine for the resynthesis of the native inhibitor, the appropriate label can be specifically incorporated into the protein. The ^{13}C-enriched lysine can be synthesized

in high yield by the method of Rogers et al. (9) which uses dihydropyran and potassium [^{13}C]cyanide as the key starting materials.



References

1. M. W. Hunkapiller, S. H. Smallcombe, D. R. Whitaker, and J. H. Richards, Biochemistry, 12, 4732 (1973).

2. J. D. Robertus, J. Kraut, R. A. Alden, and J. J. Birktoft, Biochemistry, 11, 4293 (1972).

3. A. R. Fersht and Y. Requena, J. Amer. Chem. Soc., 93, 7079 (1971).

4. Part II, This Thesis.

5. M. Laskowski, Jr., and R. W. Sealock, The Enzymes, Third Edition, 3, 376 (1971).

6. J. P. Vincent and M. Lazdunski, Biochemistry, 11, 2967 (1972).

7. A. Ruhlmann, D. Kukla, P. Schwager, K. Bartels, and R. Huber, J. Mol. Biol. 77, 417 (1973).

8. R. W. Sealock and M. Laskowski, Jr., Biochemistry, 9, 3703 (1969).

9. R. A. Rogers, R. D. Emmick, L. W. Tyran, L. L. Phillips, A. A. Levine, and N. D. Scott, J. Amer. Chem. Soc., 71, 1837 (1949).

PROPOSITION II

The mechanism of the cooperative interaction between thymus-derived (T) and bone marrow-derived (B) lymphocytes which triggers antibody production by the B cells in response to antigenic challenge is the subject of considerable study (1). Although both cell types possess specific surface receptors which recognize the antigen, T cells are apparently the mediators of the control process which regulates the antibody response by the B cells (2). Several studies suggest that this role may be fulfilled by T cells acting in three ways: (i) specific antigen presentation to the B cells (3, 4); (ii) release of a specific or nonspecific mediator which transmits a signal to the B cells (5, 6); or (iii) a combination of i and ii (7). There is considerable evidence (8) that correct antigen presentation is critical to the ability of the B cell to recognize the foreign substance, and T cells may function to concentrate the antigen on their receptors so as to optimally orient the antigenic determinants to the B cells. This presentation to the B cell could occur directly between the surfaces of the T and B cells or after release of the complexed antigen from the T cells. There are indications that released, complexed antigen may first be concentrated on the surface of macrophages from where it is then detected by the B cells (9). There are also many reports of nonspecific factors (1, 5, 6) released by the T cells upon their recognition of antigen which stimulate B cell activity. These factors, unlike the specific T cell receptor/antigen complexes which would presumably only trigger specific

B cells, are capable of inducing blast transformation and antibody production by B cells which recognize unrelated antigens. Although data from several workers are ambiguous and even contradictory, there are reports that the activity in some systems is associated with a proteinaceous (trypsin-sensitive) material of molecular weight 150,000-300,000 (10). The exact nature and function of this nonspecific mediator has not been established, however, since it has not been separated from the crude T cell extract which contains a variety of substances, some of which actually inhibit B cell response (11). It also appears that the activating factor does not stimulate B cells unless intact, though irradiated, T cells are present in the culture (irradiation destroys the ability of the T cell to synthesize the nonspecific mediator but apparently does not block the specific antigen presentation role) (7). Thus, proper T cell action may require both specific and nonspecific roles to be functional.

One of the primary difficulties plagueing attempts to elucidate the role of the soluble mediators (either specific or nonspecific has been the complexity of the systems used. The lymphocyte cultures used have included a broad spectrum of antigen specificites, and only a small fraction of the lymphocytes respond specifically to the antigen being studied. Supernatants from these cultures necessarily contain, therefore, such a wide range of substances that defining the properties of the few produced in response to a given antigen is difficult. For this reason, a study of lymphocyte cultures containing only B and/or T cells specific for a given antigen

should prove useful in elucidating the role of the proposed chemical mediators, and such a study is outlined below.

