I. INHIBITION OF ELASTASE BY PEPTIDE CHLOROMETHYL KETONES

II. MODIFICATION OF CHYMOTRYPSIN BY AN ARYL CYANATE REAGENT

A THESIS

Presented to

The Faculty of the Division of

Graduate Studies and Research

by

Peter Mirko Tuhy

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in the School of Chemistry

Georgia Institute of Technology

January, 1975

Original Page Numbering Retained.

I. INHIBITION OF ELASTASE BY PEPTIDE CHLOROMETHYL KETONES

II. MODIFICATION OF CHYMOTRYPSIN BY AN ARYL CYANATE REAGENT

Approved:

Charles L. Liotta, Chairman

Herbert O. House

Sheldon W. May

James C. Powers, Advisor

Date approved by Chairman: 27 Feb 75

ACKNOWLEDGMENTS

I would like to express deep gratitude toward my research advisor, Dr. Jim Powers, for his valuable help, advice, ideas, and support in the chemistry laboratory and in all other areas of academic concern. I feel that I owe to him as my mentor not just the knowledge but also the attitudes I will need in my future career as a research biochemist. It has been a great privilege and pleasure to be associated with this fine scientist, teacher, and mountaineer during my stay at Georgia Tech. I also want to thank Dr. Spicer and Dr. Bertrand, as directors of the School of Chemistry, for providing my financial support in the form of a NSF Traineeship (1970-72) and a NDEA Traineeship (1972-74), and Dr. Powers for providing a Research Assistantship (1974-75). In addition I acknowledge the help and suggestions of Dr. Liotta, Dr. House, and Dr. May as members of my research advisory committee. Finally I wish to thank an assortment of research lab associates, faculty members, family members, school friends, camping partners, and girlfriends for making the student life more enjoyable and interesting, and of course God for making everything possible.

In connection with the experimental work I wish to express thanks to Dr. Akhtar Ali, formerly at the University of Washington, for synthesizing a number of peptide acids

used as peptide chloromethyl ketone precursors, and to Dr. Aaron Janoff at the State University of New York at Stony Brook for providing the 1.6 mg of human leukocyte elastase used in inhibition studies. Special permission was received from the Division of Graduate Studies to vary the headings of this thesis from that specified in the thesis manual, in order that these headings might conform to those found in the biochemical literature in which this dissertation has been published.

I. INHIBITION OF ELASTASE BY PEPTIDE CHLOROMETHYL KETONES

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

SUMMARY

A series of P_1-alanine peptide chloromethyl ketones, six tripeptides and five tetrapeptides, were synthesized for use as inhibitors of elastase in kinetic studies and structure-reactivity correlations. Most of them are effective, active-site-directed, irreversible inhibitors, fairly reactive and highly specific for both porcine pancreatic elastase and human leukocyte elastase. A typical reagent, Ac-Ala-Ala-Ala-AlaCH_2Cl, is specific for elastolytic proteases since it virtually does not inhibit related chymotrypsin or trypsin under identical conditions. The tetrapeptides are markedly more reactive than the tripeptides in general according to relative $k_{obsd}/[I]$ and k_3/K_I values, caused by tighter binding (smaller K_I) plus a faster rate of inactivation (larger k_3), leading to irreversible enzyme inhibition. The most effective elastase inhibitor is Ac-Ala-Ala-Pro-AlaCH_2Cl, and its isomer Ac-Ala-Pro-Ala-AlaCH_2Cl is a non-inhibitor for pancreatic elastase but a good inhibitor for leukocyte elastase, showing that the two enzymes are different and distinct. For optimum reactivity a leukocyte elastase inhibitor should possess a P_1 alanine, a P_2 proline or leucine, no P_3 proline (for tripeptides), and preferably a P_4 residue (i.e. a tetrapeptide). The behavioral similarity of elastase toward peptide substrates and inhibitors

implies that they are both interacting with the same extended binding site region in the enzyme. Human leukocyte elastase reacts less rapidly overall with the set of peptide chloromethyl ketones, and it is now implicated in the pathological processes of pulmonary emphysema, arthritis, arteritis, and related elastic tissue diseases. The peptide chloromethyl ketones are presently being used in investigations of the physiological and pathological functions of elastolytic proteases and eventually may become valuable in treating these diseases.

CHAPTER I

INTRODUCTION

Proteolytic enzymes, or protein-hydrolyzing enzymes, are among the most widespread, common, and important types of enzymes found in living systems from humans to bacteria. Because of their major roles in life processes as well as their availability they have undergone extensive and intensive investigation. Proteolytic enzyme chemistry is presently at the forefront of basic research efforts in biochemical and biomedical studies. Some of the human biological processes in which these enzymes are involved include blood coagulation, food digestion, ovum fertilization, hormone production, immunological response, zymogen activation, and tissue inflammation. They are becoming utilized in industrial processes for breaking down protein raw materials, and studied in medical science due to their implication in tissue-destruction diseases. Since 1960, proteolytic enzymes have been discovered to play a role in an ever-increasing number of human physiological and pathological functions.

Among the proteolytic enzymes is a rather large family called the "serine proteases" which is characterized by the reactivity of a serine group at the active site. The well-known enzymes chymotrypsin, subtilisin, trypsin, and elastase,

as well as plasmin and thrombin, belong to this family. A wealth of information is known about the serine proteases, including the detailed three-dimensional crystal structures for four species, complete amino acid sequences for many species, individual primary substrate specificities, and a generally accepted theory for the mechanism of catalytic action. The differences between these enzymes arise mainly from their distinct substrate specificities for particular kinds of amino acids. In general they are amenable to detailed analysis since they possess no allosteric activity, coenzymes, polymeric units, etc. Most of the characterization studies of the enzymes have been based on their hydrolytic activity toward synthetic substrates, e.g. small peptides and peptide esters and amides. The serine proteases are all believed to operate by an identical mechanism of action during peptide hydrolysis as in eq 1 (Bender and Kezdy, 1964). Initially a substrate molecule binds to

$$E + S \rightleftharpoons E\cdot S \rightarrow E\text{-}S' + P_1 \rightarrow E + P_1 + P_2 \qquad (1)$$

$$E + I \rightleftharpoons E\cdot I \rightarrow E\text{-}I' + P_3 \qquad (2)$$

the enzyme to form a reversible enzyme-substrate complex E·S, then the active-site serine group cleaves the proper peptide bond and forms an acyl-enzyme intermediate E-S′ (Figure 1A) (usually the rate-limiting step), and finally a water molecule is used to deacylate the intermediate and regenerate the

active enzyme. In the general case of an irreversible inhibitor, eq 2, an inhibitor molecule binds to the enzyme to form an enzyme-inhibitor complex E·I, but then one of the active-site groups attacks to make a covalent bond with the inhibitor and forms an inhibited enzyme derivative E-I′ (Figure 1B), and the enzyme becomes inactivated. X-Ray structural investigations, sequence analyses, and various kinetic studies have demonstrated a remarkable amount of homology among the serine proteases. For example, chymotrypsin, subtilisin, trypsin, and elastase all possess the same catalytic residues at the active site: serine, histidine, and aspartic acid, in a charge relay system with virtually identical spatial arrangements. This charge relay system confers unusually high nucleophilic activity upon the active-site serine residue. These findings of homology among the serine proteases indicate that they have catalytic properties and mechanisms of action which are nearly identical despite their different biological functions. A combination of gene duplication and divergent evolution is commonly invoked to rationalize this enzymatic similarity.

Elastase is a general name for the serine proteases which possess elastolytic activity, including the well-known pancreatic elastase and a newly-found leukocyte elastase. All of the characterization work on this enzyme has been carried out on porcine pancreatic elastase (EC 3.4.21.11). It is secreted by the pancreas in zymogen form, pro-elastase,

which is activated to elastase by a tryptic split. The enzyme consists of a single polypeptide chain with 240 amino acids and 4 disulfide bridges, has a molecular weight of 25,900, and operates at a pH optimum of pH 8.5 (Hartley and Shotton, 1971). The complete amino acid sequence has been established and the essential active-site residues are Ser-188 and His-45. X-Ray crystallographic investigations have determined the three-dimensional structure of tosyl-elastase and native elastase to a resolution of 3.5 Å (Shotton and Watson, 1970; Shotton et al, 1971). Because of the extensive homology with other serine proteases, the mechanism of elastase activity is usually considered to be identical with that for chymotrypsin (Hess, 1971; Hartley and Shotton, 1971). One physiological function of elastase is the proteolytic digestion of elastin, the elastic fibrous protein of connective tissue, found in the lungs, arteries, ligaments, etc. Corresponding to the composition of elastin, the substrate specificity of elastase is for a few residues with small alkyl uncharged side chains, e.g. alanine, serine, and valine (Hartley and Shotton, 1971). Like the other serine proteases, elastase reacts with a variety of synthetic substrates and inhibitors, e.g. Ac-Ala-Ala-Ala-OMe, but unlike them its reactivity is highly dependent on the peptide length of substrate or inhibitor. Also like its relatives, the solid-state structure of elastase correlates well with its solution kinetic properties (Shotton et al, 1971).

Another kind of elastolytic protease has recently come into the picture, a leukocyte elastase which is separate and distinct from pancreatic elastase. The differences deal with the variability of conditions under which the two kinds of enzyme exhibit elastolytic activity (Janoff and Scherer, 1968). The leukocyte elastase was isolated from human polymorphonuclear (PMN) leukocyte granule fractions and then purified by affinity chromatography (Janoff and Scherer, 1968; Janoff, 1973; Folds et al, 1972; Ohlsson and Olsson, 1973; Sweetman and Ornstein, 1974). This human leukocyte elastase is presently being characterized in terms of its chemical properties and physiological and pathological functions. It is optimally active at physiological pH (7-8) and exhibits esterase activity toward synthetic substrates e.g. BOC-Ala-ONp and Ac-Ala-Ala-Ala-OMe. Also it is known to degrade human lung elastin, arterial walls and basement membrane, digest bacterial cell walls and bacterial proteins, induce cellular surface changes correlated with cancerous loss of growth control, and undergo inhibition by α_1-antitrypsin and peptide chloromethyl ketones (Janoff et al, 1972; Janoff, 1970a, 1972a; Janoff and Blondin, 1973, 1974; Mosser et al, 1973; Janoff, 1972b; and Ohlsson, 1971). Indirect experimental evidence has intimated that human leukocyte elastase may be involved in pathological processes associated with elastic tissue destruction. Thus, this enzyme and related neutral proteases are implicated in the pathogenesis

of pulmonary emphysema, acute arteritis, and rheumatoid arthritis, as well as in the tumor-producing action of co-carcinogenic substances (Janoff, 1970a,b, 1972a,b; Janoff et al, 1972; Galdston et al, 1973; Mittman, 1972; Mosser et al, 1973). It should be recognized that a number of leukocyte proteases are connected with these inflammatory diseases, including at least three leukocyte elastase isoenzymes, as well as some collagenases, neutral proteases, thiol cathepsins, and other lysosomal proteases (Janoff et al, 1972).

As an illustration, human leukocyte elastase is believed to be a mediator of pulmonary emphysema thru the uninhibited proteolysis of elastic lung tissue (Mittman, 1972). Normally the lungs are protected from proteolytic digestion during phagocytosis by the protease inhibitor α_1-antitrypsin. Certain persons with a genetic deficiency of α_1-antitrypsin are predisposed toward chronic pulmonary lung diseases. This situation provides the incentive for the development of synthetic elastase inhibitors which one would expect to be useful reagents for the treatment of emphysema, arthritis, and related diseases. An appropriate elastase inhibitor, sufficiently reactive and specific, and capable of replacing α_1-antitrypsin, might prove to be valuable in the treatment of emphysema, arteritis, arthritis, etc. Furthermore this kind of reagent would certainly be helpful in the elucidation of other biological functions of human leukocyte elastase. Clearly these elastase inhibitors would be important

and relevant reagents for continuing biochemical and biomedical studies of elastolytic proteases.

A variety of non-specific synthetic inhibitors have been found to react with elastase stoichiometrically and irreversibly, including diisopropyl fluorophosphate (DFP), p-nitrophenyl diethyl phosphate, and sulfonyl fluorides such as tosyl fluoride (Tos-F). These inhibitors are known to react specifically with the active-site serine residue of all the serine proteases, including Ser-188 of elastase (Hartley and Shotton, 1971). Recently 1-bromo-4-(2,4-dinitrophenyl) butan-2-one was shown to inhibit pancreatic elastase by alkylation of the carboxyl group of a glutamic acid residue, possibly Glu-6 (Visser et al, 1971). However, this compound was incapable of inhibiting a human leukocyte elastase (Janoff, 1969). Similarly, n-butyl isocyanate was shown to inhibit elastase by alkylcarbamylation of active-site Ser-188, but the reagent was not specific for elastase since it inhibited chymotrypsin in addition (Brown and Wold, 1973a,b). Thus at the beginning of this work there were no suitable inhibitors which were both reactive and specific for elastase.

It appeared that certain peptide chloromethyl ketones might be likely reagents for use as potential elastase inhibitors. These compounds are relatives of Tos-PheCH_2Cl and Tos-LysCH_2Cl, the prototype active-site specific inhibitors of chymotrypsin and trypsin (Shaw, 1970). If they are properly designed to resemble a substrate they can be specific

inhibitors for serine proteases with similar specificities (Shaw, 1970). Simple amino-acid chloromethyl ketones such as Tos-AlaCH_2Cl and Tos-ValCH_2Cl were found to be incapable of inhibiting elastase (Kaplan et al, 1970), but peptide chloromethyl ketones held promise as better inhibitors. Ac-Ala-PheCH_2Cl was made for chymotrypsin (Powers and Wilcox, 1970), and Z-Ala-PheCH_2Cl was made for subtilisin (Morihara and Oka, 1970). The interactions of serine proteases with peptide chloromethyl ketones were investigated by the x-ray structural determinations of the binding modes of these inhibitors to chymotrypsin A_γ (Segal et al, 1971a,b) and to subtilisin BPN′ (Robertus et al, 1972; Kraut et al, 1971). These enzyme-inhibitor interactions are illustrated in Figures 1B, 2, & 4. The inhibitors were attached to the enzymes via a covalent bond between the methylene part of the chloromethyl ketone moiety and the imidazole ring of the active-site histidine residue. There was also an extended binding region where the peptide chain of an extended inhibitor and a 3-residue section of the enzyme's backbone formed an antiparallel β-sheet structure. The inhibition via alkylation is irreversible, and acylation of active-site serine in the inactivated derivative would not be productive (Figure 1B). Further studies on the interactions of serine proteases with peptide chloromethyl ketones were made by investigating the solution inhibition kinetics of chymotrypsin A_α (Kurachi et al, 1973) and of subtilisin BPN′ (J.C. Powers

and J.T. Tippett, unpublished observations). In general, a speedier rate of inhibition reaction was correlated with a greater interaction between enzyme and inhibitor, at both the primary specificity site and at distant binding subsites.

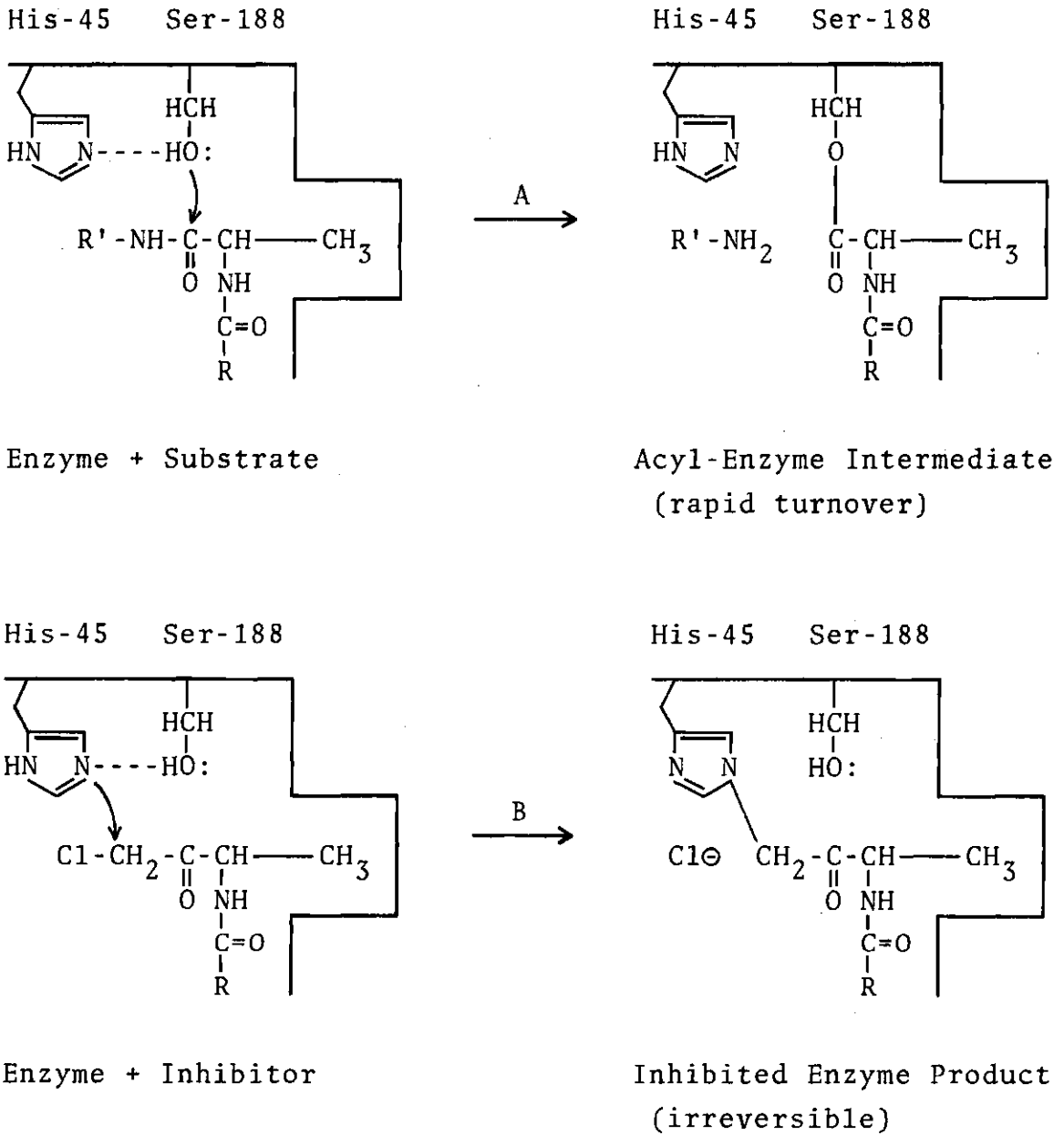
Our peptide chloromethyl ketones were designed to be both reactive and specific inhibitors for elastase by incorporating the structural features of an alanine residue at the "P_1" position plus an extended peptide chain. The exemplary structure of Ac-Ala-Ala-Pro-AlaCH_2Cl is shown in Figure 3. First, the compounds have an alanine residue at the P_1 position (notation of Schechter and Berger, 1967) because elastase has demonstrated a specificity for alanine residues. It is established that the enzyme attacks substrates and most readily cleaves peptide bonds with alanine on the carboxyl side, from studies with protein substrates (Narayanan and Anwar, 1969; Atlas et al, 1970) and peptide substrates (Geneste and Bender, 1969; Kaplan et al, 1970; Atlas and Berger, 1972). Second, the inhibitors have an extended peptide chain to enhance their reactivity thru greater contact with the enzyme surface near the catalytic region. This increases the resemblance of the inhibitors to natural elastin. It was demonstrated that the rate of elastase hydrolysis of substrates is highly dependent on the peptide chain length, from studies with synthetic peptide substrates (Atlas et al, 1970; Atlas and Berger, 1972; Thompson and Blout, 1970, 1973c). It is likely that secondary binding

contacts are essential for elastase proteolytic activity.

The peptide chloromethyl ketones were anticipated to inhibit elastase rapidly and irreversibly, and also selectively in the presence of related serine proteases. By analogy they were expected to bind tightly at the extended binding site of elastase and inactivate it by alkylation of the active-site His-45. If an initial reagent, e.g. Ac-Ala-Ala-AlaCH_2Cl, was successfully found to be an effective elastase inhibitor, then a series of well-varied compounds could be used in an investigation of structure-reactivity relationships. This kind of study is applicable to the development of optimum elastase inhibitors for testing the biological activity of elastolytic enzymes.

This Thesis, Part I, is a report on the synthesis and reactivity of a series of peptide chloromethyl ketones which are both reactive and specific elastase inhibitors. They have been used to explore the nature of the catalytic activity and extended binding site of pancreatic elastase and human leukocyte elastase. The work has already been published as a preliminary communication and a complete article (Powers and Tuhy, 1972, 1973), and additional work on human leukocyte elastase has also appeared in short articles (Tuhy and Powers, 1975; Powers et al, 1975). Two other research groups have subsequently and independently reported similar studies on elastase-inhibitor interactions (Thompson and Blout, 1973a; Thomson and Denniss, 1973). There have also

been several reports on the biological activity of human leukocyte elastase with the use of our peptide chloromethyl ketones, mainly by Janoff and coworkers. The results will help to define the structural features necessary for an effective elastase inhibitor and may lead to a treatment for emphysema and related diseases.