The lymphocytes which respond to the dinitrophenyl (DNP) hapten can be fractionated from the total lymphocyte population by specific absorption onto nylon fibers coated with DNP-derivatized gelatin (12). T cells can be separated from B cells by treating the lymphocytes with serum complement and specific antisera to either the B or T cells (13). Further, cells with high affinity surface receptors (whose numbers increase dramatically after initial antigen challenge in vivo) can be separated from those with relatively lower affinity receptors (whose numbers do not seem to increase after immunization) by inhibiting binding of the high affinity cell to the DNP-gelatin with dilute (4 μg/ml) solutions of DNP-bovine serum albumin (DNP-BSA) (13). The several classes of bound cells can be removed from the gelatin by plucking the taut fibers in a medium containing fetal calf serum. The released cells are viable (> 90% as measured by trypan blue exclusion) and can be used to reconstitute a response to the DNP-hapten in irradiated, syngeneic mice (12).

Attempts can be made to isolate soluble mediators from T cell fractions by incubating the lymphocytes with antigen (such as DNP-BSA) and testing the supernatant for activity capable of replacing the T cell function in cultures containing only B cells or cultures of B cells with macrophages and/or irradiated T cells. Antibody production by transformed B cells is assayed by the Jerne plaque assay (14). If soluble mediators are found, their chemical nature

can be studied by observing their stability to trypsin, DNase, and RNase. Their specificity can be studied by determining their ability to initiate antibody production by B cells specific for other antigens. The presence of complexed antigen in the mediator can be tested by treatment of the active supernatant with specific DNP-BSA antisera. Finally, attempts can be made to isolate active material(s) from the supernatant by classical fractionation methods (ammonium sulfate precipitation, ion exchange chromatography, affinity chromatography, gel filtration, gel electrophoresis, etc.). Purification would permit more precise chemical characterization of the material (amino acid, nucleic acid, and carbohydrate content and composition).

References

1. D. H. Katz and B. Benaceraff, Adv. Immuno., 15, 1 (1972).
2. M. C. Raff, Nature, 242, 19 (1973).
3. N. A. Mitchison, in "Mediators of Cellular Immunity," edited by H. S. Lawrence and M. Landy, Academic Press, New York, 1969, p. 97.
4. M. Feldman, J. Exp. Med., 136, 737 (1972).
5. R. M. Gorczynski, R. G. Miller, and R. A. Phillips, J. Immuno., 108, 547 (1972).
6. M. Feldman and A. Basten, Nature New Biology, 237, 13 (1972).
7. R. M. Gorczynski, R. G. Miller, and R. A. Phillips, J. Immuno., 111, 900 (1973).

8. M. Feldman and A. J. Basten, J. Exp. Med., 134, 103 (1971).

9. M. Feldman, J. Exp. Med., 135, 1049 (1972).

10. R. M. Gorczynski, Ph. D. Thesis, University of Toronto, 1972.

11. R. M. Gorczynski, R. G. Miller, and R. A. Phillips, J. Immuno., 110, 968 (1973).

12. U. Rutishauser, P. D'Eustachio, and G. M. Edelman, Proc. Nat. Acad. Sci. U. S., 70, 3894 (1973).

13. G. M. Edelman, Science, 180, 830 (1973).

14. M. B. Rittenberg and K. L. Pratt, Proc. Soc. Exp. Biol. Med., 132, 575 (1969).

PROPOSITION III

Optimal humoral response to a small group of antigens can apparently develop without participation of thymus-derived (T) lymphocytes. Included in this group are pneumococcal polysaccharide (1), polymerized flagellin of Salmonella adelaide (2), Escherichia coli polysaccharide (3), polyvinylpyrrollidone (3), and MS2 phage (4). The common feature of these substances is a structure which consists of identical units arranged in a repeating, linear sequence. This property apparently favors a positive immunogenic signal upon direct interaction between antigen and bone marrow-derived (B) lymphocyte surface receptors. High doses of antigen, however, can render the B cells specifically unresponsive for prolonged periods of time to doses of antigen that are normally immunogenic (5, 6). Since T cells do not seem to be essential to the response to these antigens and since the response does not (as is usually the case for T cell-mediated responses) seem to be subject to low-dose induced tolerance (7), the high-dose induced tolerance probably results from a direct interaction between the antigen and B cell (8).

The interactions between surface receptors and antigens which trigger blast transformation and antibody production at certain antigen concentrations and induce tolerance at higher concentrations of antigen are not well understood. Techniques using fluorescence- and radio-labeled antigens have revealed interesting phenomena which occur on the B cell surface upon

antigen binding, however, and these phenomena are probably related to the transformation and tolerance-inducing responses. Early studies (9, 10) using fluorescein-labeled antibodies specific for the surface receptors on B cells showed that initially the bound label appears randomly distributed over the cell surface. However, the bound antibody and its attached receptors soon aggregate in patches which collect in a "cap" covering only a small polar region of the cell. The complexed receptors are subsequently cleared from the cell surface and do not seem to reappear. Diener and Paetkau (11) have used tritium-labeled polymerized flagellin (S. adelaide) to show that this capping phenomenon is also observed when specific antigen binds to the surface receptors at immunogenic doses. However, in this case the receptors are rapidly replaced, and further incubation with antigen results in continued cap development and an overall increase in total bound antigen. Incubation with tolerogenic doses of flagellin leads to initial cap formation, but the subsequent increase in receptor density apparently results in a degree of cross-linking by antigen which restricts further capping.

The relation of these phenomena to immunity/tolerance induction is not yet clear, but the sensity of the radio-labeled-antigen binding technique should permit valuable visual observation of the cell surface dynamics relating to antigen recognition. In particular, a study of the effect of chemical substances which can, under certain conditions, break the tolerant state should be useful.

Synthetic polyanions, including the polynucleotyde poly A-poly U (12), dextran sulfate (13, 14), and polyacrylic acid (13, 14) can serve this function, and they appear to act directly on B cells. Though their mode of action is not known, they also seem to replace the normal T cell stimulatory function in T cell- dependent B cell antibody production (12-14).

The heterogeneity of the B cell population that responds to the polymerized flagellin hinders the interpretation of quantitative aspects of antigen binding in the sustem studied by Diener and Paetku (11). This problem can be averted by studying the humoral response by BALB/c mice to phosphorylcholine (PPC). The response to this antigen, which is a constituent of the C-polysaccharide from the rough strain of pneumoccus R36A, appears to be monoclonal in BALB/c mice (15). Immunization with this hapten, whether as pneumoccal polysaccharide or as p-azophenylphosphorylcholine-derivatized carrier proteins, elicits production of a single PPC antibody which is identical to the TEPC-15 myeloma protein (16). Spleen cells from immunized mice could be fractionated by the nylon fiber binding technique of Edelman (17). Nylon fibers coated with PPC-derivatized gelatin should specifically bind the PPC-sensitive clone. These can be removed with high recovery of viable cells by plucking the taut fibers in a medium containing fetal calf serum to yield a highly-enriched PPC-sensitive cell culture. The tritium-labeled pneumoccal polysaccharide can be produced by culturing the bacteria in a medium containing [^{3}H]glucose (19). Binding of the antigen to the surface receptors on

lymphocytes can be studied by autoradiography (11). The binding and capping process can be studied as a function of antigen concentration, temperature, and time. The influence of polyanions such as polyacrylic acid or dextran sulfate can be studied by observing the antigen binding in the presence of these compounds. Radio-labeled polyanions could be used to detect specific binding of these compounds to the B cell surface. Furthermore, the in vitro studies with the specific B cells can be compared to in vivo phenomena and in vitro cultures containing mixed lymphocyte populations to determine other factors which might influence the B cell response.