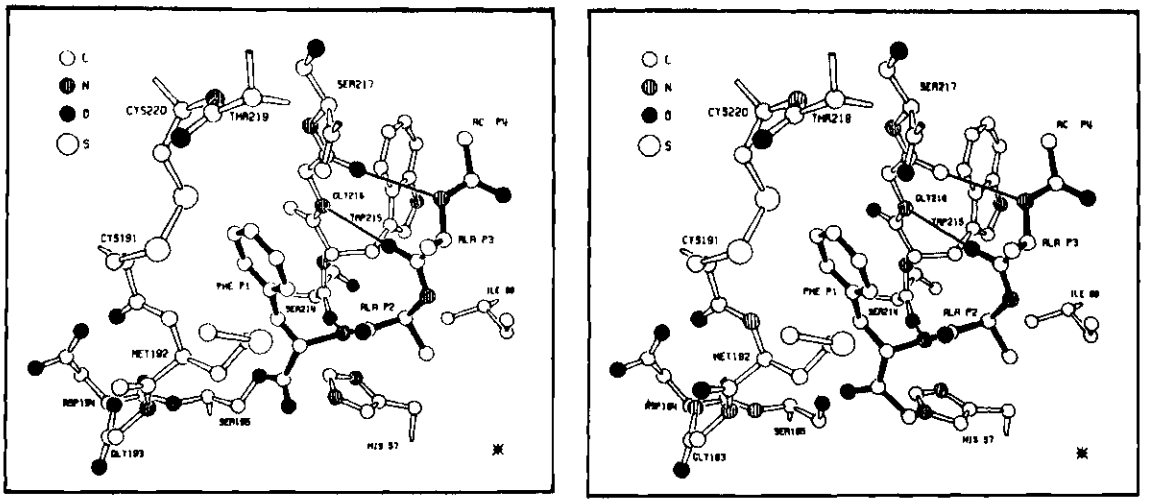
Enzyme + Substrate

Acyl-Enzyme Intermediate
(rapid turnover)

Enzyme + Inhibitor

Inhibited Enzyme Product
(irreversible)

Figure 1. Diagram of Pancreatic Elastase Showing the Active Site and Binding Site Regions. Note: A comparison of the reaction pathways for the enzymatic reaction with (A) a normal peptide substrate, and (B) a peptide chloromethyl ketone inhibitor (proposed).



A B

Figure 2. Perspective Drawings of Serine Protease Models Showing the Active Site Region and the Extended Binding Site. Note: (A) Chymotrypsin A with its Ser-195 acylated by the substrate Ac-Ala-Ala-Phe-OMe; (B) Chymotrypsin A with His-57 alkylated by the inhibitor Ac-Ala-Ala-PheCH_2Cl. Two hydrogen bonds are formed between the bound peptide and the extended binding site of the enzyme. These drawings were deduced from x-ray crystallographic studies. From Segal, D.M., Powers, J.C., Cohen, G.H., Davies, D.R., and Wilcox, P.E. (1971), Biochemistry 10, 3728.

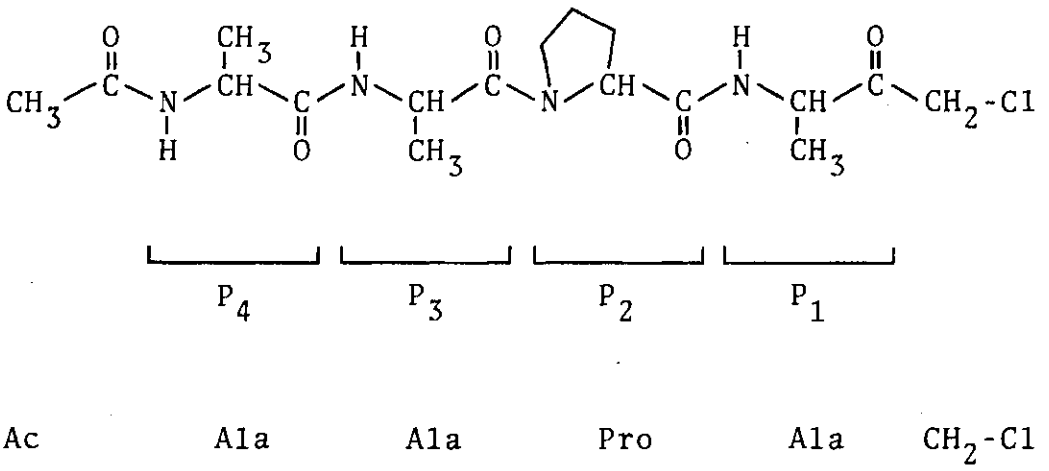


Figure 3. Structure of the Typical Peptide Chloromethyl Ketone Ac-Ala-Ala-Pro-AlaCH_2Cl. Note: The residues P_1-P_4 are designated according to the notation of Schechter and Berger (1967). This is an effective elastase inhibitor.

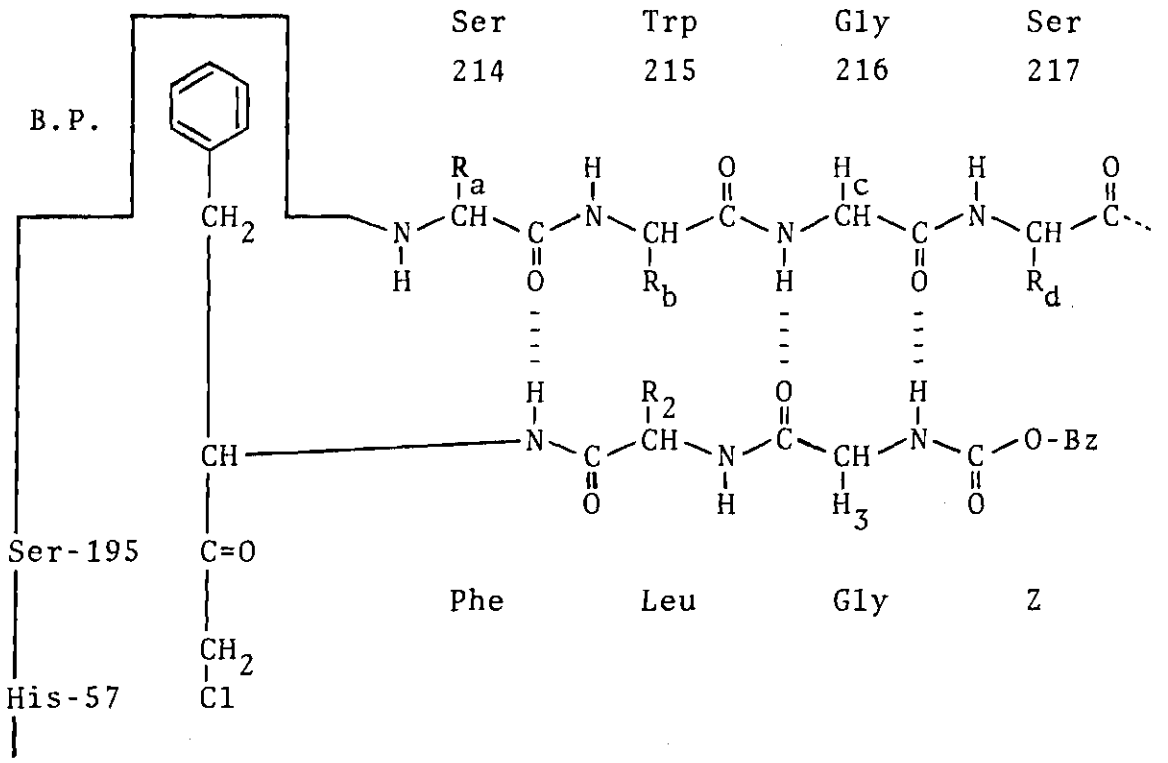


Figure 4. Diagram of Chymotrypsin A Showing the Extended Binding Site Region. Note: Chymotrypsin (above) and the inhibitor Z-Gly-Leu-PheCH_2Cl (below) form an anti-parallel β-sheet structure involving at least three hydrogen bonds. This interaction is also a binding feature of elastase.

CHAPTER II

METHODS

Reagents and Materials. Porcine pancreatic elastase (batch 24202) was obtained from Whatman Biochemicals Ltd. and used without further purification; its substrate, BOC-Ala-ONp, was purchased from Sigma Chemical Co. Human leukocyte elastase (1.6 mg) was kindly provided upon isolation by Dr. Aaron Janoff at S.U.N.Y. at Stony Brook; it was 85% pure as determined by a gel densitometry scan and contained one major and two minor elastase isoenzymes. Chymotrypsin A_α (lot CDIOBK) was obtained from Worthington Biochemical Co., and its substrate, Ac-Tyr-OEt, was synthesized by standard methods and had mp 80-81°. Trypsin (lot 102C-1920) and its substrate, Bz-Arg-OEt, were obtained from Sigma Chemical Co. The tripeptide acids Ac-Ala-Ala-Ala-OH, Ac-Ala-Ala-Pro-OH, Ac-Ala-Ala-Phe-OH, and Ac-Ala-Pro-Ala-OH were synthesized by Dr. Akhtar Ali. The peptide chloromethyl ketones Z-Gly-Leu-AlaCH_2Cl and Ac-Ala-Gly-AlaCH_2Cl were synthesized by Mr. Clint Joiner; Z-Gly-Leu-PheCH_2Cl and Ac-Phe-Gly-Ala-LeuCH_2Cl were synthesized by Mr. Mark Lively. Thin-layer chromatography was performed using Merck silica gel G plates. All other reagents and solvents used were analytical grade. Mass spectra were taken on a Varian M-66 instrument and nuclear

magnetic resonance (nmr) spectra were taken on a Varian T-60 instrument. Enzyme inhibition kinetics were performed on a Beckman DB-GT uv spectrophotometer and a Radiometer automated pH-stat (model TTT 11).

N-Benzyloxycarbonyl-L-alanine Chloromethyl Ketone, Z-AlaCH_2Cl. The diazo-ketone intermediate Z-AlaCHN_2 was prepared from Z-Ala-OH (13.4 g, 60 mmol) and diazomethane (100-120 mmol) according to a mixed anhydride method (Penke et al, 1970). Anhydrous HCl was bubbled thru a solution of Z-AlaCHN_2 (ca. 14.8 g, 60 mmol) in 300 ml of ether-tetrahydrofuran at 5° until it turned colorless. Work-up of the reaction mixture involved evaporation of the ether solvent at 25°, extraction of the residue into ethyl acetate, washing with citric acid and $NaHCO_3$ solutions, and drying over anhydrous $MgSO_4$. Evaporation led to an oil which crystallized upon standing. The product was recrystallized from ethyl acetate-cyclohexane (1:3) to give 9.60 g (63%) of a white solid, mp 88-89°, $[\alpha]_D^{25} = -42°$ (c 1.8, CH_3OH), R_F 0.84 ($CHCl_3$-CH_3OH, 9:1); Thompson and Blout (1973a) report mp 87-88°, $[\alpha]_D^{25} = -43.6°$ (c 2.8, CH_3OH). The mass spectrum had major peaks at m/e 178 ($C_6H_5CH_2OCONH\text{-}CH(CH_3)^+$), 134 ($C_6H_5CH_2OCONHCH(CH_3)^+\text{-}CO_2$), 107 ($C_6H_5CH_2O^+$), and 91 ($C_6H_5CH_2^+$); no M^+ or M-HCl peaks were observed. The nmr spectrum ($CDCl_3$) had peaks at δ 7.3 (5H, s, C_6H_5), 5.3 (1H, b, NH), 5.1 (2H, s, $C_6H_5\underline{CH_2}$), 4.7 (1H, q, CH), 4.2 (2H, s, CH_2Cl), and 1.4

(3H, d, CH_3). Anal. Calcd for $C_{12}H_{14}ClNO_3$: C, 56.37; H, 5.52; N, 5.48. Found: C, 56.27; H, 5.34; N, 5.60.

L-Alanine Chloromethyl Ketone Hydrobromide, HBr·AlaCH_2Cl. Z-AlaCH_2Cl (5.11 g, 20 mmol) was deblocked by dissolving in 6 ml of a 32% solution of hydrogen bromide in acetic acid, and within 3 min the product crystallized out and was washed with 100 ml of ether. The product was recrystallized from acetone-ethyl acetate (3:2) to give 2.53 g (62%) of a hygroscopic white solid. It was immediately dissolved in 10 ml of dry dimethylformamide (DMF) and used in subsequent coupling reactions.

N-Acetyl-L-alanyl-L-alanyl-L-alanine Chloromethyl Ketone, Ac-Ala-Ala-AlaCH_2Cl, was prepared according to a regular mixed anhydride coupling method. The dipeptide acid Ac-Ala-Ala-OH was synthesized from Ac-Ala-OH and HCl·Ala-OMe by standard peptide coupling methods. Thus, a solution of Ac-Ala-Ala-OH (1.21 g, 6 mmol) in 50 ml of dimethylformamide was stirred at -20°, while N-methylmorpholine (0.66 ml, 6 mmol) and isobutyl chloroformate (0.78 ml, 6 mmol) were added. After 5 min, the HBr·AlaCH_2Cl (1.31 g, 6.5 mmol, in 10 ml DMF) and N-methylmorpholine (0.66 ml, 6 mmol) were added, and the mixture was allowed to warm to 25° while stirring for 4 hr. The solvent was removed in vacuo and the product was isolated from the residue after extraction of all other components into 60 ml of hot acetone. The product was recrystallized from 95% ethanol to give 0.45 g (25%) of a white

solid, mp 194-195°, R_F 0.70 ($CHCl_3$-CH_3OH, 4:1). The mass spectrum had major peaks at m/e 305 (M^+), 269 (M-HCl), 256 (Ac-Ala-Ala-Ala$^+$), 228 (Ac-Ala-Ala-NHCH(CH_3)$^+$), 185 (Ac-Ala-Ala$^+$), 157 (Ac-Ala-NHCH(CH_3)$^+$), 114 (Ac-Ala$^+$ or -Ala-NHCH-(CH_3)$^+$), and 86 (Ac-NHCH(CH_3)$^+$). Anal. Calcd for $C_{12}H_{20}ClN_3O_4$: C, 47.14; H, 6.59; N, 13.74. Found: C, 46.72; H, 6.53; N, 13.53.

N-Acetyl-L-alanyl-L-prolyl-L-alanine Chloromethyl Ketone, Ac-Ala-Pro-AlaCH_2Cl, was prepared similarly to Ac-Ala-Ala-AlaCH_2Cl from Ac-Ala-Pro-OH (1.15 g, 5 mmol) (made by standard methods) and HBr·AlaCH_2Cl (5 mmol). In this case, however, the product was isolated from the residue by chromatography on a 30 x 2 cm silica gel column, eluting with $CHCl_3$-CH_3OH (19:1). The product was recrystallized from ethanol to give 0.72 g (43%) of a white solid, mp 172-173°, R_F 0.73 ($CHCl_3$-CH_3OH, 4:1); Thompson and Blout (1973a) report mp 176-180°. The mass spectrum had major peaks at m/e 331 (M^+), 295 (M-HCl), 254 (Ac-Ala-Pro-NHCH(CH_3)$^+$), 211 (Ac-Ala-Pro$^+$), 183 (Ac-Ala-$C_4H_7N^+$), 140 (-Ala-$C_4H_7N^+$), 114 (Ac-Ala$^+$), and 86 (Ac-NHCH(CH_3)$^+$). The nmr spectrum (DMSO-d6) had peaks at

$$C_4H_7N^+ = \overline{\text{N-(CH}_2\text{)}_3\text{-CH}}^+$$

∂ 8.0-8.4 (2H, m, NH), 4.2-4.6 (3H, m, CH) including 4.5 (2H, s, CH_2Cl), 3.4-3.7 (2H, b, NCH_2), 1.7-2.1 (4H, b, CH-CH_2CH_2) including 1.8 (3H, s, CH_3CO), and 1.2 (6H, d, $CHCH_3$).

The ir spectrum (nujol mull) showed a carbonyl band at 1740 cm^{-1}. Anal. Calcd for $C_{14}H_{22}ClN_3O_4$: C, 50.68; H, 6.68; N, 12.66. Found: C, 50.80; H, 6.86; N, 12.51.

N-Acetyl-L-prolyl-L-alanyl-L-alanine Chloromethyl Ketone, Ac-Pro-Ala-AlaCH_2Cl, was prepared similarly to Ac-Ala-Pro-AlaCH_2Cl from Ac-Pro-Ala-OH (1.15 g, 5 mmol) (made by standard methods) and HBr·AlaCH_2Cl (5 mmol). The product was isolated by chromatography and recrystallized from ethyl acetate to give 0.92 g (55%) of a white solid, mp 142-143°, R_F 0.73 ($CHCl_3$-CH_3OH, 4:1). The mass spectrum had major peaks at m/e 311 (M^+), 295 (M-HCl), 254 (Ac-Pro-Ala-$NHCH(CH_3)^+$), 211 (Ac-Pro-Ala^+), 183 (Ac-Pro-$NHCH(CH_3)^+$), 140 (Ac-Pro^+ or -Pro-$NHCH(CH_3)^+$), and 112 (Ac-$C_4H_7N^+$). Anal. Calcd for $C_{14}H_{22}ClN_3O_4$: C, 50.68; H, 6.68; N, 12.66. Found: C, 50.78; H, 6.73; N, 12.63.

N-Acetyl-L-alanyl-L-alanyl-L-alanyl-L-alanine Chloromethyl Ketone, Ac-Ala-Ala-Ala-AlaCH_2Cl, was prepared similarly to Ac-Ala-Ala-AlaCH_2Cl from Ac-Ala-Ala-Ala-OH (1.64 g, 6 mmol) and HBr·AlaCH_2Cl (ca. 7 mmol). The product was isolated by extraction and recrystallized from methanol to give 1.28 g (57%) of a white solid, mp 252-253°, R_F 0.60 ($CHCl_3$-CH_3OH, 4:1). The mass spectrum had major peaks at m/e 340 (M-HCl), 299 (Ac-Ala-Ala-Ala-$NHCH(CH_3)^+$), 256 (Ac-Ala-Ala-Ala^+), 228 (Ac-Ala-Ala-$NHCH(CH_3)^+$), 185 (Ac-Ala-Ala^+), 157 (Ac-Ala-$NHCH(CH_3)^+$), 141 ((-Ala-Ala^+)-H), and 114 (Ac-Ala^+); no M^+ peak was observed. The ir spectrum (nujol mull) showed

carbonyl bands at 1690 and 1740 cm^{-1}. Anal. Calcd for $C_{15}H_{25}ClN_4O_5$: C, 47.81; H, 6.69; N, 14.87. Found: C, 48.20; H, 6.83; N, 14.54.

N-Acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanine Chloromethyl Ketone, Ac-Ala-Ala-Pro-AlaCH_2Cl, was prepared similarly to Ac-Ala-Ala-AlaCH_2Cl from Ac-Ala-Ala-Pro-OH (1.79 g, 6 mmol) and HBr·AlaCH_2Cl (ca. 6.5 mmol). The product was isolated by extraction and recrystallized from ethanol-acetone to give 0.48 g (20%) of a white solid, mp 187-188°, R_F 0.69 ($CHCl_3$-CH_3OH, 4:1). The mass spectrum had major peaks at m/e 366 (M-HCl), 325 (Ac-Ala-Ala-Pro-$NHCH(CH_3)^+$), 282 (Ac-Ala-Ala-Pro^+), 254 (Ac-Ala-Ala-$C_4H_7N^+$), 185 (Ac-Ala-Ala^+), 167 ((-Ala-Pro^+)-H), 157 (Ac-Ala-$NHCH(CH_3)^+$), and 114 (Ac-Ala^+); no M^+ peak was observed. The nmr spectrum (DMSO-d6) had peaks at δ 8.0-8.4 (3H, m, NH), 4.2-4.6 (4H, m, CH) including 4.5 (2H, s, CH_2Cl), 3.4-3.6 (2H, b, NCH_2), 1.8-2.1 (4H, b, $CHCH_2CH_2$) including 1.8 (3H, s, CH_3CO), and 1.2 (9H, d, $CHCH_3$). The uv absorption in H_2O showed a λ_{max} at 220 nm. Anal. Calcd for $C_{17}H_{27}ClN_4O_5$: C, 50.68; H, 6.76; N, 13.91. Found: C, 50.48; H, 6.79; N, 13.52.

N-Acetyl-L-alanyl-L-alanyl-L-phenylalanyl-L-alanine Chloromethyl Ketone, Ac-Ala-Ala-Phe-AlaCH_2Cl, was prepared similarly to Ac-Ala-Ala-AlaCH_2Cl from Ac-Ala-Ala-Phe-OH (1.05 g, 3 mmol) and HBr·AlaCH_2Cl (ca. 3.3 mmol). The product was isolated by extraction and recrystallized from methanol to give 0.56 g (41%) of a white solid, mp 236-237°, R_F

0.76 ($CHCl_3$-CH_3OH, 4:1). The mass spectrum had major peaks at m/e 416 (M-HCl), 375(Ac-Ala-Ala-Phe-$NHCH(CH_3)^+$), 332 (Ac-Ala-Ala-Phe^+), 304 (Ac-Ala-Ala-$NHCH(CH_2Ph)^+$), 217 ((-Ala-Phe^+)-H), 185 (Ac-Ala-Ala^+), 157 (Ac-Ala-$NHCH(CH_3)^+$), and 114 (Ac-Ala^+); no M^+ peak was observed. Anal. Calcd for $C_{21}H_{27}ClN_4O_5$: C, 55.69; H, 6.45; N, 12.37. Found: C, 55.33; H, 6.33; N, 12.20.

N-Acetyl-L-alanyl-L-prolyl-L-alanyl-L-alanine Chloromethyl Ketone, Ac-Ala-Pro-Ala-AlaCH_2Cl, was prepared similarly to Ac-Ala-Pro-AlaCH_2Cl from Ac-Ala-Pro-Ala-OH (2 x 1.35 g, 4.5 mmol) and HBr·AlaCH_2Cl (2 x 5 mmol). The product was isolated by chromatography and recrystallized from ethanol-ethyl acetate to give 0.27 g (15%) of a white solid, mp 173-174°, R_F 0.69 ($CHCl_3$-CH_3OH, 4:1). The mass spectrum had major peaks at m/e 366 (M-HCl), 325 (Ac-Ala-Pro-Ala-$NHCH(CH_3)^+$) 282 (Ac-Ala-Pro-Ala^+), 254 (Ac-Ala-Pro-$NHCH(CH_3)^+$), 211 (Ac-Ala-Pro^+), 183 (Ac-Ala-$C_4H_7N^+$), 167 ((-Pro-Ala^+)-H), and 114 (Ac-Ala^+); no M^+ peak was observed. Anal. Calcd for $C_{17}H_{27}ClN_4O_5$: C, 50.68; H, 6.76; N, 13.91. Found: C, 50.43; H, 6.87; N, 13.42.

Enzyme Activity Assays. Elastase activity was measured by the spectrophotometric assay method using BOC-Ala-ONp as the substrate (Visser and Blout, 1972). This is described more completely in the Appendix. Chymotrypsin was assayed using Ac-Tyr-OEt as the substrate (Wilcox, 1970) and trypsin was assayed using Bz-Arg-OEt as the substrate (Walsh

and Wilcox, 1970), both on the pH-stat.

Reaction of Pancreatic Elastase with Inhibitors. Inhibition of pancreatic elastase with the series of peptide chloromethyl ketones was carried out under pseudo-first-order conditions in solutions which contained at least a tenfold excess of inhibitor over enzyme. The inhibition kinetics were monitored on the uv spectrophotometer. Two sets of inhibition conditions were employed: pH 6.5 (0.1 M phosphate buffer) with 100-fold inhibitor excess for "slow" inhibitors, and pH 5.0 (0.1 M acetate buffer) with 10-fold inhibitor excess for "fast" inhibitors. Stock solutions were prepared as follows. An elastase solution was made up in 1 mM HCl, stored at 4^{o} for use over 60 days, and had a concentration of 9.3 μM or 0.23 mg/ml (by uv absorbance). In the fixed-concentration runs an inhibitor solution was directly prepared, but in the concentration-dependent runs an inhibitor stock solution was prepared in methanol, ca. 1-10 mM, and used up within four days. The tetrapeptide compounds were relatively insoluble in water and soluble in methanol only to ca. 1-2 mM. 10 ml of an inhibitor solution was made up in pH 6.5 or 5.0 buffer containing 10% (v/v) methanol, and had a concentration of 1.0 mM or 0.1 mM (fixed-concentration runs) or a graded series (a set of [I], concentration-dependent runs). All inhibitor solutions were prepared 60 min before the start of the inhibition run at t=0.

The inhibition reaction was started by mixing 1 ml of

the inhibitor solution with 1 ml of the enzyme solution, and proceeded at 30° thru at least two half-lives. Initial concentrations were as follows: elastase, 4.7 μM; inhibitor, 0.5 mM or 0.05 mM (fixed-concentration runs) or a graded series (concentration-dependent runs); methanol, 5% (v/v); in a total volume of 2 ml. In the concentration-dependent runs the variable inhibitor concentration range was such that the highest value, usually less than 1 mM, was five times larger than the lowest value. The reaction was monitored by removing several aliquots of 100 μl (each containing 12.5 μg of enzyme) from the inhibition mixture at periodic time intervals. At each point the residual elastase activity was measured by enzymatic assay, so forming a set of regularly decreasing rates. Control experiments identical with inhibition runs but lacking the inhibitor showed that the active enzyme concentration did not decrease more than 3% in 90 min. For each inhibition reaction the values of the kinetic parameters k_{obsd} and $k_{2nd} = k_{obsd}/[I]$ were calculated from eq 3 with the aid of a standard line-fitting computer program.

$$V = k_{obsd}\,[E] = k_{2nd}\,[I]\,[E] \qquad (3)$$

For inhibitors that underwent concentration-dependent inhibition reactions the values of the kinetic parameters k_3, K_I, and k_3/K_I were calculated from eq 4 with the aid of a second linear computer program. Correlation coefficients of better than 0.993, usually 0.998, were maintained for the

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_3}\frac{1}{[I]} + \frac{1}{k_3} \qquad (4)$$

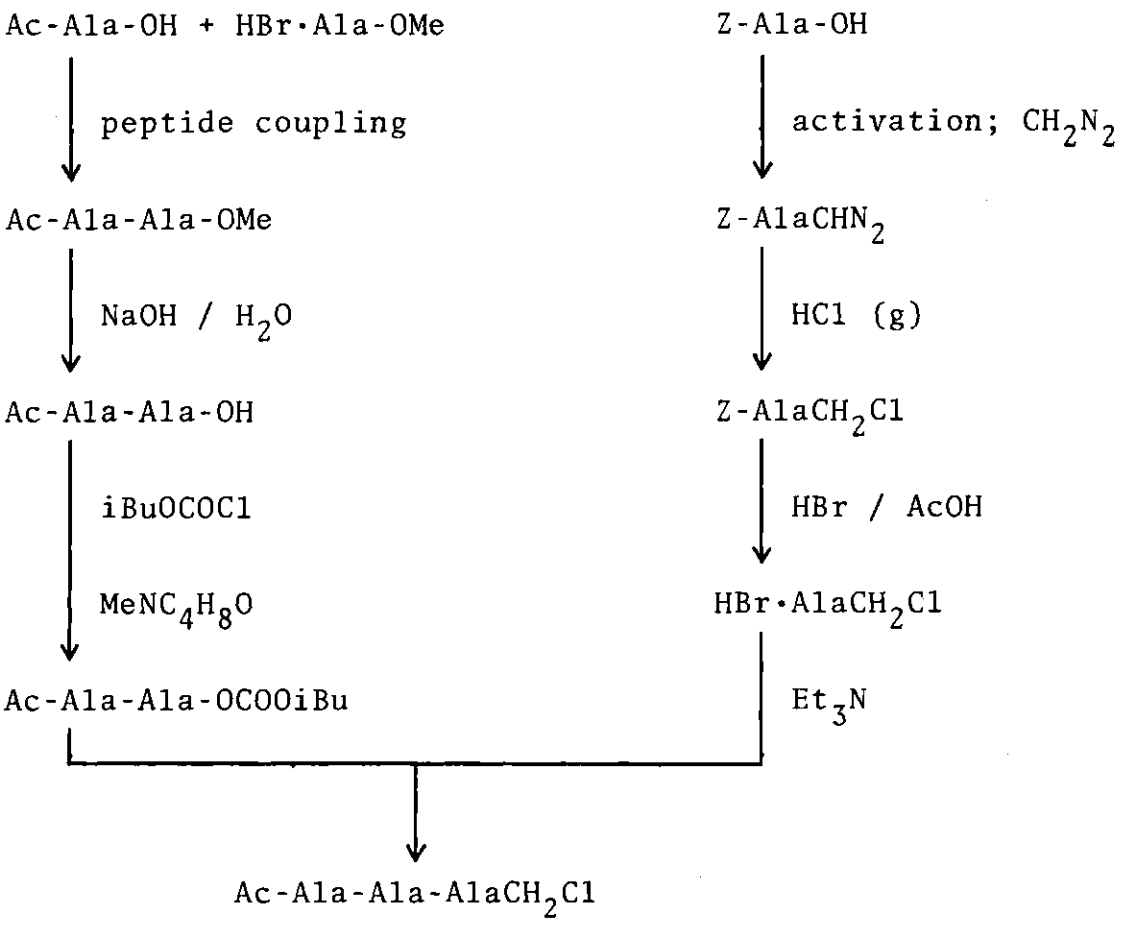
linear plots throughout.

The experimental data were processed by computer programs as follows. For each inhibition reaction at its set value of [I], several data points [time, velocity] were plotted as ln (velocity) *vs* time *via* a linear (non-weighted) least-squares program, and k_{obsd} was given as the negative of the slope of this line, while the half-life was gotten from pseudo-first-order kinetics, half-life = $(\ln 2)/k_{obsd}$. A further analysis was performed for concentration-dependent runs. For a series of inhibition reactions at varying values of [I], eight data points [[I], k_{obsd}] were plotted as $1/k_{obsd}$ *vs* $1/[I]$ *via* another linear least-squares program, and k_3/K_I was given as the inverse of the slope of this line while k_3 was given as the inverse of its y-intercept if non-zero.

Reaction of Chymotrypsin and Trypsin with Inhibitors. Inhibition experiments with chymotrypsin and trypsin were carried out under conditions virtually identical with those employed in the inhibition runs with pancreatic elastase. The inhibition kinetics were monitored on the pH-stat. Stock solutions of enzymes and inhibitors were made up to the correct concentrations as required and used quickly. The inhibition reaction was started by mixing 0.5 ml of the inhib-

itor solution, 0.5 ml of the enzyme solution, and 9 ml of buffer, and proceeded at 30° for 4-6 hr. Initial concentrations were as follows: enzyme, 5 μM; inhibitor, 0.5 mM or 0.1 mM; methanol 5% (v/v); in a total volume of 10 ml. Periodic enzyme assays and kinetic analyses were performed as before.

Reaction of Leukocyte Elastase with Inhibitors. Inhibition experiments with leukocyte elastase were carried out under conditions identical with those employed in the inhibition runs with pancreatic elastase, except on a smaller scale. The inhibition kinetics were monitored on the uv spectrophotometer. Stock solutions of enzyme and inhibitors were made up to the correct concentrations as required and used immediately. The inhibition reaction was started by mixing 0.16 ml of the inhibitor solution with 0.15 ml of the enzyme solution, and proceeded at 25° and pH 6.5 thru at least two half-lives. Initial concentrations were as follows: elastase, ca. 10 μM; inhibitor, 1 mM or 0.2 mM; $CaCl_2$, 0.1 mM; methanol, 5% (v/v); in a total volume of 0.31 ml. Periodic enzyme micro-assays and kinetic analyses were performed as before.



Peptide Chloromethyl Ketone

Scheme 1. A Synthetic Preparation of Ac-Ala-Ala-AlaCH$_2$Cl.

CHAPTER III

RESULTS

Synthesis of the Inhibitors. A series of nine alanine peptide chloromethyl ketone inhibitors was readily prepared using Z-AlaCH_2Cl as the key intermediate for the final coupling step. This compound by itself is not an elastase inhibitor. The synthesis of Z-AlaCH_2Cl utilized a new mixed anhydride procedure (Penke et al, 1970) which proceeded easily and in better yield (63%) than the usual acid chloride method. This compound was deblocked and coupled with a selected set of blocked peptide acids to produce the peptide chloromethyl ketone inhibitors listed in Tables 1 and 2. All new compounds were identified and characterized by mass spectra, tlc, combustion analysis, and sometimes also by nmr, ir, and uv data. They were all further assumed to retain their optical purity. Mass spectra were especially valuable for confirming assigned structures since the inhibitors displayed highly characteristic peptide fragmentation patterns. For example, the two isomeric compounds Ac-Ala-Ala-Pro-AlaCH_2Cl and Ac-Ala-Pro-Ala-AlaCH_2Cl may be distinguished on the basis of their mass spectral data.

All of the peptide chloromethyl ketones were relatively easy to prepare and handle with the exception of minor

difficulties in some recrystallizations. Due to several peptide (amide) bonds and a lack of large alkyl or phenyl groups they are highly polar and non-hydrophobic in nature. The compounds all exist as white crystalline solid materials, moderately soluble in methanol, but only slightly soluble in water and insoluble in benzene. In the solid state they are stable and maintain their activity for at least two years, and may be stored for indefinite periods. However in aqueous solution the chloromethyl ketones undergo slow hydrolysis, probably to hydroxymethyl ketones, with a half-life of ca. 30-40 hr. It appears that the compounds are non-toxic and unharmful in normal handling, but caution in their use is advised.

Elastase Inhibition Studies. The series of peptide chloromethyl ketones acted for the most part as very fine irreversible inhibitors of pancreatic elastase and leukocyte elastase. Good pseudo-first-order kinetics, i.e. in which $[I]/[E] \geq 10$, were observed for practically all inhibition reactions. The specific activity of elastase, expressed as Δ absorbance/min/μg in the initial assay, was 0.010 for the pancreatic enzyme and 0.006 for the leukocyte enzyme. The inhibitor solutions were freshly prepared since the peptide chloromethyl ketones underwent a slow hydrolysis upon standing in buffered aqueous solution as measured by a decreased k_{obsd} of inhibition. Also inhibitor solutions contained 5% (v/v) methanol to help dissolve some sparingly soluble com-

pounds such as Ac-Ala-Ala-Ala-AlaCH$_2$Cl. The reaction conditions were selected in order to obtain feasible reaction rates; both the inhibitor concentration and pH were decreased to levels as low as practicable so that the rates could be measured easily and accurately. Values of k_{obsd} were measured and corrected for background hydrolysis. In all experiments the reaction was allowed to proceed thru ca. two half-lives, and the half-lives were adjusted to range from 5 to 60 min.

Table 1 shows the results for the fixed-concentration experiments in which pancreatic elastase was inhibited by five tripeptide and four tetrapeptide chloromethyl ketones. The tripeptide compounds were noted to be "slow" inhibitors and were run at pH 6.5 using a 100-fold excess of inhibitor, while the tetrapeptide compounds were "fast" inhibitors and were run at pH 5.0 using a 10-fold excess of inhibitor. Although the pH optimum for elastase (esterase) activity is pH 8.5, fairly rapid inhibition occurred at pH 6.5, and even lower at pH 5.0 with the "fast" inhibitors. One inhibitor, Ac-Ala-Ala-AlaCH$_2$Cl, was run at both pH 6.5 and 5.0 at the same inhibitor concentration (0.5 mM), to establish a general correlation between the two solution conditions. This compound had a $k_{obsd}/[I]$ value which was 4.9 times faster at pH 6.5. The second-order rate constant $k_{obsd}/[I]$ is suitable only for purposes of approximate reactivity comparisons among the series of inhibitors (see below). The best pan-

creatic elastase inhibitor is Ac-Ala-Ala-Pro-AlaCH_2Cl; remarkably, its isomer Ac-Ala-Pro-Ala-AlaCH_2Cl is a non-inhibitor.

Table 2 shows the results for the fixed-concentration experiments in which leukocyte elastase was inhibited by five tripeptide and five tetrapeptide chloromethyl ketones. Here the reaction conditions were virtually identical with the preceding runs in concentration but much smaller in amount in order to minimize the enzyme consumed. The tripeptide compounds were generally noted to be "slow" inhibitors and were run at a 100-fold excess of inhibitor, while the tetrapeptide compounds were "fast" inhibitors and were run at a 20-fold excess of inhibitor, all at pH 6.5. Two compounds were tested, Z-Gly-Leu-PheCH_2Cl and Ac-Phe-Gly-Ala-LeuCH_2Cl, which did not match the substrate specificity of elastase. Due to the little amount of enzyme available (1.6 mg), it was impossible to perform concentration-dependent inhibition studies to determine k_3/K_I and K_I values. Slight problems of reproducibility arose from a lack of enough enzyme for duplicating all inhibition runs. The best leukocyte elastase inhibitor is Ac-Ala-Ala-Pro-AlaCH_2Cl, while its isomer Ac-Ala-Pro-Ala-AlaCH_2Cl is also an inhibitor. Table 7 is a tabulation of the pertinent elastase inhibition kinetic results.

Kinetics of Inhibition. The kinetics of inhibition of certain enzymes by irreversible inhibitors reveal the

existence of a reversible complex between enzyme and inhibitor preceding covalent bond formation. The reaction pathway for this inhibition reaction is expressed by eq 5 and 6. An initial binding step, an equilibrium process, is followed by the inactivation step, a dynamic rate process. Here E·I

$$E + I \overset{K_I}{\rightleftharpoons} E\cdot I \xrightarrow{k_3} E\text{-}I \qquad (5)$$

$$K_I = [E][I]/[E\cdot I] \qquad (6)$$

represents the noncovalently-bound enzyme-inhibitor complex, E-I is the covalently and irreversibly inhibited enzyme product, K_I is the dissociation constant of the E·I complex, and k_3 is the limiting rate of inactivation. Pseudo-first-order kinetics are achieved for the inhibition reaction if the initial inhibitor concentration is sufficiently greater than the total enzyme concentration. In this case k_{obsd}, the observed first-order rate constant, is given by eq 7 and 8

$$k_{obsd} = \frac{k_3[I]}{K_I + [I]} \qquad (7)$$

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_3}\frac{1}{[I]} + \frac{1}{k_3} \qquad (8)$$

(Kitz and Wilson, 1962; Kurachi et al, 1973).

The most appropriate parameter to compare the relative reactivity of the various inhibitors is the "inhibition constant" k_3/K_I analogous to the catalytic constant k_{cat}/K_M.

It is comparable to a second-order rate constant (k_{2nd}). If the inhibitor concentrations used are equal or close to K_I ($[I] \cong K_I$), then $k_{obsd}/[I]$ will vary over a range of inhibitor concentrations and the kinetic constants k_3/K_I, k_3, and K_I may be determined from a double-reciprocal plot of $1/k_{obsd}$ vs $1/[I]$. But if the inhibitor concentrations used are much smaller than K_I ($[I] \ll K_I$), then eq 7 reduces to eq 9, $k_{obsd}/[I]$ will remain constant over a range of inhibitor

$$\frac{k_{obsd}}{[I]} = \frac{k_3}{K_I} \qquad (9)$$

concentrations, and the kinetic constant k_3/K_I but not k_3 or K_I may be determined.

In the absence of enough information to determine the inhibition constant k_3/K_I, it is possible to compare the reactivity of a series of inhibitors on the basis of their relative $k_{obsd}/[I]$ values. This is shown in Tables 1 and 2 for the fixed-concentration experiments. The differences in the magnitude of $k_{obsd}/[I]$ for related inhibitors reflect chiefly the effect of structural changes on the binding of inhibitor to enzyme (K_I) and on the rate of reaction within the E·I complex (k_3). However, $k_{obsd}/[I]$ values are subject to distortion from non-linear concentration effects when the inhibitor concentration is close to the inhibitor K_I value ($[I] \cong K_I$), as is the case for many of the inhibitors. Nonetheless the $k_{obsd}/[I]$ values listed are useful for providing

a rough guide to the relative reactivities of structurally related inhibitors.

Concentration-Dependent Studies. Table 3 shows the results for the concentration-dependent experiments in which pancreatic elastase was inhibited by four peptide chloromethyl ketones in a graded series of [I] concentrations. For each compound a set of eight inhibition runs was carried out at different concentrations extending over a five-fold range, with the highest [I] limited by rapidity of reaction or solubility. For all compounds except Ac-Ala-Ala-Pro-AlaCH_2Cl the $k_{obsd}/[I]$ values decreased regularly as [I] increased over the range of [I], which shows that the [I] values of the inhibitors were large enough to be within the region of K_I ($[I] \geq 0.1\ K_I$) (eq 7). Pseudo-first-order kinetics were observed for Ac-Ala-Ala-Pro-AlaCH_2Cl for up to two reaction half-lives although the ratio of [I]/[E] < 10. Yet, second-order rate constants k_{2nd} were calculated from the data obtained with this inhibitor and are listed in Table 3 in parentheses. Again one inhibitor, Ac-Ala-Ala-AlaCH_2Cl, was run at both pH 6.5 and 5.0 over a suitable [I] range, to establish a general correlation between the two solution conditions. This compound had a k_3/K_I value which was 5.6 times faster at pH 6.5.

The effectiveness of these four inhibitors may now be compared on the basis of their relative k_3/K_I values, which are superior to $k_{obsd}/[I]$ values for the purpose of corre-

lating the effect of structural changes on reactivity. This is shown in Table 4 for the concentration-dependent experiments. It is apparent from the k_3/K_I values that the tetrapeptide inhibitors are more reactive than the tripeptide inhibitors by a factor of 10 or more. In general the longer peptide chain length and increased reactivity of these inhibitors are directly related due to an enzymatic condition. The best pancreatic elastase inhibitor in this series is again Ac-Ala-Ala-Pro-AlaCH$_2$Cl. The k_3/K_I value for this compound was assumed to be equal to the second-order rate constant k_{2nd} for the reaction, which is valid only under the condition that $[I] \ll K_I$. Since the K_I value is likely to be close to that obtained for Ac-Ala-Ala-Ala-AlaCH$_2$Cl (K_I = 0.39 mM), this condition was met in the range of inhibitor concentrations used with Ac-Ala-Ala-Pro-AlaCH$_2$Cl ($[I] \leq 0.04$ mM). This is included only to base its reactivity on the more rigorous kinetic constant k_{2nd}.

The inhibitor constants K_I were obtained for three inhibitors including Ac-Ala-Ala-Ala-AlaCH$_2$Cl, verifying the formation of strong enzyme-inhibitor complexes. These constants were measurable since $k_{obsd}/[I]$ varied evenly with [I] over a range of inhibitor concentrations where $[I] \geq 0.1$ K_I for almost all [I]. Standard deviations of 5% to 40% were maintained. No K_I value was obtained for Ac-Ala-Ala-Pro-AlaCH$_2$Cl because its rapid inhibition rate necessitated the use of small values of [I] such that $[I] \ll K_I$, <u>i.e.</u>

[I] < 0.1 K_I. Along with K_I were found k_3 values, the rate of the actual alkylation step.