References

1. J. H. Humphrey, D. M. V. Parrot, and J. East, Immunology, 7, 419 (1964).
2. W. D. Armstrong, E. Diener, and G. R. Shellam, J. Exp. Med., 129, 383 (1969).
3. B. Andersson and H. Blomgren, Cell. Immunol., 2, 411 (1971).
4. R. S. Basch, Int. Arch. Allergy Appl. Immunol., 30, 105 (1966).
5. M. Feldman, Nature New Biol., 231, 21 (1971).
6. D. W. Dresser and N. A. Mitchison, Advan. Immunol., 8, 129 (1968).

7. N. A. Mitchison, in "Cell Interactions and Receptor Antibodies in Immune Responses," edited by O. Makela, A. Cross, and T. Kosunen, Academic Press, New York, 1971, p. 249.

8. D. H. Katz and B. Benaceraff, Adv. Immunol., 15, 1, (1972).

9. R. B. Taylor, W. P. H. Duffus, M. C. Raff, and S. de Petris, Nature New Biol., 233, 225 (1971).

10. E. R. Unanue, W. D. Perkins, and M. J. Karnovsky, J. Immunol., 108, 569 (1972).

11. E. Diener and V. H. Paetku, Proc. Nat. Acad. Sci. U. S., 69, 2364 (1972).

12. P. A. Campbell and P. Kind, J. Immunol., 107, 1719 (1971).

13. T. Diamantstein, B. Wagner, J. L'Age-Stehr, I. Beyse, M. V. Odenwald, and G. Schultz, Eur. J. Immunol., 1, 301 (1971).

14. T. Diamantstein and B. Wagner, Nature New Biol., 241, 117 (1973).

15. W. L. Lee, H. Cosenza, and H. Kohler, Nature, 247, 57 (1974).

16. M. Potter and R. Libermann, J. Exp. Med., 132, 737 (1970).

17. U. Rutishauser, P. D'Eustachio, and G. M. Edelman, Proc. Nat. Acad. Sci. U. S., 70, 3894 (1973).

18. A. M. Pappenheimer, Jr., W. D. Reed, and R. J. Brown, J. Immunol., 100, 1237 (1968).

PROPOSITION IV

Immunoglobulins are complex, antigen-binding proteins which consist of four (or more) polypeptide chains linked by disulfide bonds and noncovalent forces (1). Chemical studies have shown that they consist of dimeric units, each of which contains paired light (MW 23,000) and heavy (MW 53,000-70,000) chains (2). Further, the chains contain internal homologous units or domains: four in the heavy chain and two in the light chain (3). Although antibodies produced by any single organism are amazingly heterogeneous, most of the sequence diversity is located in the amino terminal domains (the variable regions) in the light and heavy chains, with the remainder (the constant regions) of the molecules essentially homologous. Further, within the variable regions of each chain exist short sequences which display extreme amino acid diversity from one antibody family to another. These so-called hypervariable regions are thought to form the antigen recognition or binding site of the immunoglobulin (3). Indirect evidence which included affinity labeling studies suggested that these sequence regions were at or near the antigen binding site, and recent x-ray crystallographic studies have confirmed that these areas combine to form a shallow cleft at one end of the protein (4).

There is as yet only limited understanding of the structural features of the antibodies which are responsible for their specificity. A major difficulty retarding efforts to characterize the binding sites is the extreme heterogeneity of antibodies produced in response

even to simple, well defined antigens. However, a major breakthrough came with the discovery that myeloma protein secreted by lymphomas which can readily be induced in a laboratory strain of mice (BALB/c) are normal, functional immunoglobulins (5). These proteins thus serve as a ready supply of easily purified antibody since they can comprise up to 95% of the circulating antibody in mice with the tumors (3).

Several of these proteins have been shown to exhibit high affinity for low molecular weight chemical haptens, and several groups have used reagents similar to these haptens, but containing also reactive groups capable of forming covalent bonds with side chains of amino acid residues, to chemically modify the antigenic binding sites (6-13). The chemical nature of these reagents, which have included diazonium, bromoacetyl, azide, and diazoketone reactive groups, is such that almost the entire hapten is left covalently attached to the protein after reaction. Thus, the ability of the antibody to bind ligands is generally abolished, although it is difficult to determine whether the effect is due to steric blocking of the recognition site by the bulky reagent or to specific disruption of a crucial interaction between hapten and antibody amino acid residue. Efforts to chemically modify the antibodies with haptens containing reacting groups which leave only small moieties attached to the protein would be useful in this determination, and studies of this type are proposed below.

The chemical modification studies reported to date indicate that tyrosine and lysine residues are frequently constituents of the

binding site (4). The nucleophilic side chains of these amino acids should prove particularly amenable to modification by suitable labeling reagents. Lysine residues, for instance, can be converted to homoarginine by reaction with S-methyl- or O-methylisourea at alkaline pH (14, 15). Attachment of the isourea to specific hapten moieties (such as dinitrophenyl or phosphorylcholine groups) should produce useful affinity labels. Alternatively, specific modification of critical lysine residues might be achieved by first blocking the surface lysines with a reagent such citraconic anhydride (16) in the presence of hapten to protect the critical residue. Removal of hapten would then expose the binding site lysine to reaction with O-methylisourea. Finally, removal of the citraconyl groups could be effected by overnhight incubation of the protein at pH 3.5, room temperature, to yield a protein modified only at specific lysine residues in the binding site.

Aryl cyanates are another potentially useful class of labeling reagents (17). They have been used to effect specific carbamylation of an amino terminal and active site serine hydroxyl group in chymotrypsin, and their electrophilicity is readily controlled by the nature of the substituents on the aromatic ring (18). Furthermore, they are readily synthesized by treatment of phenols with cyanogen bromide, and they may be effective reagents for the modification of phenolic hydroxyl and lysine amino groups of the antibodies.

Bender (19) has shown that an aryl sulfonate, methyl p-nitrobensulfonate, specifically methylates the active site histidine residue in chymotrypsin. Since one of the reactive antibody tyrosine

residues is replaced by histidine in some human myeloma immunoglobulins (4), this and similar reagents might be used to label the binding sites of these proteins.

Since the moieties left attached to the antibody groups by reaction with these reagents are relatively small, they should serve as excellent probes for the dynamic and structural features of the antigen binding sites. In particular, it might be possible to specifically introduce a ^{13}C label into the antibodies using appropriate ^{13}C-enriched affinity labels of the types described above. The nature and environment of these probes in the free and antigen-complexed antibodies could then be studied using carbon magnetic resonance techniques. Such studies could be coupled with classical methods (such as equilibrium dialysis and fluorescence quenching) for measuring binding affinities and perturbations on the antibody induced by ligand binding to gain valuable insight into the antigen binding process.

References

1. G. M. Edelman, Ann. N. Y. Acad. Sci., 190, 5 (1971).
2. G. M. Edelman, Biochemistry, 9, 3197 (1970).
3. L. Hood, Stadler Symp., 5, 73 (1973).
4. R. J. Poljak, L. M. Amzel, H. P. Avey, B. L. Chen, R. P. Phizackerley, and F. Saul, Proc. Nat. Acad. Sci. U.S., 70, 3305 (1973).
5. H. G. Kunkel, Harvey Lect., 59, 219 (1965).

6. S. J. Singer, N. Martin, and N. O. Thorpe, Ann. N. Y. Acad. Sci., 190, 342 (1971).

7. E. J. Goetzl and H. Metzger, Biochemistry, 9, 3862 (1970).

8. F. Franek, Eur. J. Biochem., 19, 176 (1971).

9. A. Ray and J. J. Cebra, Biochemistry, 11, 3647 (1972).

10. D. Givol, P. H. Strausbauch, E. Hurwitz, M. Wilchek, J. Haimovich, and H. N. Eisen, Biochemistry, 10, 3461 (1971).

11. G. W. J. Fleet, J. R. Knowles, and R. R. Porter, Biochem. J., 128, 499 (1972).

12. B. Chesbro and H. Metzger, Biochemistry, 11, 766 (1972).

13. M. Yoshioka, J. Lifter, C. L. Hew, C. A. Converse, M. Y. K. Armstrong, W. H. Konigsberg, and F. F. Richards, Biochemistry, 12, 4679 (1973).