It should be stated here that one inhibitor, Ac-Ala-Pro-AlaCH_2Cl, was synthesized in order to compare our results with those reported by Thompson and Blout (1973a). However, we were unable to reproduce their k_3/K_I value of 35 M^{-1} sec^{-1}; our experimental value is 3.8 M^{-1} sec^{-1} at pH 6.5. The most likely reason for non-agreement involves differences in the procedures used for quenching the inhibition reaction points and for measuring the residual elastase activity. In their experiments, aliquots were first quenched by dilution into cold buffer followed by measuring of enzymatic activity...incomplete quenching of the inhibition reaction or enzyme adsorption onto glass walls could lead to high values for k_{obsd}. In our experiments, aliquots were withdrawn directly from the reaction mixture for measuring of enzymatic activity...slow inhibitor hydrolysis in aqueous solution or a slight degree of inhibitor racemization could lead to low values for k_{obsd}.

Studies with Other Enzymes. Table 5 shows the results for the experiments in which chymotrypsin and trypsin were reacted with four peptide chloromethyl ketones in attempted inhibition runs. These proteases are homologous with elastase but possess different substrate specificities. Since the reaction conditions were virtually identical with those used for pancreatic elastase, a direct comparison of

results is possible. All inhibition reactions (except one) produced only slight inactivation over a 4-6 hr period for both chymotrypsin and trypsin, and no kinetic constants could be determined. Control experiments without inhibitor showed that both chymotrypsin and trypsin remained almost fully active during this time period. One inhibitor, Ac-Ala-Pro-AlaCH_2Cl, actually did inhibit chymotrypsin with $k_{obsd}/[I] = 0.12\ M^{-1}\ sec^{-1}$ and half-life = 3.2 hr under the conditions employed. However, this inhibition rate is only 3% of that with pancreatic elastase. In general the compounds were found to inhibit chymotrypsin and trypsin at rates of 1% or less of the rates at which they inhibit elastase under identical conditions. The result is probably also valid at higher pH values, e.g. physiological pH levels.

In connection with the specificity studies, leukocyte elastase was reacted with two peptide chloromethyl ketones corresponding to different protease primary specificities. The two compounds possessed at P_1 a phenylalanine and a leucine instead of the alanine residue usually recognized there. The chymotrypsin inhibitor Z-Gly-Leu-PheCH_2Cl did not have any inhibitory effect on leukocyte elastase, while the subtilisin inhibitor Ac-Phe-Gly-Ala-LeuCH_2Cl was found to inactivate the enzyme with $k_{obsd}/[I] = 0.60\ M^{-1}\ sec^{-1}$ and half-life = 97 min under the conditions employed. Although the P_1-leucine inhibitor is the slowest-acting of the tetrapeptide compounds, its inhibition rate is still twice that of

Ac-Ala-Ala-AlaCH_2Cl. Also, the two inhibitors would probably interact with pancreatic elastase in a similar fashion, although the experiments were not gone back and carried out.

Table 1. Inhibition of Pancreatic Elastase with Peptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	pH	[I] x 10^4 (M)	k_{obsd} x 10^4 (sec^{-1})[b]	Half-life (min)	$k_{obsd}/[I]$ (M^{-1} sec^{-1})	$k_{obsd}/[I]$ (rel.)[c]
Ac-Ala-Ala-AlaCH_2Cl	6.5	5.0	13.1	9	2.6	1.0
Ac-Ala-Ala-AlaCH_2Cl	5.0	5.0	2.6	44	0.53	0.2
Ac-Ala-Pro-AlaCH_2Cl	6.5	5.0	20.3	6	4.1	1.6
Ac-Pro-Ala-AlaCH_2Cl	6.5	5.0	0	-	-	-
Ac-Ala-Gly-AlaCH_2Cl	6.5	5.0	2.3	50	0.47	0.2
Z-Gly-Leu-AlaCH_2Cl	5.0	5.0	5.0	23	1.0	1.9
Ac-Ala-Ala-Ala-AlaCH_2Cl	5.0	0.5	4.9	24	9.8	18.4
Ac-Ala-Ala-Pro-AlaCH_2Cl	5.0	0.5	18.9	6	37.8	71.
Ac-Ala-Pro-Ala-AlaCH_2Cl	6.5	5.0	0	-	-	-
Ac-Ala-Ala-Phe-AlaCH_2Cl	5.0	0.5	4.2	27	8.4	16.0

[a]Elastase concentration 5 μM, 5% (v/v) methanol, at 30°. [b]Averages of at least three runs with a maximum spread of ± 5%. [c]These relative values were calculated for pH 6.5 assuming that $k_{obsd}/[I]$ measured at pH 5.0 for some of the inhibitors would be *ca.* 20% of $k_{obsd}/[I]$ at pH 6.5.

Table 2. Inhibition of Leukocyte Elastase with Peptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	[I] x 10^3 (M)	k_{obsd} x 10^4 (sec^{-1})	Half-life (min)	$k_{obsd}/[I]$ (M^{-1} sec^{-1})	$k_{obsd}/[I]$ (rel.)	$k_{obsd}/[I]$ (rel.)[c]
Ac-Ala-Ala-AlaCH_2Cl	1.0	2.8[b]	42	0.28	1.0	1.0
Ac-Ala-Pro-AlaCH_2Cl	1.0	10.9	11	1.1	4.0	1.6
Ac-Pro-Ala-AlaCH_2Cl	1.0	0	-	-	-	-
Z-Gly-Leu-AlaCH_2Cl	1.0	20.2	6	2.0	7.3	1.9
Z-Gly-Leu-PheCH_2Cl	1.0	0	-	-	-	
Ac-Ala-Ala-Ala-AlaCH_2Cl	0.2	2.0[b]	59	0.97	3.5	18.4
Ac-Ala-Ala-Pro-AlaCH_2Cl	0.2	8.7[b]	13	4.4	15.8	71.
Ac-Ala-Pro-Ala-AlaCH_2Cl	0.2	4.4	26	2.2	8.1	-
Ac-Ala-Ala-Phe-AlaCH_2Cl	0.2	2.6	44	1.3	4.8	16.0
Ac-Phe-Gly-Ala-LeuCH_2Cl	0.2	1.2	97	0.60	2.2	

[a]Elastase concentration 10 μM, 5% (v/v) methanol, at 25° and pH 6.5. [b]Average of two inhibition runs. [c]Data for pancreatic elastase (from Table 1).

Table 3. Concentration-Dependent Inhibition of Pancreatic Elastase with Peptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	pH	[I] x 10^4 (M)	k_{obsd} x 10^4 (sec^{-1})	$k_{obsd}/[I]$ (M^{-1} sec^{-1})
Ac-Ala-Ala-AlaCH_2Cl	6.5	1.0	3.97	3.97
		1.25	4.85	3.88
		1.67	6.08	3.64
		2.0	7.23	3.62
		2.5	8.98	3.59
		3.0	10.88	3.62
		3.75	13.06	3.48
		5.0	15.75	3.15
Ac-Ala-Ala-AlaCH_2Cl	5.0	4.0	2.24	0.559
		5.0	2.64	0.529
		6.7	3.25	0.485
		8.0	3.58	0.448
		10.0	3.90	0.390
		12.0	4.34	0.361
		15.0	5.08	0.338
		20.0	5.80	0.290
Ac-Ala-Pro-AlaCH_2Cl	6.5	0.8	2.94	3.68
		1.0	3.46	3.46
		1.33	4.21	3.17
		1.6	5.09	3.18
		2.0	6.26	3.13
		2.4	7.44	3.10
		3.0	10.16	3.39
		4.0	12.50	3.13

Table 3. Concentration-Dependent Inhibition of Pancreatic Elastase with Peptide Chloromethyl Ketones.[a] (Continued)

Inhibitor P_4 P_3 P_2 P_1	pH	[I] x 10^4 (M)	k_{obsd} x 10^4 (sec^{-1})	$k_{obsd}/[I]$ (M^{-1} sec^{-1})
Ac-Ala-Ala-Ala-$AlaCH_2Cl$	5.0	0.4	3.35	8.37
		0.5	3.87	7.74
		0.67	4.82	7.20
		0.8	5.69	7.12
		1.0	7.01	7.01
		1.2	8.73	7.27
		1.5	10.14	6.76
		2.0	11.93	5.96
Ac-Ala-Ala-Pro-$AlaCH_2Cl$	5.0	0.08	2.44	30.6 (42.2)[b]
		0.10	3.10	31.0 (39.9)
		0.13	4.28	32.2 (38.6)
		0.16	5.02	31.4 (36.6)
		0.20	6.42	32.1 (36.3)
		0.24	7.76	32.3 (35.7)
		0.30	10.90	36.3 (39.6)
		0.40	13.65	34.1 (36.5)

[a]Elastase concentration 5 μM, 5% (v/v) methanol, at 30°. [b]Values in parentheses are second-order rate constants k_{2nd}. Average k_{2nd} = 38.2 M^{-1} sec^{-1}.

Table 4. Concentration-Dependent Inhibition of Pancreatic Elastase with Peptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	pH	k_3/K_I[b] (M^{-1} sec^{-1})	k_3/K_I (rel.)[c]	$k_3 \times 10^3$ (sec^{-1})	K_I[d] (mM)	[I] range (mM)
Ac-Ala-Ala-AlaCH_2Cl	6.5	4.2	1.0	6	1.5 (.27)	0.10 - 0.50
Ac-Ala-Ala-AlaCH_2Cl	5.0	0.75	-	0.9	1.2 (.06)	0.40 - 2.00
Ac-Ala-Pro-AlaCH_2Cl	6.5	3.8	0.9	5	1.2 (.48)	0.08 - 0.40
Ac-Ala-Ala-Ala-AlaCH_2Cl	5.0	8.9	11.8	3	0.4 (.09)	0.04 - 0.20
Ac-Ala-Ala-Pro-AlaCH_2Cl	5.0	38.[e]	51.	-	-	.008 - .040

[a]Elastase concentration 5 μM, 5% (v/v) methanol, at 30°. [b]Maximum standard deviations of ± 5%. [c]These relative values were calculated for pH 6.5 assuming that k_3/K_I measured at pH 5.0 for some of the inhibitors would be ca. 18% of k_3/K_I at pH 6.5. [d]Values in parentheses are standard deviations. [e]Average second-order rate constant k_{2nd} from Table 3.

Table 5. Inhibition of Chymotrypsin and Trypsin with Peptide Chloromethyl Ketones.[a]

Inhibitor					[I] x 10^4	Chymotrypsin		Trypsin	
P_4	P_3	P_2	P_1		pH	(M)	Time (hr)	act. (%)[b]	Time (hr)	act. (%)[b]
Ac-Ala-	Ala-	Ala-	$AlaCH_2Cl$		6.5	5.0	6	62	4	92
Ac-Ala-	Pro-	Ala-	$AlaCH_2Cl$		6.5	5.0	6	28	4	87
Ac-Ala-	Ala-	Ala-	$AlaCH_2Cl$		5.0	1.0	6	100	4	92
Ac-Ala-	Ala-	Pro-	$AlaCH_2Cl$		5.0	1.0	6	100	4	88
None					6.5	-	6	100	4	91

[a]Enzyme concentration 5 μM, 5% (v/v) methanol, at 30°, for both. [b]Enzyme activity after t (hr) measured as percent of initial activity at t=0.

CHAPTER IV

DISCUSSION

A selected variety of peptide chloromethyl ketones were tested as inhibitors of both porcine pancreatic elastase and human leukocyte elastase mostly with excellent results. The correlation between inhibitor structure and inhibitory ability against elastase is the primary finding of this work and the basis for further chemical inquiry.

Development of Elastase Inhibitors. As revealed by x-ray crystallographic investigations, the active-site regions of the serine proteases chymotrypsin, subtilisin, trypsin, and elastase all show a remarkable degree of homology in structure (Segal et al, 1971a; Robertus et al, 1972; Stroud et al, 1971; Shotton et al, 1971). This provides the basis for the proposed uniformity in their mechanism of action. The catalytic centers of the enzymes are essentially identical in composition and conformation, all containing a charge relay system composed of an activated serine, a histidine, and an aspartic acid residue (Blow et al, 1969). Each enzyme contains a pronounced binding pocket adjacent to the catalytic residues, whose dimensions and chemical characteristics determine the primary substrate specificity. Peptide chloromethyl ketones containing a phenylalanine at P_1 (see

below) have been shown to react specifically with chymotrypsin and subtilisin by alkylation of the active-site histidine residue (Segal et al, 1971a; Robertus et al, 1972). The phenyl group of the inhibitor P_1 residue is located inside the hydrophobic S_1 binding pocket of chymotrypsin and subtilisin. Also, there is an extended binding region in which the peptide chain of a tripeptide inhibitor and a three-residue section of the enzyme's backbone form an antiparallel β-sheet structure. In elastase the S_1 binding pocket is partially filled by the alkyl side chains of Val-216 and Thr-226 (Shotton and Watson, 1970). It is thus smaller-sized as compared to those in chymotrypsin and subtilisin, but the extended binding site is presumably similar. Finally, the reactivity in solution between chymotrypsin and peptide chloromethyl ketones has been correlated well with the crystal structure of the inhibited enzyme derivatives (Kurachi et al, 1973).

To clarify matters in this section, the use of the term "elastase" will refer to porcine pancreatic elastase unless otherwise stated. Near the end of the section is a sub-section which deals with the results obtained for human leukocyte elastase. In the discussion of the structure-reactivity relationships of various inhibitors, the notation of Schechter and Berger (1967) is adopted for describing peptide binding subsites for proteolytic enzymes. The amino-acid residues of a substrate (or inhibitor) are designated

P_1, P_2, etc., while the corresponding subsites of the enzyme are designated S_1, S_2, etc. The numbering starts with that substrate residue just beyond which the peptide bond is cleaved by the enzyme and proceeds in the N-terminal direction. For the alanine peptide chloromethyl ketones listed in Tables 1 and 2, the alanine chloromethyl ketone moiety -AlaCH_2Cl is taken to be the P_1 residue. In addition, the N-terminal blocking group Z, as in Z-Gly-Leu-AlaCH_2Cl, is considered as a P_4 residue.

The rationale for designing peptide chloromethyl ketones to be both reactive and specific inhibitors of elastase was to make them resemble normal peptide substrates such as Ac-Ala-Ala-Ala-OMe. Most of the inhibitors contain at P_1 an alanine residue because elastase has demonstrated a primary specificity for alanine residues. In addition, most of the inhibitors contain only alanine and proline residues at P_2-P_4 since elastin, the natural substrate of elastase, has a high content of alanine, proline, and glycine. The inhibitors were expected to react with elastase by alkylation of His-45, the active-site histidine residue, analogous to the earlier studies with chymotrypsin and subtilisin. Finally, an important feature of the peptide chloromethyl ketones is their extended peptide chain length which is supposed to enhance their reactivity thru an increased interaction with the extended binding site of elastase. Similar results have been obtained for chymotrypsin and phenylala-

nine peptide chloromethyl ketones (Kurachi et al, 1973).

Effectiveness of Elastase Inhibitors. The experimental results demonstrate that our strategy was successful for designing P_1-alanine peptide chloromethyl ketones to be reactive and specific inhibitors of elastase. Most of the synthesized compounds were found to be highly reactive inhibitors of elastase, and acted both rapidly and irreversibly even at low pH. The best inhibitor, Ac-Ala-Ala-Pro-AlaCH_2Cl, has a second-order reaction constant of k_3/K_I = 166 M^{-1} sec^{-1} at 30° and pH 6.5. No direct evidence was produced to indicate that peptide chloromethyl ketones actually react with His-45, the active-site histidine residue of elastase. However, it is almost certain that alkylation of His-45 does take place by analogy with related serine proteases. It is verified elsewhere that these inhibitors do react with a histidine residue of elastase (Thompson and Blout, 1973a). Furthermore, the peptide chloromethyl ketones were found to be highly specific for elastase since they were virtually inactive toward the closely related enzymes chymotrypsin and trypsin. In support of this, two inhibitors known to be specific for chymotrypsin and subtilisin were shown to be much less active toward leukocyte elastase. This specificity renders the inhibitors useful in physiological experiments which require the selective inhibition of elastolytic proteases in preference to related enzymes. These properties may help to establish the compounds as potential bio-

medical reagents.

Structure-Reactivity Relationships. From kinetic studies, the overall reaction rate between an enzyme and an irreversible inhibitor is dependent on both the amount of E·I complex present at equilibrium (i.e. the strength of binding), and the limiting rate of inactivation within the E·I complex to give an E-I product (eq 2). The strength of binding, measured by K_I, is determined by the increase in

$$E + I \rightleftharpoons E \cdot I \rightarrow E\text{-}I' + P_3 \qquad (2)$$

entropy upon inhibitor binding due to the disruption of water structure in the enzyme. The velocity of inactivation, measured by k_3, is influenced by the stereoelectronic relationship between enzyme and inhibitor within the E·I complex. It is apparent that structural differences in a series of inhibitors could affect both the inhibitor binding (K_I) and the rate of inactivation (k_3), although these two factors may not be affected in a self-consistent way. Certain characteristics of the inhibitors will act to increase their inhibitory capability. The structure-reactivity relationships among peptide chloromethyl ketones may be used to investigate how certain structural features affect the general reactivity of these compounds with elastase. A primary purpose of these studies is to learn how to design an optimum elastase inhibitor having a maximum activity. The inhibitor structures were regularly varied thru substitutions of res-

idues, at P_1 and P_2 to confirm the enzyme's primary and secondary specificity, and at P_3 and P_4 to examine the enzyme's extended binding site. The inhibitor reactivities are readily compared on the basis of relative second-order rate constants, $k_{obsd}/[I]$ and k_3/K_I, which reflect the combined effects of both inhibitor binding and enzymatic inactivation. For four key inhibitors the values of K_I and k_3 have been individually determined to distinguish the separate effects of these two factors. Finally it should be noted that the relative values of $k_{obsd}/[I]$ and k_3/K_I in Tables 1-4 are somewhat different at pH 6.5 and 5.0 and thus are classified as pH-dependent. This indicates that the subsite interactions between enzyme and inhibitor, as well as the extent of protonation of His-45, are pH-dependent.

P_4 Residues. The first notable outcome is that the reactivity of peptide chloromethyl ketones toward elastase is strongly affected by the number of residues in the peptide chain of the inhibitor. Those inhibitors which possess a P_4 residue (tetrapeptides) are found to be in general 10-50 times more reactive than those without (tripeptides). For example, from k_3/K_I values, Ac-Ala-Ala-Pro-AlaCH_2Cl is 50 times more reactive than its tripeptide analog while Ac-Ala-Ala-Ala-AlaCH_2Cl is 12 times more reactive than its tripeptide analog (Table 4). This substantial difference in the rates of inhibition is the evidence for an interaction between the inhibitors and an extended binding site in elas-

tase. It is noteworthy that an interaction at P_4-S_4, this distant (> 10 Å) from the active site of the enzyme, should have such a marked effect on the inhibition rate. The addition of a P_4 residue onto the inhibitor thus acts to increase its k_3/K_I value, and both K_I and k_3 are affected by the elongation. The K_I value decreases: the K_I of Ac-Ala-Ala-Ala-AlaCH_2Cl is three times lower than that of the tripeptide analog. This tighter binding of the bound complex is probably caused by increased hydrogen-bonding and/or hydrophobic contacts between the P_4 residue of inhibitor and the S_4 binding subsite of elastase. The k_3 value increases: the k_3 for Ac-Ala-Ala-Ala-AlaCH_2Cl is three times higher than that for the tripeptide analog. This acceleration of the rate of inactivation within the bound complex is somehow caused by the interaction at P_4-S_4, either by minutely changing the enzyme's active-site geometry or by slightly altering the inhibitor's conformation. Thus the increase in the rate of inhibition due to a distant interaction at P_4-S_4 is caused by both stronger binding and an increased rate of alkylation within the E·I complex. With chymotrypsin it was observed that generally the larger the number of interactions of a peptide chloromethyl ketone with the enzyme, the larger the overall rate of inhibition (Kurachi et al, 1973).

Extended Binding Site. A number of studies with peptide substrates have shown that elastase possesses an extended substrate binding site composed of at least five sub-

sites (S_1' and S_1-S_4) near the catalytic residues. The substrates employed were peptide acids, esters, and amides (Atlas et al, 1970; Atlas and Berger, 1972; Thompson and Blout, 1970, 1973c). Since there is a large incremental difference in reactivity between tri- and tetrapeptide chloromethyl ketones as well as tri- and tetrapeptide substrates (Thompson and Blout, 1973c), it is evident that the inhibitors are interacting with the same extended substrate binding site of the enzyme. Whereas peptide chloromethyl ketones are excellent inhibitors, simpler analogs e.g. Tos-AlaCH_2Cl and Tos-ValCH_2Cl are incapable of inhibiting elastase (Kaplan et al, 1970). Thus it appears that the occupation of a substantial portion of the extended binding site by an inhibitor is not simply helpful but obligatory in order to allow inhibition to take place. An extended peptide inhibitor presumably binds at this site almost entirely in a productive binding mode leading to a maximum inhibition rate.

An explanation for the surprising increase in reactivity of elastase toward peptide amide substrates, caused by the addition of a P_4 residue, has been offered (Thompson and Blout, 1970). The extended binding site is believed to play a key role in this phenomenon. The binding pocket at the S_1 subsite of elastase is smaller than in chymotrypsin and results in poorer enzyme-substrate binding since its occupation by a P_1 residue cannot correctly orient the scissile bond as well within the active site. It appears that

the extended binding site interactions, especially at P_4-S_4, act to properly position the susceptible part of a substrate or inhibitor for attack within the active site of the enzyme, possibly thru a conformational change. Thus the filling of the S_4 subsite by a P_4 residue yields an anchoring effect. An "induced fit" enzyme model may be invoked to explain the enzymatic mechanism for intramolecular transformation of a distant binding interaction into an increased total reaction rate (Koshland and Neet, 1968). A conformational change triggered by binding contacts at an adjacent site can improve the orientation and fit of a substrate or inhibitor at the active site of the enzyme.

P_3 Residues. Two peptide chloromethyl ketones, Ac-Ala-Pro-Ala-AlaCH_2Cl and Ac-Pro-Ala-AlaCH_2Cl, were discovered to be completely unreactive toward elastase under the standard inhibition conditions. Since both compounds have a proline as the P_3 residue, it would seem to create an unfavorable interaction with the S_3 subsite of elastase. Thus an inhibitor with a proline at P_3 is incapable of productive binding to the enzyme, or else it might bind weakly but not in a conformation leading to enzymatic inhibition. This could be attributed to the abnormal geometry of proline and the consequent disruption of stereoelectronic stabilization in the binding site. Peptide amide substrates were also found to be unhydrolyzed by elastase and incapable of productive binding when a proline at P_3 would interact with the

S_3 subsite of the enzyme (Thompson and Blout, 1973b). In a study of chymotrypsin hydrolysis of a series of peptide esters it was noted that a proline at P_3 caused a 30-fold increase in K_M relative to an isomeric substrate with a proline at P_2 (Segal, 1972). This effect is likely caused by a situation more complex than simple steric interference. One explanation for this poor binding is that a proline at P_3 could disrupt the β-sheet hydrogen-bonding formed between an extended peptide substrate or inhibitor and a section of chymotrypsin's backbone (Segal et al, 1971a,b). At present it can only be speculated that a similar structural situation underlies the inability of elastase to accept a proline in its S_3 binding subsite.

P_2 Residues. The reactivity of peptide chloromethyl ketones toward elastase is partially affected by the steric size and character of the P_2 residue of the inhibitor. The following approximate relative rate factors are calculated from the data in Table 1. Among the tripeptide inhibitors, the $k_{obsd}/[I]$ is approximately equal whether P_2 is alanine (Ac-Ala-Ala-AlaCH_2Cl) or proline (Ac-Ala-Pro-AlaCH_2Cl), meaning that the two have a similar effect at P_2; relative to P_2 = alanine, when P_2 is leucine (Z-Gly-Leu-AlaCH_2Cl) $k_{obsd}/[I]$ rises by a factor of 2, but when P_2 is glycine (Ac-Ala-Gly-AlaCH_2Cl) it drops by a factor of 5. Among the tetrapeptide inhibitors, relative to $k_{obsd}/[I]$ for P_2 = alanine (Ac-Ala-Ala-Ala-AlaCH_2Cl) as the standard, when P_2

is proline (Ac-Ala-Ala-Pro-AlaCH_2Cl) $k_{obsd}/[I]$ increases by a factor of 4, but when P_2 is phenylalanine (Ac-Ala-Ala-Phe-AlaCH_2Cl) it is virtually unchanged. Hence the relative reactivity of inhibitors with a variety of P_2 residues can be approximately represented as follows: Gly < Ala < Pro, Leu > Phe. The homologous protease chymotrypsin possesses a secondary specificity for substrates or inhibitors with P_2 residues, especially leucine (Boc-Gly-Leu-PheCH_2Cl), which have medium-sized alkyl side chains, due to hydrophobic interaction of the P_2 residue with Ilu-199 at the enzyme's S_2 subsite (Kurachi et al, 1973). Our results suggest that elastase may also exhibit a secondary specificity for inhibitors with P_2 residues, e.g. proline (Ac-Ala-Ala-Pro-AlaCH_2Cl) and leucine (Z-Gly-Leu-AlaCH_2Cl), which have medium-sized alkyl side chains. Elastase appears to manifest a definite preference for such P_2 residues which may be large enough to provide a hydrophobic interaction but not too large to foster steric repulsion. Further studies are necessary to explore the nature and cause of any secondary specificity under more inclusive and invariant conditions. According to our limited results, a proline appears to be optimal for the P_2 residue of an elastase inhibitor, and a leucine at P_2 seems to be almost as advantageous. At this juncture, for optimum reactivity, an elastase inhibitor should contain a P_1 alanine, a P_2 proline or leucine, no P_3 proline, and preferably a P_4 residue.

Comparison of Inhibitors with Substrates. It is worthwhile now to compare certain properties of binding and reactivity toward elastase for a series of peptide chloromethyl ketones with the complementary peptide amides. Some kinetic parameters for inhibitors and substrates are listed in Table 6 (data from Thompson and Blout, 1973b,c). It may be assumed that K_I and K_M values are largely pH-independent, since the K_M for Bz-Ala-OMe is constant from pH 5 to 10 (Hartley and Shotton, 1971). However, k_3/K_I and k_{cat}/K_M values are highly pH-dependent over this pH range. The K_I values for the inhibitors and the K_M values for the substrates are similar overall both in magnitude and the direction of change upon going from tripeptides to tetrapeptides. This indicates that both kinds of compounds are probably binding to elastase in an identical manner. The relative k_3/K_I and k_{cat}/K_M values for the inhibitors (at pH 6.5) and substrates (at pH 9.0) also display parallel general trends in their reactivity. In going from tripeptides to tetrapeptides, however, the inhibition parameter k_3/K_I increases by a factor of ca. 20-30, but the catalytic parameter k_{cat}/K_M increases by a factor of over 100. This indicates that the addition of a P_4 residue has a less sensitive effect on the alkylation of His-45 by peptide chloromethyl ketones than the acylation of Ser-188 by peptide amides. In general, distant interactions again play their part in the binding and reactivity of both inhibitors and substrates with elas-

tase in a similar manner.

From x-ray crystallographic investigations, the extended binding regions (in addition to the active-site regions) of chymotrypsin and subtilisin show a remarkable degree of homology (Segal et al, 1971a; Kraut et al, 1971; Robertus et al, 1972). It is interesting to speculate that the extended binding site of elastase is homologous with those of the other two enzymes. This leads to the proposal for a common binding scheme among the family of serine proteases, including elastase too. Our results are consistent with the formation of β-sheet hydrogen-bonding between a peptide inhibitor and the extended binding site of elastase, analogous in relative orientation and conformation to that in chymotrypsin and subtilisin. However, a much different binding scheme is postulated for elastase based on the x-ray structures of elastase-substrate complexes at 3.5 Å resolution (Shotton et al, 1971). So, an x-ray crystallographic investigation of the structural features of elastase inhibited by a peptide chloromethyl ketone seems absolutely necessary for answering many questions about elastase itself and its relation to familial proteases. These include extended binding site interactions, primary and secondary specificity, and active-site and binding-site homologies with other proteases. Some work on the x-ray structure of an inhibited elastase derivative was started by D.M. Shotton in 1973 but unfortunately it has not been completed yet.

Human Leukocyte Elastase. A human leukocyte elastase has been recently isolated and partially characterized, and it is implicated in the pathology of emphysema, arteritis, arthritis, and related tissue diseases (Janoff, 1972a). For the continuing studies of human leukocyte elastase and its biological behavior, our peptide chloromethyl ketone inhibitors have proven very useful. In addition to their fine reactivity the inhibitors are highly specific for elastolytic proteases in the presence of other PMN leukocyte enzymes including collagenases and other neutral proteases. Therefore a variety of specific new functions of human leukocyte elastase may now be scrutinized. For example, the digestion of elastin-rich human lung tissue and rat aortic tissue by a human granulocyte elastase *in vitro* is completely inhibited by Ac-Ala-Ala-AlaCH_2Cl (Janoff, 1972a). Additionally, the esterolytic activity of human leukocyte neutrophils containing three major elastase-like esterases is *ca.* 80% inhibited by Ac-Ala-Ala-AlaCH_2Cl (Sweetman and Ornstein, 1974). This inhibitor was also used for the location of elastolytic enzymes on electrophoretic cationic zymograms. The lysing action by a human granulocyte elastase upon peptidoglycan-containing cell walls of autoclaved bacteria *in vitro* is prevented by Ac-Ala-Ala-AlaCH_2Cl (Janoff and Blondin, 1973). Furthermore, the digestion of phagocytosed *E. coli* proteins by a human granulocyte elastase is inhibited by Ac-Ala-Ala-Pro-AlaCH_2Cl and Ac-Ala-Ala-AlaCH_2Cl (Janoff and Blondin,

1974). The ability of human leukocyte lysosomes containing an elastase to induce cell surface changes in mouse fibroblasts associated with loss of growth control is depressed by Ac-Ala-Ala-Pro-AlaCH_2Cl (Mosser et al, 1973). A number of further studies on the physiological and pathological functions of human leukocyte elastase using peptide chloromethyl ketones are currently in progress.

In closely related work, various newly-found proteases other than elastolytic enzymes are being investigated with the aid of peptide chloromethyl ketones. A lysosomal thiol protease, human cathepsin B1, was shown to degrade native collagen, and the collagenase and amidase activity is inhibited by Ac-Ala-Ala-AlaCH_2Cl and Ac-Ala-Ala-Pro-AlaCH_2Cl (Barrett, 1973; Burleigh et al, 1974). This implies that human cathepsin B1 exhibits a rather broad substrate specificity. However, a specific rabbit fibroblast collagenase which also degrades collagen is not inhibited by Ac-Ala-Ala-Ala-AlaCH_2Cl or Ac-Ala-Ala-Pro-AlaCH_2Cl (Werb and Burleigh, 1974).

Structure-Reactivity Relationships. Another set of experiments was run by us to determine the structure-reactivity relationships among peptide chloromethyl ketones in their interaction with human leukocyte elastase. A slightly expanded group of inhibitors was employed and reactivities are compared only on the basis of relative $k_{obsd}/[I]$ values. This kinetic study establishes that human leukocyte elastase

is distinctly different from porcine pancreatic elastase in several aspects of its reactivity with the inhibitors. The results may help to optimize the compounds for eventual use as potential biomedical reagents.

The tetrapeptides, possessing a P_4 residue, are in general 3-4 times more reactive than the tripeptides; for example, both Ac-Ala-Ala-Ala-AlaCH_2Cl and Ac-Ala-Ala-Pro-AlaCH_2Cl are four times more reactive than their tripeptide analogs from $k_{obsd}/[I]$. Hence the longer the peptide chain the higher the reactivity (especially P_3 to P_4), which is evidence for an interaction between the inhibitors and an extended binding site in leukocyte elastase. Although no information was obtainable, the addition of a P_4 residue onto the inhibitor probably acts to decrease K_I and increase k_3 the same as before. Almost all of the inhibitors contain an alanine at P_1, e.g. Ac-Ala-Ala-Ala-AlaCH_2Cl, corresponding to the substrate specificity of elastase for alanine residues. If P_1 is phenylalanine as in the chymotrypsin inhibitor Z-Gly-Leu-PheCH_2Cl, no leukocyte elastase inhibition is observed, but if P_2 is leucine as in the subtilisin inhibitor Ac-Phe-Gly-Ala-LeuCH_2Cl, a small rate of elastase inhibition takes place. This reinforces the idea that inhibitors with an alanine at P_1 are fairly specific for elastolytic proteases. The following approximate relative rate factors are calculated from the data in Table 2. Among the tripeptide inhibitors, relative to $k_{obsd}/[I]$ for P_2 = ala-

nine (Ac-Ala-Ala-AlaCH_2Cl), when P_2 is proline (Ac-Ala-Pro-AlaCH_2Cl) $k_{obsd}/[I]$ increases by a factor of 4, and when P_2 is leucine (Z-Gly-Leu-AlaCH_2Cl) it increases by a factor of 7. Among the tetrapeptide inhibitors, relative to $k_{obsd}/[I]$ for P_2 = alanine (Ac-Ala-Ala-Ala-AlaCH_2Cl), when P_2 is proline (Ac-Ala-Ala-Pro-AlaCH_2Cl) $k_{obsd}/[I]$ increases by a factor of 4, but when P_2 is phenylalanine (Ac-Ala-Ala-Phe-AlaCH_2Cl) it is virtually unchanged. Thus leukocyte elastase may also possibly exhibit a secondary specificity for inhibitors with P_2 residues, e.g. proline (Ac-Ala-Pro-AlaCH_2Cl) and leucine (Z-Gly-Leu-AlaCH_2Cl), which have medium-sized alkyl side chains. If P_3 is proline as in the tripeptide Ac-Pro-Ala-AlaCH_2Cl, leukocyte elastase inhibition is totally blocked, but if P_3 is proline as in the tetrapeptide Ac-Ala-Pro-Ala-AlaCH_2Cl, a medium rate of inhibition does take place. Recall that Ac-Ala-Pro-Ala-AlaCH_2Cl is a non-inhibitor toward porcine pancreatic elastase. Two inhibitors, Z-Gly-Leu-AlaCH_2Cl and Ac-Phe-Gly-Ala-LeuCH_2Cl, containing at P_4 a phenylalanine or benzyloxycarbonyl with a benzyl side chain, are somewhat more reactive than normally expected. In the P_4 position of an inhibitor a large hydrophobic group, e.g. benzyl, appears to be beneficial although further studies will be necessary for verification. In conclusion, for optimum reactivity, a leukocyte elastase inhibitor should contain a P_1 alanine, a P_2 proline or leucine, no P_3 proline (for tripeptides), and preferably a P_4 residue.

Comparison of Two Kinds of Elastase. It is informative at this point to examine certain inhibition characteristics of human leukocyte elastase as compared to the well-known porcine pancreatic elastase (refer to Table 7). The nature of the differences may provide some insight into their separate biological functions. First of all, leukocyte elastase reacts substantially less rapidly overall with this group of peptide chloromethyl ketones than does pancreatic elastase as indicated by smaller values of $k_{obsd}/[I]$ for inhibition, which is most likely due to the lesser specific activity of the enzyme sample. For leukocyte elastase the increase in reactivity toward inhibitors is ca. 10-fold smaller in going from tripeptides to tetrapeptides, which may indicate differences in the conformation of the extended binding sites and the effect of the individual subsites on the catalytic activity of the two enzymes. Secondly, the structure-reactivity relationships are generally parallel between the two kinds of elastase and the inhibitors in regard to the structural features required for enhancement of reactivity for enzymatic inhibition. In particular, the increase in reactivity toward inhibitors with an extra P_4 residue, the narrow primary specificity for P_1 residues such as alanine, and the secondary specificity for P_2 residues such as proline and leucine, are common features of both enzymes. Finally, Ac-Ala-Ala-Pro-AlaCH_2Cl is the most effective inhibitor tested for both leukocyte elastase and pancreatic

elastase, and represents our "best" inhibitor. Unusually, its isomer Ac-Ala-Pro-Ala-AlaCH_2Cl is a relatively good inhibitor for leukocyte elastase but is completely inactive against pancreatic elastase, which clearly implies that these two kinds of enzyme are actually distinct entities. Further comparisons of the properties of the two elastase species must await the availability of leukocyte elastase of higher purity and quantity.

Table 6. Comparison of Kinetic Parameters for a Series of Inhibitors and Substrates.[a]

Peptide	Chloromethyl Ketone ($-CH_2Cl$)		Amide ($-NH_2$)	
P_4 P_3 P_2 P_1	k_3/K_I[b] (M^{-1} sec^{-1})	K_I (mM)	k_{cat}/K_M (M^{-1} sec^{-1})	K_M (mM)
Ac-Ala-Ala-Ala—	4.2 (1.0)[d]	1.5	13[e] (1.0)[d]	2.5
Ac-Ala-Pro-Ala—	3.8 (0.9)	1.2	21[e] (1.6)	4.2
Ac-Ala-Ala-Ala-Ala—	50.[c] (12)	0.4	2070[f] (160)	2.9
Ac-Ala-Ala-Pro-Ala—	166.[c] (51)	-	2900[f] (220)	2.1

[a]For reaction with pancreatic elastase. [b]Values at pH 6.5. [c]Values adjusted to pH 6.5. [d]Values in parentheses are relative constants. [e]pH 9.0 (Thompson and Blout, 1973c). [f]pH 9.0 (Thompson and Blout, 1973b).

Table 7. Inhibition of Leukocyte and Pancreatic Elastase with Peptide Chloromethyl Ketones.[a]

Inhibitor	I	Leukocyte Elastase		Pancreatic Elastase		
		$k_{obsd}/[I]$	$k_{obsd}/[I]$	$k_{obsd}/[I]$	$k_{obsd}/[I]$	$k_{obsd}/[I]$
P_4 P_3 P_2 P_1	M.W.	(M^{-1} sec^{-1})	(rel.)[b]	(M^{-1} sec^{-1})	(rel.)	(rel.)[b]
Ac-Ala-Ala-AlaCH_2Cl	305.5	0.28	1.0	2.6	1.0	9.5
Ac-Ala-Pro-AlaCH_2Cl	331.5	1.1	4.0	4.1	1.6	14.7
Ac-Pro-Ala-AlaCH_2Cl	331.5	-	-	-	-	-
Ac-Ala-Gly-AlaCH_2Cl	291.5			0.47	0.2	1.7
Z-Gly-Leu-AlaCH_2Cl	425.5	2.0	7.3	4.9	1.9	17.9
Z-Gly-Leu-PheCH_2Cl	501.5	-	-			
Ac-Ala-Ala-Ala-AlaCH_2Cl	376.5	0.97	3.5	48.	18.4	175.
Ac-Ala-Ala-Pro-AlaCH_2Cl	402.5	4.4	15.8	187.	71.	677.
Ac-Ala-Pro-Ala-AlaCH_2Cl	402.5	2.2	8.1	-	-	-
Ac-Ala-Ala-Phe-AlaCH_2Cl	452.5	1.3	4.8	42.	16.0	151.
Ac-Phe-Gly-Ala-LeuCH_2Cl	480.5	0.60	2.2			

[a]Elastase concentration 5-10 μM, inhibitor concentration 0.05-1.0 mM, 5% (v/v) methanol, at 30° and pH 6.5. [b]These two sets of results are on an identical scale.

CHAPTER V

CONCLUSIONS

In conclusion, the kinetic results demonstrate that P_1-alanine peptide chloromethyl ketones are fairly reactive and specific inhibitors for both porcine pancreatic elastase and human leukocyte elastase. They are reactive and specific because of their P_1-alanine chloromethyl ketone moiety plus their substrate-like extended peptide structure. In addition they are shown to be remarkably specific for elastolytic proteases in preference to chymotrypsin, trypsin, or collagenase-like enzymes. The elastase inhibitors have been analyzed for structure-reactivity relationships to gather important information on the reactivity, specificity, and extended binding site of elastase and on structural homologies with related proteases. The tetrapeptides are markedly more reactive (by k_{2nd}) than the tripeptides as irreversible elastase inhibitors, due to increased binding (K_I) plus a faster rate of inactivation (k_3) from interaction with the enzyme's extended binding site. It appears that elastase exhibits a secondary specificity for inhibitors with P_2 residues, e.g. proline and leucine, having medium-sized alkyl side chains. The most effective elastase inhibitor is found to be Ac-Ala-Ala-Pro-AlaCH_2Cl, and its isomer Ac-Ala-Pro-Ala-

$AlaCH_2Cl$ is a non-inhibitor for pancreatic elastase but a good inhibitor for leukocyte elastase, implying that the two enzymes are different and distinct. From the limited results, for optimum reactivity, a leukocyte elastase inhibitor should contain a P_1 alanine, a P_2 proline or leucine, no P_3 proline (for tripeptides), and preferably a P_4 residue (i.e. a tetrapeptide). The behavioral analogies of elastase toward peptide substrates and inhibitors support the hypothesis that the enzyme-inhibitor binding mode leading to alkylation of His-45 is identical to the productive substrate binding mode leading to acylation of Ser-188. An x-ray structural determination of an elastase derivative inhibited by a peptide chloromethyl ketone will be required to establish the interactions between enzyme and inhibitor. Human leukocyte elastase has recently been implicated in the pathological processes associated with pulmonary emphysema, rheumatoid arthritis, acute arteritis, and other related elastic tissue diseases. A number of investigations using peptide chloromethyl ketones are presently helping to elucidate the character of these elastolytic proteases and their many physiological and pathological functions. Eventually the inhibitors may become valuable in the treatment of these diseases.

CHAPTER VI

RECOMMENDATIONS

A profitable extension of this work would be to prepare and isolate an inhibited elastase derivative, modified by attaching a peptide chloromethyl ketone at the active site. Thus, 20 mg of pancreatic elastase could be 100% inhibited by a 10-fold excess of Ac-Ala-Ala-Pro-AlaCH_2Cl at 25° and pH 6.5 within 1 hr, and the derivative isolated by gel filtration or chromatography. This product would be crystallized and used for x-ray structural determination of the exact modes of interaction between the enzyme and inhibitor. A crystallographic investigation may then be a valuable complement to those already done for the related proteases chymotrypsin and subtilisin. Another area for further efforts is a continuation of kinetic studies on the inhibition of elastase with the use of improved, related, or different kinds of inhibitors. For example, Ac-Phe-Ala-Pro-AlaCH_2Cl may be an extremely good elastase inhibitor as it incorporates many positive features. The beneficial effect of valine at P_1 ought to be examined since elastase appears to exhibit a substrate specificity for valine at P_1. The relative effect of various residues at P_1, P_2 and P_4, and leaving groups at P_1', on the ability to promote enzymatic

inhibition needs to be evaluated more rigorously. It is probably also a good idea to seek to improve the synthetic procedure and yields for making peptide chloromethyl ketones, especially Ac-Ala-Ala-Pro-AlaCH_2Cl. The inhibitors could then be prepared in large quantities for testing the elastolytic activity of several newly-found leukocyte proteases. Finally, the detailed medical effects of peptide chloromethyl ketones on the biological processes in mammalian organisms must be ascertained carefully and thoroughly. The inhibitors may subsequently be tested as drug-type agents in humans against emphysema and related tissue diseases. There is strong belief that the compounds are reactive enough to inhibit leukocyte elastase *in vivo* if they can be applied to this task only, so the biggest problem is to ensure that they are specific enough not to interfere with other critical biological functions in side reactions. The whole field of enzyme inhibition chemistry has valuable application to research in medical science. These biological studies can be performed *e.g.* by the USFDA to determine the effectiveness, safety, and ultimate utility of the elastase inhibitors.