14. W. L. Hughes, H. A. Saroff, and A. L. Carney, J. Amer. Chem. Soc., 71, 2476 (1949).

15. B. Kassell and R. Chow, Biochemistry, 5, 3449 (1966).

16. H. B. J. Dixon and R. N. Perham, Biochem. J., 109, 312 (1968).

17. E. Grigat and R. Putter, Angew. Chem. Int. Ed. Engl., 6, 206 (1967).

18. G. T. Robillard, J. C. Powers, and P. E. Wilcox, Biochemistry, 11, 1773 (1972).

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

PROPOSITION V

Important, though as yet undertermined, aspects of pepsin-catalyzed hydrolysis of amide and ester bonds are the existence and characteristics of any covalent intermediates that might form during the catalytic reaction. Attempts to isolate possible intermediates of the acyl (anhydride) or amino (amide) type have failed (1). Further, the failure of the covalent amino-enzyme hypothesis to explain pepsin-catalyzed transpeptidation has led to a questioning of the often accepted need for such an intermediate (2).

Attempts to identify catalytically important groups on the enzyme have been more successful. The pH dependence of the hydrolysis of neutral dipeptide substrates suggest that at least two catalytic groups with pK_a's of 1.1 and 4.7 are essential for catalysis (3). Chemical modification studies using active site-directed, irreversible inhibitors have shown that at least two, and possibly three, enzymic carboxyl groups are necessary for enzymic activity (4, 5). The function of these groups is still somewhat uncertain, but kinetic evidence suggests that one acts as a carboxylate anion and one as a neutral carboxylic acid group. The involvement of an important electrophilic component has been suggested by the equivalence of the rates for pepsin-catalyzed hydrolysis of peptide (amide) and depsipeptide (ester) substrates which might suggest that the carboxyl group with pK_a 4.7 may form a hydrogen bond or donate a proton (6, 7). However, solvent isotope effects

(k_{H_2O}/k_{D_2O}) have been observed only for N-trifluoroacetyl-L-phenylalanine (8) and Gly-Gly-Gly-L-(p-NO_2)Phe-L-PheOCH_3 (9); they are absent for other peptides and all sulfite esters (10, 11).

Nucleophilic attack by the carboxylate anion (pK_a 1.1) on the substrate carbonyl group to yield some type of tetrahedral intermediate is generally thought to be the first step in hydrolysis. This attack may be facilitated by concerted electrophilic attack by the neutral carboxyl group (pK_a 4.7) on the carbonyl oxygen. However, the mechanism by which this intermediate breaks down to release products remains unknown. Two aspects of the reaction process, in particular, require further examination: (i) the order of product release and (ii) the source of the oxygen atom added to the acyl moiety of the substrate to produce the carboxyl product. Knowles (12, 13) has suggested that product release is ordered with the carboxylic acid, as the anion, being the first product to leave the enzyme. However, the observation that pepsin can catalyze transpeptidation by acyl transfer as well as by amino transfer suggests a more random mechanism (14), and the apparent order of product release may, in fact, be pH dependent (15). There is one report that an oxygen atom may be transferred from an enzymic carboxyl group to the sulfur atom during hydrolysis of p-bromophenyl methyl sulfite (16), but analogous studies with other substrates have not been performed.