APPENDIX

Elastase Activity Assay Procedure. The decreasing activity of pancreatic elastase during an inhibition run was measured according to the general assay method of Visser and Blout (1972). The substrate solution consists of 0.20 mM BOC-Ala-ONp in 0.05 M phosphate (pH 6.5) containing 2% dioxane, good for up to 3 hr, and 3.0 ml of solution in a standard uv cell is used for each assay point. A rate of substrate background hydrolysis is determined beforehand, usually ca. $\Delta A = 0.003\ min^{-1}$. After the elastase and inhibitor solutions are mixed together to start the inhibition reaction, 6 to 8 aliquots are removed beginning at 1.0 min and then at regular time intervals thru two reaction half-lives. Each 100 µl aliquot containing 12.5 µg of enzyme is mixed rapidly into the substrate solution and the release of p-nitrophenol is followed at 345 nm for 1.0-1.2 min on the uv spectrophotometer. A typical assay pattern for an inhibition run with half-life $t_{½}$ = 16 min would be at t=1 ($\tan\theta \approx 2.5$), 4½, 8, 12, 17, 22, 28, and 35 min. The rates of hydrolysis as expressed by tan θ of the linear traces are proportional to the concentration of residual active enzyme. The procedure for leukocyte elastase is identical in relative concentration but smaller in amount, with 5 or 6 50 µl aliquots each containing 12 µg of enzyme assayed against 1.2 ml portions of

substrate solution. In either case the inhibition reaction is instantly quenched upon dilution and the initial rate of hydrolysis is virtually constant.

Reference:

Visser, L., and Blout, E.R. (1972), Biochim. Biophys. Acta 268, 257.

REFERENCES

Atlas, D., and Berger, A. (1972), Biochemistry 11, 4719.

Atlas, D., Levit, S., Schechter, I., and Berger, A. (1970), FEBS (Fed. Eur. Biochem. Soc.) Lett. 11, 281.

Barrett, A.J. (1973), Biochem. J. 131, 809.

Bender, M.L., and Kezdy, F.J. (1964), J. Amer. Chem. Soc. 86, 3704.

Blow, D.M., Birktoft, J.J., and Hartley, B.S. (1969), Nature (London) 221, 337.

Brown, W.E., and Wold, F. (1973a), Biochemistry 12, 828.

Brown, W.E., and Wold, F. (1973b), Biochemistry 12, 835.

Burleigh, M.C., Barrett, A.J., and Lazarus, G.S. (1974), Biochem. J. 137, 387.

Folds, J.D., Welsh, I.R.H., and Spitznagel, J.K. (1972), Proc. Soc. Exp. Biol. Med. 139, 461.

Galdston, M., Janoff, A., and Davis, A.L. (1973), Am. Rev. Respir. Dis. 107, 718.

Geneste, P., and Bender, M.L. (1969), Proc. Nat. Acad. Sci. U.S. 64, 683.

Hartley, B.S., and Shotton, D.M. (1971), Enzymes, 3rd Ed., 3, 323.

Hess, G.P. (1971), Enzymes, 3rd Ed., 3, 213.

Janoff, A. (1973), Lab. Invest. 29, 458.

Janoff, A. (1972a), Amer. J. Pathol. 68, 579.

Janoff, A. (1972b), Am. Rev. Respir. Dis. 105, 121.

Janoff, A. (1970a), Lab. Invest. 22, 228.

Janoff, A. (1970b), Ser. Haemat. 3, 96.

Janoff, A. (1969), Biochem. J. 114, 157.

Janoff, A., and Basch, R.S. (1971), Proc. Soc. Exp. Biol. Med. 136, 1045.

Janoff, A., and Blondin, J. (1974), Proc. Soc. Exp. Biol. Med. 145, 1427.

Janoff, A., and Blondin, J. (1973), Lab. Invest. 29, 454.

Janoff, A., Sandhaus, R.A., Hospelhorn, V.D., and Rosenberg, R. (1972), Proc. Soc. Exp. Biol. Med. 140, 516.

Janoff, A., and Scherer, J. (1968), J. Exp. Med. 128, 1137.

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

Kitz, R., and Wilson, I. (1962), J. Biol. Chem. 237, 3245.

Koshland, D.E. Jr., and Neet, K.E. (1968), Ann. Rev. Biochem. 37, 359.

Kraut, J., Robertus, J.D., Birktoft, J.J., Alden, R.A., Wilcox, P.E., and Powers, J.C. (1971), Cold Spring Harbor Symp. Quant. Biol. 36, 117.

Kurachi, K., Powers, J.C., and Wilcox, P.E. (1973), Biochemistry 12, 771.

Mittman, C., Ed. (1972), Pulmonary Emphysema and Proteolysis, New York, N.Y., Academic Press.

Morihara, K., and Oka, T. (1970), Arch. Biochem. Biophys. 138, 526.

Mosser, A.G., Janoff, A., and Blondin, J. (1973), Cancer Res. 33, 1092.

Narayanan, A.S., and Anwar, R.A. (1969), Biochem. J. 114, 11.

Ohlsson, K. (1971), Scand. J. Clin. Lab. Invest. 28, 251.

Ohlsson, K., and Olsson, I. (1973), Eur. J. Biochem. 36, 473.

Penke, B., Czombos, J., Balaspiri, L., Petres, J., and Kovacs, K. (1970), Helv. Chim. Acta 53, 1057.

Powers, J.C., Carroll, D.M., and Tuhy, P.M. (1975), Ann. N.Y. Acad. Sci., in press.

Powers, J.C., and Tuhy, P.M. (1973), Biochemistry 12, 4767.

Powers, J.C., and Tuhy, P.M. (1972), J. Amer. Chem. Soc. 94, 6544.

Powers, J.C., and Wilcox, P.E. (1970), J. Amer. Chem. Soc. 92, 1782.

Robertus, J.D., Alden, R.A., Birktoft, J.J., Kraut, J., Powers, J.C., and Wilcox, P.E. (1972), Biochemistry 11, 2439.

Schechter, I., and Berger, A. (1967), Biochem. Biophys. Res. Commun. 27, 157.

Segal, D.M. (1972), Biochemistry 11, 349.

Segal, D.M., Cohen, G.H., Davies, D.R., Powers, J.C., and Wilcox, P.E. (1971b), Cold Spring Harbor Symp. Quant. Biol. 36, 85.

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

Shaw, E. (1970), Enzymes, 3rd Ed., 1, 91.

Shotton, D.M., and Watson, H.C. (1970), Nature (London) 225, 811.

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

Stroud, R.M., Kay, L.M., and Dickerson, R.E. (1971), Cold Spring Harbor Symp. Quant. Biol. 36, 125.

Sweetman, F., and Ornstein, L. (1974), J. Histochem. Cytochem. 22, 327.

Thompson, R.C., and Blout, E.R. (1970), Proc. Nat. Acad. Sci. U.S. 67, 1734.

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

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

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

Thomson, A., and Denniss, I.S. (1973), Eur. J. Biochem. 38, 1.

Tuhy, P.M., and Powers, J.C. (1975), FEBS (Fed. Eur. Biochem. Soc.) Lett., in press.

Visser, L., and Blout, E.R. (1972), Biochim. Biophys. Acta 268, 257.

Visser, L., Sigman, D.S., and Blout, E.R. (1971), Biochemistry 10, 735.

Walsh, K.A., and Wilcox, P.E. (1970), Methods Enzymol. 19, 37.

Werb, Z., and Burleigh, M.C. (1974), Biochem. J. 137, 373.

Wilcox, P.E. (1970), Methods Enzymol. 19, 73.

II. MODIFICATION OF CHYMOTRYPSIN BY AN ARYL CYANATE REAGENT

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

SUMMARY

A novel aryl cyanate, Ac-Phe(OCN)-OEt (N-acetyl-p-cyanato-L-phenylalanine ethyl ester), was synthesized as an intended reagent for altering the primary substrate specificity of chymotrypsin. This reagent is an effective, active-site-directed, irreversible inhibitor, moderately reactive and unusually specific in its modification of chymotrypsin. The reaction of chymotrypsin A_α with Ac-Phe(OCN)-OEt yields two derivatives, a N-tyrosyliminomethyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_α (60%), and a further product, a partially trisubstituted chymotrypsin A_α (40%). The active-site Ser-195 is modified by carbamylation resulting in total inhibition of enzymatic activity, and N-terminal Cys-1 is modified by isourea formation with inhibitor, but the binding pocket remains unchanged. Thus the enzyme reaction is selective in being largely limited to only two reaction sites, and its course is virtually identical to the known reaction with p-nitrophenyl cyanate. However, the initial attempt at enzyme engineering has failed to create a new and different species of active enzyme (by carbamylation of Ser-189 or Ser-190), with a modified binding pocket and an altered substrate specificity. This result may be caused by a lack of enzyme-reagent binding with a concurrent bimolecular reaction, or an extreme reactivity difference in

the active-site and binding-site residues. The principal finding is the necessity to protect the reactive active-site residues during selective enzyme modification procedures intended elsewhere, e.g. with the aid of a reversible blocking technique like an appropriate acyl-enzyme species. The reaction of chymotrypsin with Ac-Phe(OCN)-OEt still shows that aryl cyanates may be established as versatile and valuable reagents in protein chemistry.

CHAPTER I

INTRODUCTION

This endeavor marks the entrance into a new area of enzyme chemistry called "enzyme engineering" which deals with the modification of proteins to suit a specific purpose. Our approach was to try to selectively convert chymotrypsin into another enzyme with altered properties. Refer to the Introduction of this Thesis, Part I, for a background of proteolytic enzymes and serine proteases, including a description of the mechanism of catalytic action. These enzymes are responsible for cleaving peptide bonds at certain points on polymeric protein substrates.

The serine protease family, containing such enzymes as chymotrypsin, subtilisin, trypsin, and elastase, possesses a surprising amount of homologous features. This includes similarities in crystal structure, active-site geometry, extended binding site, amino-acid sequence, catalytic mechanism, and biological function. However, the enzymes may be separated and classified according to the type of peptide bonds which they preferentially hydrolyze. The differences among the serine proteases arise principally from the variations in substrate specificity for particular kinds of amino acids at the substrate cleavage po-

sition. Each enzyme contains a pronounced pocket adjacent to the catalytic residues having special dimensions and steric and electrostatic properties. This binding pocket, or "tosyl hole" (Steitz et al, 1969) at the enzymes's S_1 subsite (notation of Schechter and Berger, 1967) determines its primary substrate specificity. The enzymes show structural differences at their primary binding sites to accommodate different kinds of substrate P_1 residues. These enzyme-substrate interactions are illustrated in Figures 1A and 2. Thus, in chymotrypsin, the binding pocket is hydrophobic and slot-shaped to envelop fully a phenyl group, which results in specificity for residues with aromatic side chains, e.g. Phe, Tyr, and Trp. In trypsin, at the rear of the binding pocket is located the anionic carboxylate group of Asp-177, which results in specificity for residues with positively-charged side chains, e.g. Lys and Arg. In elastase, the binding pocket is partially filled with the alkyl group of Val-216 and hence is smaller in size, which results in specificity for residues with small alkyl side chains, e.g. Ala, Ser, and Val. Finally, in subtilisin, the binding pocket is generally more flexible and larger in size, which results in wider specificity for residues with aromatic or large alkyl side chains, e.g. Phe and Leu. This information is based on x-ray structural studies of bound enzyme derivatives. It should be noted that an enzyme's overall specificity is determined by the total interaction

with a substrate including binding contacts along an extended binding site to aid in binding the peptide portion of the substrate thru hydrogen-bonding.

Chymotrypsin is a very well-characterized serine protease, and most of the work on this enzyme has been carried out on bovine pancreatic chymotrypsin A (EC 3.4.21.1). It is secreted by the pancreas in zymogen form, chymotrypsinogen which is activated to chymotrypsin by tryptic splits. The essential active-site residues in the charge relay system are Ser-195, His-57, and Asp-102. X-Ray crystallographic investigations have determined the three-dimensional structure of tosyl-chymotrypsin and native chymotrypsin A (Blow et al, 1969; Blow, 1971; Birktoft and Blow, 1972). Further investigations have studied the three-dimensional structure of chymotrypsin A with a variety of bound substrates and inhibitors (Steitz et al, 1969; Henderson, 1970; Segal et al, 1971a,b). The mechanism of chymotrypsin activity has become almost conclusively established thru a combination of structural and kinetic studies (Henderson et al, 1971; Hess, 1971). Because of extensive homology this mechanism is believed to be operative among the entire serine protease family. As mentioned before, chymotrypsin exhibits a substrate specificity for aromatic residues, and the phenyl group of a substrate P_1 residue fits snugly into the enzyme's binding pocket. The affinity for the P_1 residue comprises the primary substrate specificity. This binding pocket or

"tosyl hole" is a depression ca. 12 x 6.5 x 4 Å in size on the enzyme surface. It is surrounded mainly by sections of the enzyme's peptide backbone and the side chains of few interior residues e.g. Ser-189 and Ser-190 (Steitz et al, 1969) (Figure 2). Near the entrance to the binding pocket is located Met-192, an exterior residue susceptible to modification. The solid-state structure of chymotrypsin also correlates well with its solution kinetic properties (Kurachi et al, 1973).

The term "enzyme engineering" may be applied to describe the specific chemical transformation of certain regions of an enzyme in order to change some aspect of its activity or function. The concept involves the creation of a new, different, active enzyme from a suitable existing enzyme by means of selective chemical modification. In general the purpose is to modify a key but non-essential secondary site, other than the active site, which could influence enzyme function. One practical application of this idea is the alteration of particular residues at the primary binding site while preserving the integrity of the catalytic residues at the active site. The desired result is the formation of a new enzyme having an altered substrate specificity yet with a retained catalytic activity. This route was taken experimentally in an attempt to engineer a new kind of enzymatic species originating from chymotrypsin. Such an enzyme would be not only interesting as a synthetic protein

but also useful as a catalyst for an appropriate biochemical reaction, e.g. the specific hydrolysis of a polypeptide. The possibility of altering the functional properties of proteins has relevance for both chemical and medical studies. As already stated, x-ray investigations etc. have demonstrated much homology among the serine proteases chymotrypsin, trypsin, subtilisin, and elastase. Hence, it is reasonable to expect that modifying the binding pocket of chymotrypsin would merely create a new family member, i.e. a species keeping its catalytic activity but possessing an altered substrate specificity. An assumption inherent in this type of modification is that no major conformational change would occur causing a significant change in proteolytic activity. However a slight change in catalytic abilities would probably occur, e.g. a small decrease in k_{cat}, concurrent with the alteration of specificity.

There have been relatively few previous studies whose intent was to modify the binding or specificity site of an enzyme, apart from irreversible inhibition. Moderate changes occurred in the binding and catalytic properties of chymotrypsin upon alteration of Met-192 at the entrance to the binding pocket. The changes resulted from oxidation of Met-192 to the sulfoxide by periodate, and alkylation of Met-192 by p-nitrophenyl N-bromoacetyl-α-aminoisobutyrate as well as by 2-bromomethyl-3,1-benzoxazin-4-one (Weiner et al, 1966; Kezdy et al, 1967; Alazard et al, 1973). It was

found that the binding constants K_M for specific substrates were usually increased, and upon alkylation the enzymatic acylation rates k_{cat} and deacylation rates were affected also. Related experiments have been performed with trypsin upon alteration of the carboxyl group of Asp-177 at the bottom of its binding pocket. The modification of Asp-177 was carried out by an N-ethyl-5-phenylisoxazolium salt, triethyloxonium fluoroborate, glycinamide plus carbodiimide, and p-nitrophenyl p'-(S,N^3-dimethylisothioureido) benzoate (Feinstein et al, 1969; Nakaya et al, 1970; Eyl and Inagami, 1971; Bodländer and Shaw, 1971). In most of these experiments the intent was analytical, with virtually no evaluation of the enzyme's altered binding or catalytic behavior. In a related project, the active-site residue of subtilisin was converted from serine (an alcohol) to cysteine (a thiol of resemblant properties), with the result that thiol-subtilisin lost its catalytic activity but not its binding ability towards normal substrates (Neet et al, 1968).

Another kind of reagent useful for localized modification includes the carbenes and nitrenes, photo-generated in situ from an appropriate enzyme-bound precursor. The photolysis of diazoacetyl-chymotrypsin A led to carboxymethylation by intramolecular carbene insertion into His, Ser, and Tyr probably at the active site, but only to a 3% extent (Hexter and Westheimer, 1971). Likewise, the photolysis of a phenylazido reagent bound to chymotrypsin A led

to tagging of undetermined residues probably in the binding pocket (Knowles, 1972). Although these reagents are unusually reactive they are not especially specific, and thus far they have led to the creation of no worthwhile new selectively modified enzymes.

It appeared that certain aryl cyanates might be likely reagents for use as potential specific-site chymotrypsin modifiers. These compounds have gained utility as moderately reactive electrophilic reagents in the field of synthetic organic chemistry (Grigat and Pütter, 1967; Grigat, 1972). Aryl cyanates or cyanic esters, ArOCN, react with nucleophiles to form iminocarbonic esters which may be hydrolyzed to carbamates as outlined in eq 1. One simple aryl cyanate, p-nitrophenyl cyanate, has also been

$$\text{Ar-OCN} + \text{H-X} \xrightarrow{\text{pH} < 7} \text{ArO}\overset{\overset{\text{NH}}{\|}}{\text{C}}\text{X} \xrightarrow{\text{H}_2\text{O}} \text{ArOH} + \text{NH}_2\overset{\overset{\text{O}}{\|}}{\text{C}}\text{X} \qquad (1)$$

used as an effective enzyme modification reagent. The reaction of chymotrypsin A with p-nitrophenyl cyanate led to the formation of mono- and dicarbamyl enzyme derivatives which were used in x-ray structural studies on the properties of acyl-enzymes (Robillard et al, 1972). This reagent was found to inhibit chymotrypsin rapidly and irreversibly by selective attack at only two sites including the catalytic residue Ser-195. Another related type of compound, an alkyl isocyanate, was also found to be an active-site-directed

irreversible inhibitor of chymotrypsin (Brown and Wold, 1973). The precedent showed that aryl cyanates were reasonably reactive and fairly specific as protein modification reagents.

The initial purpose of this investigation was synthetic in character--to design and create a new enzyme with a deliberately altered substrate specificity. In theory there are several basic approaches which may be taken to achieve this result. The steric requirements or the electrostatic nature (or both) of the primary binding site may be changed by placing a sizeable or polar group, respectively, inside the binding pocket. Likewise, the steric and electrostatic properties of a secondary binding site may be changed in a similar manner by chemical refinements at a distant binding site. In practice our goal was the selective modification of the binding pocket of chymotrypsin A by attaching a carbamyl group to the hydroxyl oxygen of Ser-189 or Ser-190 near the back of the pocket. A carbamyl group in this location was expected to alter both the space-filling and hydrogen-bonding character of the pocket toward substrates. We selected Ser-189 and Ser-190 inside the binding pocket as a target site for modification since they were the only feasible reactive groups to manipulate in the vicinity. Met-192 at the opening of the pocket is a target for several protein reagents but its modification cannot alter specificity in an easily regulated way.

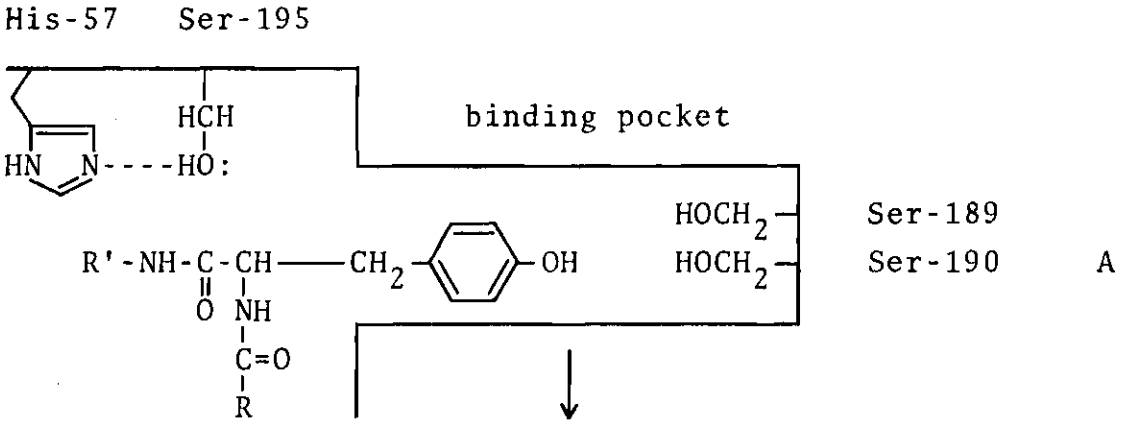
Our aryl cyanate reagent, Ac-Phe(OCN)-OEt (N-acetyl-

p-cyanato-L-phenylalanine ethyl ester), was designed to interact with chymotrypsin in a manner both reactive and specific toward the residues lining the binding pocket. In the synthesis of the substrate analog Ac-Phe(OCN)-OEt, the hydroxyl group of the substrate Ac-Tyr-OEt is replaced by a cyanate group (Figure 3). Thus Ac-Phe(OCN)-OEt is rendered both reactive and specific for chymotrypsin by incorporating the features of an aryl cyanate group plus a substrate-like structure. It was supposed to be reactive enough to interact with the unreactive binding-pocket serine groups, yet specific enough to retain intact the active-site residues. The desired outcome was the facile and single modification of a binding-pocket residue in chymotrypsin by carbamylation. The reagent was expected to bind to the enzyme with a K_M (or K_I) value similar to K_M for the substrate Ac-Tyr-OEt. Upon binding of Ac-Phe(OCN)-OEt, the cyanate group would be positioned deep within the binding pocket and rather close to Ser-189 and/or Ser-190 located back there. The low reactivity of these serine groups would be counteracted by the proximity of the reactive cyanate group. Under ideal conditions, a reaction would ensue between chymotrypsin A and reagent to yield the iminocarbonate intermediate followed by hydrolysis to the desired O-carbamyl-Ser-189 (or -190) chymotrypsin, modified by carbamylation of Ser-189 (or -190) alone. Hopefully the active-site Ser-195 and catalytic activity would remain essentially intact.

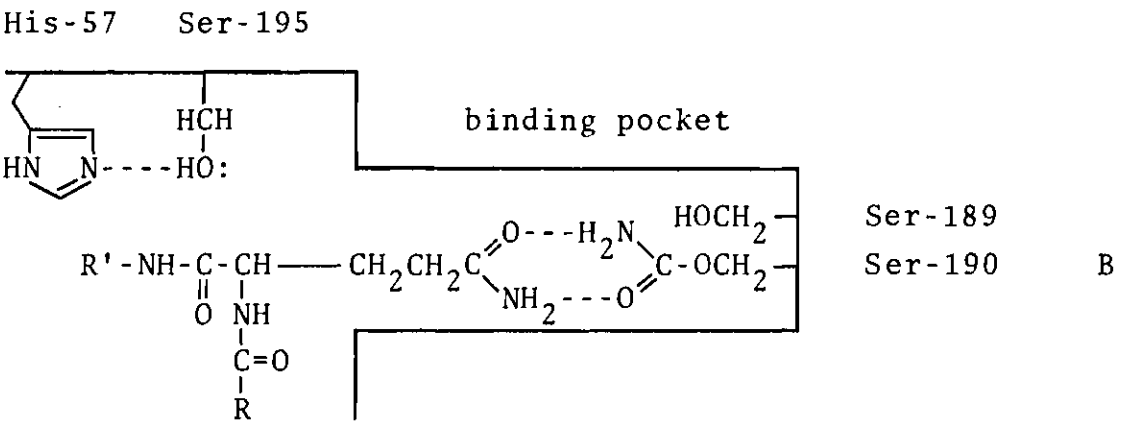
If this kind of "new" enzyme could be easily made, it was anticipated to possess a different substrate specificity due to the effect of the carbamyl group in the binding pocket. Both the steric and electrostatic character of the S_1 binding subsite would presumably be altered. The expected outcome should be the transformation of chymotrypsin into an elastase-like protease having a specificity for residues with small polar or alkyl side chains, e.g. Ser and Ala. The range of specificity would be determined by kinetic studies with a series of substrates and inhibitors. For example, the specificity might shift toward the two amide residues, Asn and Gln, because of the proper hydrogen-bonding interactions they make with the carbamyl group (Figure 1B). Otherwise, the specificity might shift toward small polar alkyl residues, e.g. Ser and Thr, due to the requirements for a sterically smaller and possibly polar side chain. If this result indeed happens then the first trial work in enzyme engineering will be successfully completed.

This Thesis, Part II, is a report on the synthesis and reactivity of the aryl cyanate reagent Ac-Phe(OCN)-OEt with chymotrypsin A and its use as an enzyme modifier. The modified enzyme derivatives have been characterized in terms of the resulting site, type, extent, and effect of reaction. The work will be published a short time after the thesis date, as a complete article (Powers, Tuhy, and Witter, 1975). The principal finding is that Ac-Phe(OCN)-OEt is an effective

inhibitor of the normal catalytic activity of chymotrypsin, but this is caused by the inactivation of the active-site Ser-195 rather than the modification of a binding-pocket serine group. Some explanations and implications for enzyme engineering are covered, which may lead to eventual mastery of this potentially powerful enzyme modification technique.

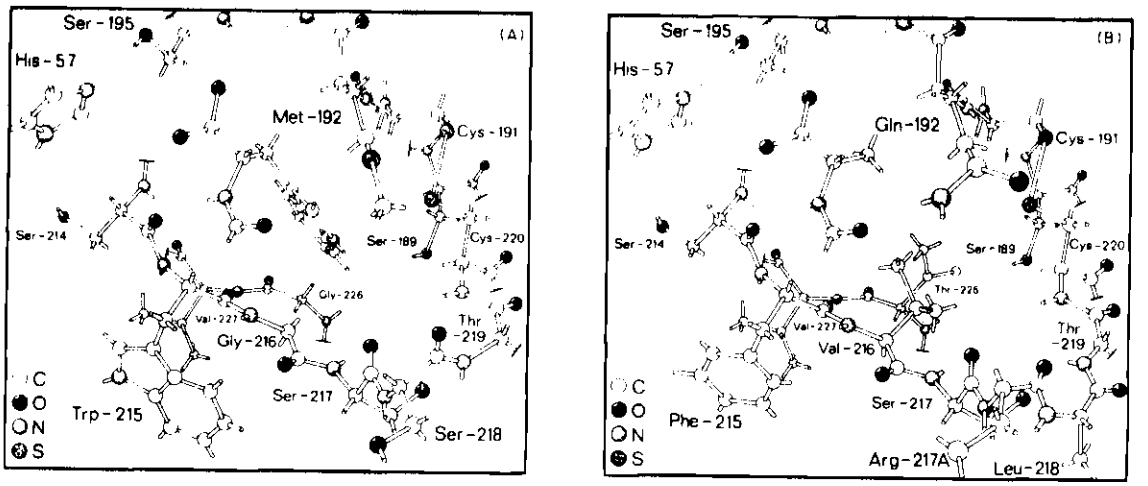


P_1 = Tyr. Normal Substrate Bound to Unmodified Enzyme.



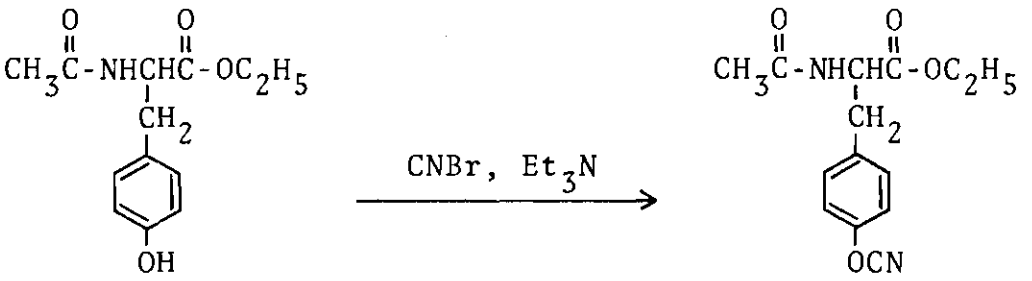
P_1 = Gln. Different Substrate Bound to Modified Enzyme.

Figure 1. Diagram of Chymotrypsin A Showing the Active Site and Binding Site Regions. Note: A comparison of the two enzymatic binding schemes in (A) native chymotrypsin, and (B) chymotrypsin modified by carbamylation of a binding pocket residue (proposed).



A B

Figure 2. Perspective Drawings of Serine Protease Models Showing the Active Site Region and the Binding Pocket Site. Note: (A) Chymotrypsin A with bound formyl-tryptophan; (B) Elastase with similarly arranged formyl-alanine. Mark how the size and shape of the substrate P_1 side chain matches the available space in the binding pocket of the enzyme. These drawings were deduced from x-ray crystallographic studies. From Hartley, B.S., and Shotton, D.M. (1971), in The Enzymes, 3rd Ed., 3, 363.



Ac-Tyr-OEt

Ac-Phe(OCN)-OEt

Figure 3. Structure and Preparation of the Aryl Cyanate Reagent Ac-Phe(OCN)-OEt. Note: This is N-acetyl-p-cyanato-L-phenylalanine ethyl ester, a chymotrypsin inhibitor.

CHAPTER II

METHODS

Reagents and Materials. Chymotrypsin A_α (lot CDI1BK) was obtained from Worthington Biochemical Co. and used without further purification; its substrate, Ac-Tyr-OEt, was synthesized by standard methods and had mp 80-81°. Chymotrypsinogen A (lot 111C-8170) and acetyl-trypsin (lot 90C-2200) were obtained from Sigma Chemical Co. and used without further purification. Diaflo ultrafilters used in enzyme concentration were purchased from Amicon Corp. Carboxymethyl cellulose, CM-52 microgranular, was purchased from Whatman Biochemicals Ltd. Sephadex G-25 and Sepharose 4B were obtained from Pharmacia Fine Chemicals. Potassium [^{14}C] cyanide (11.5 mCi/mmole, lot 553-199) and [^{14}C] diisopropyl fluorophosphate (DFP) (110 mCi/mmole, lot 669-016) were obtained from New England Nuclear. Cyanogen bromide, p-nitrophenol, 4-phenylbutylamine, and all other reagents and solvents used were analytical grade. Mass spectra were taken on a Perkin-Elmer Hitachi RMU-7L instrument and nuclear magnetic resonance (nmr) spectra were taken on a Varian T-60 instrument. Enzyme inhibition kinetics were performed on a Radiometer automated pH-stat system (model TTT 11).

p-Nitrophenyl Cyanate, NO_2-Ph-OCN. This compound -

was synthesized from p-nitrophenol (0.70 g, 5 mmol) and cyanogen bromide (0.53 g, 5 mmol) according to the method of Robillard et al (1972). The product was recrystallized from carbon tetrachloride to give 0.54 g (65%) of a light yellow solid, mp 75-76°; Robillard et al (1972) report mp 75-77°. The mass spectrum showed a major peak at m/e 164 (M^+), and the ir spectrum (nujol mull) showed a cyanide band at 2260 and 2290 cm^{-1}.

N-Acetyl-(p-cyanato)-L-phenylalanine Ethyl Ester, Ac-Phe(OCN)-OEt. Potassium cyanide (0.32 g, 5 mmol) was dissolved in 5 ml of water and added dropwise to bromine (0.26 ml, 5 mmol) under 0.5 ml water at 10°, ensuring that the bromine color was completely discharged. The cyanogen bromide was extracted into 4 x 5 ml of ether and dried over magnesium sulfate. A solution of Ac-Tyr-OEt (1.35 g, 5 mmol) in 20 ml of acetone was stirred at 0°, while a solution of cyanogen bromide (ca. 5 mmol) in 20 ml of ether was added. Triethylamine (0.70 ml, 5 mmol, in 5 ml acetone) was added dropwise over a 0.8-hr period, 5 ml of ether was then added, and the mixture stirred at 0° for 1 hr. After filtration of the reaction mixture, the filtrate was evaporated in vacuo at 25°. Treatment of the yellow oil twice by adding anhydrous ether, stirring, filtering, and evaporating the ether yielded a crude white solid. The product was crystallized with difficulty from ethyl acetate-cyclohexane (3:5-3:8) to give 0.42 g (30%) of a white solid, mp 86-87°, $[\alpha]_D^{25}$ = +16°

(c 1, C_2H_5OH). The mass spectrum showed major peaks at m/e 276 (M^+) as well as 217, 192, 189, 161, 132, 107, 102, 91 (undesignated), and the ir spectrum (nujol mull) showed a cyanide band at 2240 and 2280 cm^{-1}. The nmr spectrum ($CDCl_3$) had peaks at ∂ 7.3 (4H, s, C_6H_4), 6.3 (1H, d, NH), 4.7 (1H, m, CH), 4.2 (2H, q, CH_2CH_3), 3.2 (2H, d, CH_2Ph), 2.0 (3H, s, CH_3CO), and 1.3 (3H, t, CH_2CH_3). The uv absorption (C_2H_5OH) showed a doublet peak at 272 and 265 nm. Anal. Calcd for $C_{14}H_{16}N_2O_4$: C, 60.86; H, 5.84; N, 10.14. Found: C, 61.16; H, 5.92; N, 10.22.

[^{14}C] Ac-Phe(OCN)-OEt was prepared by an identical procedure, beginning with potassium [^{14}C] cyanide (5 mmol) (specific activity ca. 0.1 mCi/mmole). The product yield was 9.46 g (33%) of a white solid, with a specific activity of 0.088 mCi/mmole.

Enzyme Activity Assay. Chymotrypsin activity was measured by the potentiometric assay method using Ac-Tyr-OEt as the substrate (Wilcox, 1970) with 0.5 N NaOH as titrant.

Kinetics of Inhibition. The reaction of chymotrypsin with Ac-Phe(OCN)-OEt was carried out under pseudo-first-order conditions in an inhibition mixture containing enzyme, inhibitor, and substrate together. The inhibition kinetics were monitored on the pH-stat. Stock solutions were prepared as follows: Chymotrypsin, 8 μM or 0.2 mg/ml -by uv absorbance in 1 mM HCl; Ac-Phe(OCN)-OEt, 60 mM in dioxane; and Ac-Tyr-OEt, 1 M in dioxane. To 10 ml of a solution 10

mM in $CaCl_2$ and 3% (v/v) in methanol, was added 0.1 ml each of inhibitor and substrate solutions, and the pH was adjusted to 7.5 with 0.5 N NaOH. The inhibition reaction was started by adding 0.1 ml of enzyme solution to the mixture, and proceeded for 6-8 min at 25° and pH 7.5. Initial concentrations were as follows: chymotrypsin, 0.08 μM; Ac-Phe-(OCN)-OEt, 0.6 mM; Ac-Tyr-OEt, 10 mM; organic solvents, 5% (v/v); in a total volume of 10 ml. Due to the presence of substrate, a continuous monitoring of the reaction was provided on the pH-stat in the form of a decreasing exponential curve which yielded kinetic results. Control experiments were carried out with enzyme plus inhibitor, enzyme plus substrate, and inhibitor plus substrate under identical conditions. With the aid of computer programs the values of kinetic parameters, including k_3/K_I, were determined for the inhibition reaction, using as a basis the kinetic treatment of Hart and O'Brien (1973). Standard deviations of less than 5% were maintained throughout.

CM-Cellulose Chromatography. A 70 x 1.2 cm column of carboxymethyl cellulose CM-52 was re-equilibrated before each run with 1 ℓ of 0.05 M potassium phosphate (pH 6.2). All operations were performed at 4°. The protein sample was pumped onto the column and eluted at 0.5 ml/min with a linear salt gradient of increasing ionic strength. This gradient consisted of 0.6 ℓ each of 0.05 M and 0.12 M potassium phosphate (pH 6.2).

Affinity Chromatography. An affinity column support of Sepharose-4-phenylbutylamine (PBA) was prepared according to the method of Stevenson and Landman (1971) with slight modification. This is described more completely in the Appendix. The 50 x 1.2 cm column of Sepharose-PBA was re-equilibrated before each run with 1 ℓ of 0.1 M tris (pH 8.0). All operations were performed at 4°. The protein sample was pumped onto the column and eluted at 0.6 ml/min with 0.1 M tris (pH 8.0) using a peristaltic pump. Protein bound to the column was removed by eluting with 0.1 M tris (pH 8.0) containing 1 M tetraethylammonium bromide.

Reaction of Chymotrypsin A_α with p-Nitrophenyl Cyanate. O-Carbamyl-Ser-195 chymotrypsin A_α was prepared based on the general method of Robillard et al (1972). Lyophilized chymotrypsin A_α (100 mg, 4 μmol) was dissolved in 10 ml of 0.05 M acetate buffer (pH 4.5) at 25°. A solution of p-nitrophenyl cyanate (0.98 mg, 6 μmol, 1.5 equiv) in 0.30 ml of dioxane was added in increments to the enzyme solution. After the inhibition reaction proceeded for 1 hr at 25° and pH 4.5, the enzymatic activity decreased to 6.5%. The pH was raised to 7.6 and α-toluene-sulfonyl fluoride (0.70 mg, 4 μmol, 1 equiv) in 0.25 ml of dioxane was added to reach full inhibition; after 1 hr at 25° and pH 7.6, chymotrypsin was 100% inactivated. Excess reagent was removed by dialysis at pH 3. The protein mixture was promptly subjected to CM-cellulose chromatography and two major known derivatives,

O-carbamyl-Ser-195 chymotrypsin A_α (1a) and a disubstituted chymotrypsin A_α (1b), were obtained in a 50:50 ratio (Figure 4A). After concentration the proteins were dialyzed at pH 3. O-Carbamyl-Ser-195 chymotrypsin A_α (1a) was chromatographed on the affinity column in a second purification step (Figure 5A) and was isolated as 9.0 mg (9%) of a lyophilized solid.

Reaction of Chymotrypsin A_α with Ac-Phe(OCN)-OEt. Lyophilized chymotrypsin A_α (150 mg, 6 μmol) was dissolved in 10 ml of 0.05 M citrate buffer (pH 6.5) and cooled to 0°. A solution of Ac-Phe(OCN)-OEt (9.9 mg, 36 μmol, 6 equiv) in 0.30 ml of dioxane was added in increments to the enzyme solution. After the inhibition reaction proceeded for 2.5 hr at 0° and pH 6.5, the enzymatic activity decreased to 1.5%. Excess reagent was removed by dialysis at pH 3. The protein mixture was promptly subjected to CM-cellulose chromatography and two major derivatives, designated 2a and 2b, were obtained in a 60:40 ratio (Figure 4B). After concentration the proteins were dialyzed at pH 3. The two derivatives were chromatographed separately on the affinity column in a second purification step (Figure 5B) and were finally isolated as lyophilized solids, 28 mg of 2a and 16 mg of 2b.

The reaction of chymotrypsin A_α with [^{14}C] Ac-Phe-(OCN)-OEt was run in an almost identical manner. Here 7 equiv of [^{14}C] Ac-Phe(OCN)-OEt was used and the inhibition

reaction proceeded for 4 hr to reach 1.5% enzymatic activity. The two derivatives were isolated as lyophilized solids, 36 mg of [^{14}C] 2a and 16 mg of [^{14}C] 2b.

Preparation of N-Carbamyl-Cys-1 Chymotrypsin A_∂. N-Carbamyl-Cys-1 chymotrypsinogen A was synthesized on a 24 μmole scale from chymotrypsinogen A and 5 equiv of p-nitrophenyl cyanate according to the method of Robillard et al (1972). This known intermediate was isolated as a lyophilized solid. N-Carbamyl-Cys-1 chymotrypsinogen A was activated by treating the zymogen (300 mg, 50 μmol, in 14 ml) with acetyl-trypsin (12 mg in 1 ml) in 10 mM $CaCl_2$, for 1.5 hr at 0° and pH 7.6. The chymotryptic activity attained 110% by standard assay. The protein mixture was immediately subjected to CM-cellulose chromatography and a single major product was obtained in one batch which contained 70% of the protein applied. After dialysis at pH 3 the enzyme solution was concentrated to 10 mg/ml and used in subsequent inhibition reactions.

Reaction of N-Carbamyl-Cys-1 Chymotrypsin A_∂ with Ac-Phe(OCN)-OEt. Chromatographed N-carbamyl-Cys-1 chymotrypsin A_∂ (105 mg, 4 μmol) in 10 ml of solution was adjusted to pH 6.5 by adding 1 ml of 0.5 M citrate buffer (pH 6.5) and cooled to 0°. A solution of Ac-Phe(OCN)-OEt (6.8 mg, 24 μmol, 6 equiv) in 0.30 ml of dioxane was added in increments to the enzyme solution. After the inhibition reaction proceeded for 3 hr at 0° and pH 6.5, the enzymatic activity de-

creased to 1.6%. Excess reagent was removed by dialysis at pH 3. The protein mixture was promptly subjected to CM-cellulose chromatography, and a single major derivative, designated 3, was obtained in a peak with a slight left shoulder (Figure 4C). The elution gradient consisted of 0.6 ℓ each of 0.075 M and 0.10 M potassium phosphate (pH 6.2). After concentration the protein was dialyzed at pH 3. Derivative 3 was chromatographed on the affinity column to aid in its characterization (Figure 5C) and was isolated as a lyophilized solid, 30 mg of 3.

The reaction of N-carbamyl-Cys-1 chymotrypsin A_∂ with [^{14}C] Ac-Phe(OCN)-OEt was run in an almost identical manner. Here 6 equiv of [^{14}C] Ac-Phe(OCN)-OEt was used and the inhibition reaction proceeded for 3 hr to reach 1.6% enzymatic activity. The lone derivative was isolated as a lyophilized solid, 28 mg of [^{14}C] 3.

Amino Acid Analyses. A total acid hydrolysis was carried out prior to analysis by hydrolyzing 5 mg of protein sample in 2 ml of 6 N HCl for 25 hr at 110° in a deaerated, evacuated tube. The hydrolyzate was evaporated and stored in aqueous solution. Amino acid analyses were performed by Dr. Kenneth Hapner at Montana State University and Dr. Kenneth Stevenson at the University of Calgary, Alberta.