The use of ^{18}O as a tracer should help establish the detailed pathway of oxygen from water to the carbonyl group of product and, hence, shed light on reaction intermediates. In particular, such

experiments should reveal whether the oxygen that goes into the product carboxyl group comes directly from water or from one of the oxygens in the pepsin carboxyl groups. If the pepsin is incubated with a several-fold molar excess of substrate in $H_2{}^{18}O$, one or more carboxyl groups become enriched with ^{18}O (17). Removal of the products and $H_2{}^{18}O$ by dialysis should leave an enzyme which, upon incubation with a second substrate in $H_2{}^{16}O$, might transfer the ^{18}O label to the substrate. This sequence can be confirmed by isolation of the product from the second incubation and pyrolysis of the product with guanidinium hydrochloride (18) to produce CO_2 whose ^{18}O content can be determined by mass spectrometry. If positive results are obtained, the experiment can be repeated with various reaction products (virtual substrates) to determine whether they too can promote the incorporation of ^{18}O into the catalytic carboxyl groups. This approach should help elucidate the reaction sequence, and it may give clues to the identity and characteristics of intermediates.

Localization of the ^{18}O label in the enzyme can also be attempted. One approach would involve rapid denaturation of the enzyme by raising the pH to 8 to freeze any ^{18}O incorporated into catalytic groups. Enzymatic degradation of the denatured pepsin would then produce fragments whose ^{18}O content can be analyzed by decarboxylation (of the silver salt of the peptides) with bromine and mass spectroscopic analysis of the released CO_2 (17). Another approach would involve inactivation of the ^{18}O-enriched pepsin (produced by incubation of pepsin in $H_2{}^{18}O$ with a substrate or

substrate analogue) by addition of a diazo or epoxide inhibitor (19). Each of these two classes of inhibitors forms a stable ester bond with a different, essential enzymic carboxyl group. Accordingly, alkaline hydrolysis of the purified, inhibited enzyme should, by cleavage of the carbonyl carbon-oxygen bond of the ester, release inhibitor containing some of the ^{18}O originally present in the enzymic carboxyl group with which the inhibitor reacted. Pyrolysis of the inhibitor residue, thus released, with cupric chloride can then be used to convert the released oxygen to carbon dioxide for mass spectroscopic analysis (19).

References

1. G. E. Clement, Prog. Biorg. Chem., 2, 177 (1973).
2. M. S. Silver and M. Stoddard, Biochemistry, 11, 191 (1972).
3. A. J. Cornish-Bowden and J. R. Knowles, Biochem. J., 113, 353 (1969).
4. R. L. Lundblad and W. H. Stein, J. Biol. Chem., 244, 154 (1969).
5. J. A. Hartsuck and J. Tang, J. Biol. Chem., 247, 2575 (1972).
6. K. Inouye and J. S. Fruton, Biochemistry, 6, 1765 (1967).
7. J. R. Knowles, Phil. Trans. Roy. Soc. London B, 257, 135 (1970).
8. M. W. Hunkapiller and J. H. Richards, Biochemistry, 11, 2829 (1972).

9. T. R. Hollands and J. S. Fruton, Proc. Nat. Acad. Sci. U. S., 62, 1116 (1969).

10. G. E. Clement and S. L. Snyder, J. Amer. Chem. Soc., 88, 5338 (1969).

11. T. W. Reid and D. Fahrney, J. Amer. Chem. Soc., 89, 3941 (1967).

12. T. M. Kitson and J. R. Knowles, Biochem. J., 122, 241 (1971).

13. T. M. Kitson and J. R. Knowles, Biochem. J., 122, 249 (1971).

14. M. Takahashi and T. Hoffmann, Proc. Biochem. Soc., 127, 35P (1972).

15. T. M. Ginodman and N. G. Lutsenko, Biokhimiya, 37, 81 (1972).

16. T. P. Stein and D. Fahrney, Chem. Commun., 555 (1968).

17. L. S. Shkarenkova, L. M. Ginodman, L. V. Kozlov, and V. M. Orekhovich, Biokhimiya, 32, 969 (1967).

18. P. D. Boyer, D. J. Graves, C. H. Suelter, and M. E. Dempsey, Anal. Chem., 13, 1906 (1961).

19. L. M. Ginodman, T. A. Valueva, L. V. Kozlov, and L. S. Shkarenkova, Biokhimiya, 34, 171 (1969).