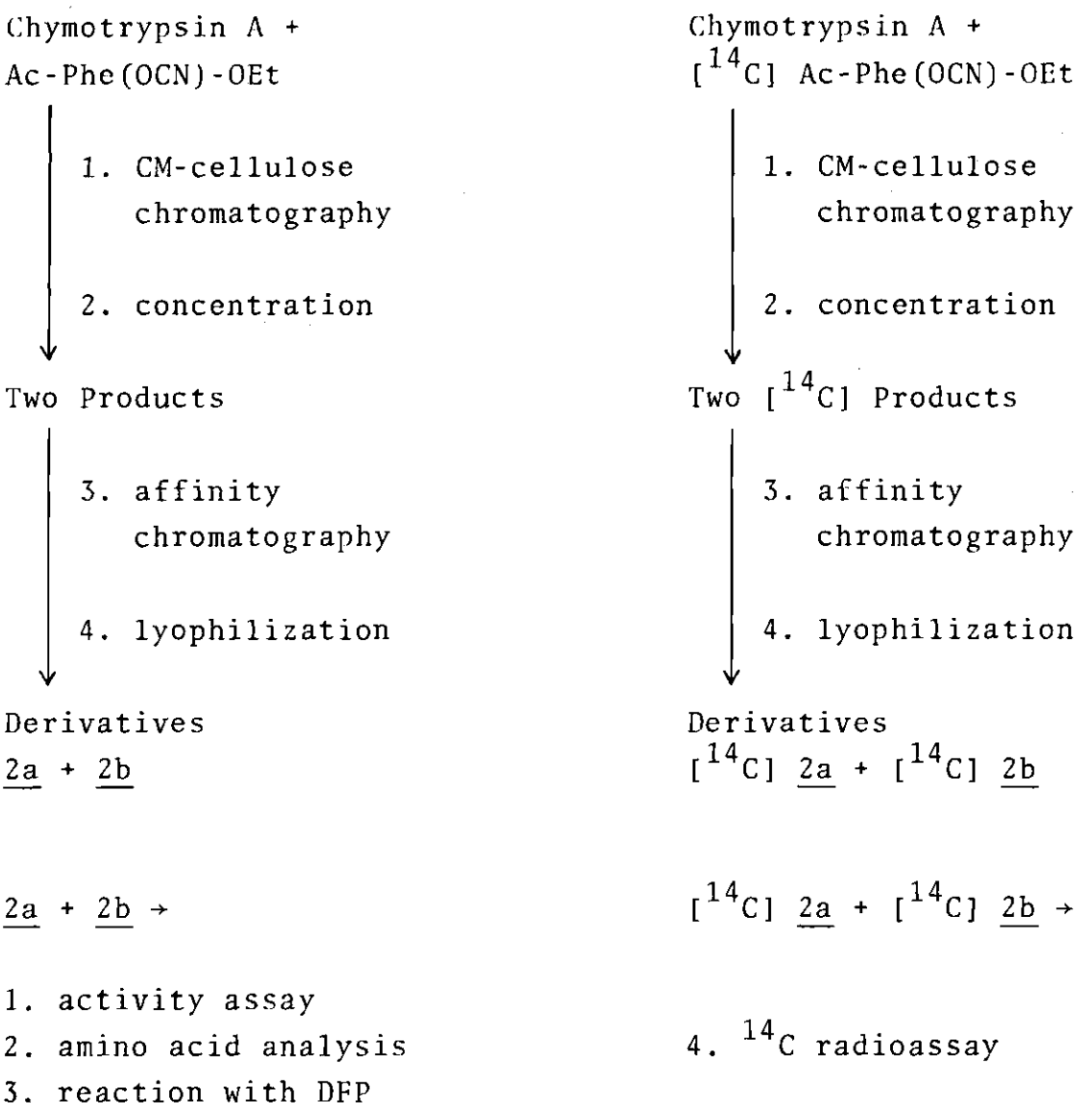
^{14}C Specific Activity Radioassay. The specific activity of ^{14}C-labeled reagents and proteins was determined by measuring the ^{14}C content of a sample using a Packard

Tri-Carb liquid scintillation spectrometer (model 3375), and relating the ^{14}C content to the sample concentration as measured by weight basis (reagents) or uv absorbance at 282 nm (proteins). An aqueous sample solution (1 ml) was added to 10 ml of scintillant and duplicate sample groups were counted at 4°; for 3, a counting rate of 5400 cpm/mg was typically observed. The scintillant used for the radioassays contained 7.50 g of 2,5-diphenyloxazole (PPO), 375 mg of 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP), and 125 g of naphthalene made up to 1.0 ℓ 1,4-dioxane. A counting efficiency of ca. 80% was always maintained as indicated by a [^{14}C] toluene internal standard.

Reaction of Chymotrypsinogen A and Enzyme Derivatives with [^{14}C] DFP. Diisopropylphosphoryl-(DIP-) chymotrypsinogen A was prepared based on the general method of Morgan et al (1972). [^{14}C] Diisopropyl fluorophosphate (DFP) possessed a nominal specific activity of 0.188 mCi/mmole. [^{14}C] DFP (7.3 mg, 40 μmol, 100 equiv) in ethylene glycol was added to a solution of chymotrypsinogen A (10 mg, 0.4 μmol) in 1 ml of 0.25 M KCl-0.05 M $CaCl_2$. The inhibition reaction was stirred for 12 hr at 25° and pH 7, the pH being kept at 7 by periodic addition of 1 N KOH. All excess [^{14}C] DFP was removed by gel filtration on a 50 x 1.2 cm column of Sephadex G-25 equilibrated with 0.02 M formate (pH 3.8). DIP-Chymotrypsinogen was isolated as 7 mg of a lyophilized solid and tested separately for ^{14}C content and activatibility.

Chymotrypsinogen A and DIP-chymotrypsinogen were activated by treating the zymogen (2.8 mg, 0.56 μmol, in 4.5 ml) with acetyl-trypsin (0.26 mg in 0.5 ml) for 2 hr at 0° in 0.1 M tris (pH 7.6). The chymotryptic activity of the former attained 110% by standard assay.

The reactions of chymotrypsin derivatives 2a and 3 with [^{14}C] DFP were run in an identical manner under these vigorous conditions. The two assumed DIP-products were isolated and tested for ^{14}C content, employing the [^{14}C] DIP-chymotrypsinogen as a standard. They were also expected to become or remain enzymatically inactive.



Scheme 1. Experimental Isolation and Analysis of 2a and 2b.

CHAPTER III

RESULTS

Inhibition of Chymotrypsin by Ac-Phe(OCN)-OEt. The aryl cyanate reagent Ac-Phe(OCN)-OEt was synthesized by reaction of Ac-Tyr-OEt with cyanogen bromide in a straightforward one-step cyanation procedure in 30% yield. Its structure was confirmed by mass spectrum, ir, nmr, uv, and combustion analysis and it was assumed to retain its optical purity. In preliminary experiments Ac-Phe(OCN)-OEt was discovered to inactivate chymotrypsin rapidly and irreversibly. It turned out to be a reasonably effective stoichiometric inhibitor of chymotrypsin, presumably by interaction of the cyanate group with the enzyme. The inhibition reaction is pH-dependent and proceeded with maximum velocity at pH 6 to 7, while the velocity dropped sharply outside pH 5 to 8. The reaction conditions could be controlled in a way to produce modified enzyme derivatives on a preparative scale.

Kinetics of Inhibition. A three-component reaction system consisting of chymotrypsin, inhibitor, and substrate together was employed to determine the inhibition kinetics due to the rapidity of the inhibition reaction. This method is based on the theoretical derivations of Main and Dauterman (1963) and Hart and O'Brien (1973). If the inhibition re-

action is preceded by a binding step with the formation of a reversible enzyme-inhibitor complex, then the overall pathway is expressed by eq 2. However, if the inhibition

$$E + I + S \begin{array}{l} \overset{K_S}{\rightleftharpoons} E\cdot S \xrightarrow{k_{cat}} E + P \\ \underset{K_I}{\rightleftharpoons} E\cdot I \xrightarrow{k_3} E\text{-}I \end{array} \qquad (2)$$

$$E + I + S \begin{array}{l} \overset{K_S}{\rightleftharpoons} E\cdot S \xrightarrow{k_{cat}} E + P \\ \searrow^{k_{2nd}} E\text{-}I \end{array} \qquad (3)$$

reaction is a simple bimolecular process without the formation of an enzyme-inhibitor complex, the overall pathway is expressed by eq 3. Here E·I and E·S are the noncovalent enzyme complexes with inhibitor and substrate, E-I is the covalently and irreversibly inhibited enzyme derivative, K_I and K_S are the dissociation constants for E·I and E·S, k_3 is the limiting rate of inactivation, and k_{2nd} is the second-order rate of inhibition. The "inhibition constant" k_3/K_I from eq 2 is the comparable quantity to k_{2nd} from eq 3. Pseudo-first-order kinetics are achieved if both the initial inhibitor and substrate concentrations are much greater than the total enzyme concentration. In this case the kinetic constants k_3/K_I, k_{2nd}, and K_I are given by eq 4-6. Here k_{obsd} is the observed first-order rate of inhibition, $\alpha = [S]/(K_S + [S])$, K_M is an approximation for K_S, and V_o and V_c are the initial velocities of substrate hydrolysis with

$$\frac{k_3}{K_I} = k_{obsd} \frac{V_c/V_o}{[I](1-\alpha)} \quad (4)$$

$$k_{2nd} = k_{obsd} \frac{1}{[I](1-\alpha)} \quad (5)$$

$$K_I = \frac{[I](1-\alpha)}{V_c/V_o - 1} \quad (6)$$

and without inhibitor present respectively. If $V_o < V_c$ the inhibition reaction proceeds via a pre-equilibrium process as in eq 2, and values for k_3/K_I, k_3, and K_I may be found. However, if $V_o \cong V_c$ the inhibition reaction is a bimolecular process as in eq 3, and only a value for k_{2nd} may be found. It is apparent that the limit of k_3/K_I is k_{2nd} as V_o approaches V_c, i.e. as V_c/V_o goes to unity.

The inhibition experiments were run under pseudo-first-order conditions in which $[I]/[E] \geq 7500$, and proceeded thru at least three half-lives. A value of K_M = 2.0 mM for Ac-Tyr-OEt was observed with our set of conditions. A control experiment with enzyme plus inhibitor alone showed that the ester group of Ac-Phe(OCN)-OEt was not undergoing appreciable hydrolysis. Other control experiments yielded values for V_c and the slow rate of background hydrolysis. In the treatment of the pH-stat data the exponential curve was fitted to a general formula (eq 7) using a non-linear (exponential) curve-fitting computer program, thereby find-

$$f(t) = a + be^{t \ln r} \quad (7)$$

ing values for a, b, and r (where $0 < r < 1$). The intermediate parameters k_{obsd} and V_o are directly related to a, b, and r, namely, $k_{obsd} = -\ln r$ and $V_o = -a \ln r$. Finally, k_{obsd} and V_o were substituted into eq 4-6 to determine values for k_3/K_I, k_{2nd}, and K_I as appropriate. The lower limit of a measurable difference between V_o and V_c was set at $V_c/V_o = 1.05$, which corresponds to an upper limit for calculable K_I at $K_I = 2.0$ mM for the inhibitor. It was observed that $V_c/V_o < 1.05$ but not $V_c/V_o = 1.00$.

For the inhibition reaction involving chymotrypsin (0.08 μM) and Ac-Phe(OCN)-OEt (0.6 mM) at 25° and pH 7.5, the kinetic constants $k_{2nd} = 4.4 \times 10^3$ $M^{-1}min^{-1}$ and half-life = 1.6 min were obtained. These kinetic results are listed in Table 1 and represent the average values. No K_I value could be reliably obtained but if existent it must be greater than 2 mM. Increasing the upper limit for calculable K_I would have required raising the inhibition concentration [I] and the reaction rate k_{obsd} beyond the range for practical kinetic analysis. The reaction is most likely a direct bimolecular process without an initial binding step, since V_c/V_o is approximately equal to 1.0. Alternatively, the reaction may proceed *via* a pre-equilibrium process but the enzyme-inhibitor complex would be relatively weak, *i.e.* $K_I > 2$ mM.

Reaction of Chymotrypsin A_α with Ac-Phe(OCN)-OEt. - Relatively mild reaction conditions were used for the preparative work to decrease the hydrolysis rate of the inhibitor's cyanate group to a carbamate and to minimize non-specific side reactions. Three criteria were stressed: low temperature, moderate pH, and a fairly small excess of inhibitor. Nevertheless, the conditions were sufficient to achieve 98% inhibition within 2.5 hr. Two modified enzyme derivatives were obtained, 2a (60%) and 2b (40%) as the only major products, and were separated by CM-cellulose chromatography and purified by affinity chromatography. They were prepared in both unlabeled and ^{14}C-labeled form in 15-30 mg batches. Derivative 2a was consistently isolated as the predominant product and was eluted from the CM-cellulose column at a somewhat higher $[K^+]$ region than 2b (Figure 4B). By analogy with O-carbamyl-Ser-195 chymotrypsin A_α (1a) and the disubstituted chymotrypsin A_α (1b), this indicates that 2a could be less highly substituted than 2b.

An experiment was carried out to determine the stability of the enzyme derivatives in solution for a 24-hr period at 25° at various pH levels. Protein samples were assayed for changes in residual enzymatic activity after 24 hr relative to initial activity. The initial activity corresponded to ca. 2% of normal chymotryptic activity. Both 2a and 2b showed little change in activity (less than 10%) upon remaining at pH 3.0 and 6.5 but not at pH 10. This indicates

irreversible inhibition, i.e. active enzyme could not be regenerated from the modified derivatives.

Amino Acid Analysis. After chymotrypsin was reacted with Ac-Phe(OCN)-OEt an amino acid analysis was performed on the enzyme derivatives to ascertain their tyrosine content. Upon total acid hydrolysis, the Ac-Phe(OCN)-OEt moiety should liberate a tyrosine residue. If the entire inhibitor were attached to the derivative somehow then the presence of at least one extra tyrosine would be revealed, whereas if only a carbamyl group were attached upon hydrolysis then no extra tyrosine would appear. As shown in Table 2 the former case is true. A complete amino acid analysis was performed in each case but the calculation for Tyr was made by comparing the size of the Phe and Tyr peaks assuming that the Phe peak corresponded to 6 residues. Chymotrypsin gave 4 tyrosines per molecule as expected (Wilcox, 1970), while derivative 2a had 5 tyrosines and 2b had an unusual 5-1/2 tyrosines. Therefore derivative 2a must possess as one of its structural features an entire inhibitor molecule linked to one site on the enzyme.

Incorporation of ^{14}C. After chymotrypsin was reacted with [^{14}C] Ac-Phe(OCN)-OEt the enzyme derivatives were radioassayed for ^{14}C content to determine the stoichiometry of reaction. The ^{14}C-labeled cyanate carbon should remain connected to the enzyme whether the entire inhibitor is attached as above or hydrolyzed to a carbamyl group. The

counting results are shown in Table 3; it turns out the approximate stoichiometry of reaction is observed to be I:E = 2:1. Both derivatives 2a and 2b had incorporated slightly more than two moles of ^{14}C-inhibitor per mole of enzyme, with the excess attributable to non-specific side reactions. Therefore derivative 2a, as well as 2b, must have resulted from reaction of the inhibitor at two different and separate sites on the enzyme. From the results it is apparent at this point that derivative 2a must possess an entire inhibitor molecule attached at one reaction site and a carbamyl group at the other site. These two sites are very likely but not necessarily the same in 2a and 2b.

Reaction of Chymotrypsin A_α with p-Nitrophenyl Cyanate. Two modified enzyme derivatives were obtained, O-carbamyl-Ser-195 ("monocarbamyl") chymotrypsin A_α (1a, 50%) and a disubstituted chymotrypsin A_α (1b, 50%) which was regarded to be N-p-nitrophenoxyiminomethyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_α, as the only major products. Derivative 1b was hydrolyzable to N-carbamyl-Cys-1 O-carbamyl-Ser-195 ("dicarbamyl") chymotrypsin A_α upon treatment at pH 10, analogous to dicarbamyl-chymotrypsin A_∂ (Robillard et al, 1972).

Affinity Chromatography. The binding properties of the enzyme derivatives upon a Sepharose 4-phenylbutylamine (PBA) affinity column were examined to determine if any

binding-pocket residues were modified by reaction with Ac-Phe(OCN)-OEt. O-Carbamyl-Ser-195 chymotrypsin A_α (1a) was prepared as a model in order to check the proper operation of the affinity chromatography. Chymotrypsinogen A, with its binding site blocked, passed thru the affinity column upon elution with 0.1 M tris (pH 8.0), while O-carbamyl-Ser-195 chymotrypsin A_α, with its binding site intact, was mostly bound to the affinity column until eluted with buffer of high ionic strength (Figure 5A). The binding is due to chymotrypsin's disposition for a phenyl group in its binding pocket. Both derivatives 2a and 2b were mainly retained on the affinity column upon elution with 0.1 M tris (pH 8.0) and were not removable until eluted with buffer of high ionic strength (Figure 5B). This is evidence that 2a and 2b possess unaltered binding sites and normal binding capacities towards substrate-like aromatic groups. Minor proportions of all the chromatographed enzyme derivatives were unretained and probably consisted of denatured or fragmented protein.

Reaction with [^{14}C] DFP. Knowing that the binding site appeared to be unaltered, it was suspected that one of the two reaction sites of chymotrypsin with Ac-Phe(OCN)-OEt might be at the active-site catalytic residue. After unlabeled derivative 2a was reacted with [^{14}C] DFP the resulting product was radioassayed for ^{14}C content to quantitate the incorporation of DFP. For serine proteases, DFP is a

powerful inhibitor which reacts solely with the active-site serine residue (Hartley, 1960) in both active enzyme and zymogen. If derivative 2a were previously modified at active-site Ser-195 then it would not react further with DFP, whereas if 2a were modified with its active site still intact then it probably would be inhibited by DFP. As shown in Table 3 the former case is true. Chymotrypsinogen A incorporated ca. 1 equiv of DFP; it reacts very slowly with DFP, $k_{2nd} = 0.105\ M^{-1}\ min^{-1}$ at 25° and pH 7 (Morgan et al, 1972). Under identical conditions, derivative 2a incorporated only 0.2 equiv of DFP, and even this is probably due to non-specific interaction. The unfortunate conclusion is that one of the two reaction sites in derivative 2a is at the active-site Ser-195.

The [^{14}C] DFP reagent was actually only 63% DFP as demonstrated by the following experiment. Chymotrypsinogen A was reacted with [^{14}C] DFP as usual and after the standard activation to chymotrypsin, it was found to be 92% inactivated by DFP. However, the same DIP-chymotrypsinogen exhibited only 58% incorporation of ^{14}C. Similar results were obtained beginning with chymotrypsin A and [^{14}C] DFP. Accordingly, the values for [^{14}C] DFP in Table 3 have been increased by a factor of 92/58 from the experimental values.

Preparation of N-Carbamyl-Cys-1 Chymotrypsin A_∂. In turn it was suspected that the second reaction site with chymotrypsin in the Ac-Phe(OCN)-OEt reaction was at the N-

terminal α-amino residue, analogous to that in derivative 1b. Hence, chymotrypsin was first protected with a N-terminal blocking group and then treated with Ac-Phe(OCN)-OEt. The expected product would arise from reaction only at Ser-195 since Cys-1 was now shielded. In order to maintain active-site Ser-195 intact, chymotrypsinogen A was modified by p-nitrophenyl cyanate yield N-carbamyl-Cys-1 chymotrypsinogen A, a previously made material (Robillard et al, 1972). Upon rapid activation with acetyl-trypsin the zymogen was converted to N-carbamyl-Cys-1 chymotrypsin A_∂ (∂-form) (Miller et al, 1971). The ∂-form differs little in reactivity at Ser-195 than the α-form used in the previous experiments.

Reaction of N-Carbamyl-Cys-1 Chymotrypsin A_∂ with Ac-Phe(OCN)-OEt. The reaction conditions were virtually identical with those used in the preparative inhibition of chymotrypsin A_α, and 98% inhibition was achieved within 3 hrs. Only one main modified enzyme derivative was obtained, 3, and again it was prepared in both unlabeled and ^{14}C-labeled form. Its CM-cellulose column elution peak (0.083 M K^+) was very near to the position for derivative 2a (0.085 M K^+) and displayed a slight left shoulder which could not be resolved despite a gradual [K^+] gradient (Figure 4C). Since one reaction site on the starting enzyme was already modified, Cys-1, there were two other probable locations left at which reaction could have occurred, in the binding pocket or at Ser-195. Derivative 3 was mainly retained on

the affinity column upon elution with 0.1 M tris (pH 8.0) (Figure 5C), which implies that, as in 2a, the binding site is unaltered.

An amino acid analysis of derivative 3 revealed that it contained 4 tyrosines per molecule (Table 2), meaning that no additional tyrosine moieties were introduced from the inhibitor. This points to a carbamyl group as a structural feature of the protein. When N-carbamyl-Cys-1 chymotrypsin A was reacted with [^{14}C] Ac-Phe(OCN)-OEt, derivative 3 was found to incorporate approximately one mole of ^{14}C-inhibitor per mole of enzyme (Table 3). In this case the inhibitor had reacted with only one site on the enzyme. When unlabeled derivative 3 was subsequently reacted with [^{14}C] DFP, the product was found to incorporate only 18% as much DFP as chymotrypsinogen A did (Table 3), much like 2a. Therefore the single reaction site in derivative 3 is clearly at the active-site Ser-195. Finally, the conclusion with respect to derivative 2a is that one of its reaction sites with the inhibitor is at the active-site Ser-195 and the other site is at the N-terminal Cys-1. For 2b the same conclusions may be drawn about two of its reaction sites.

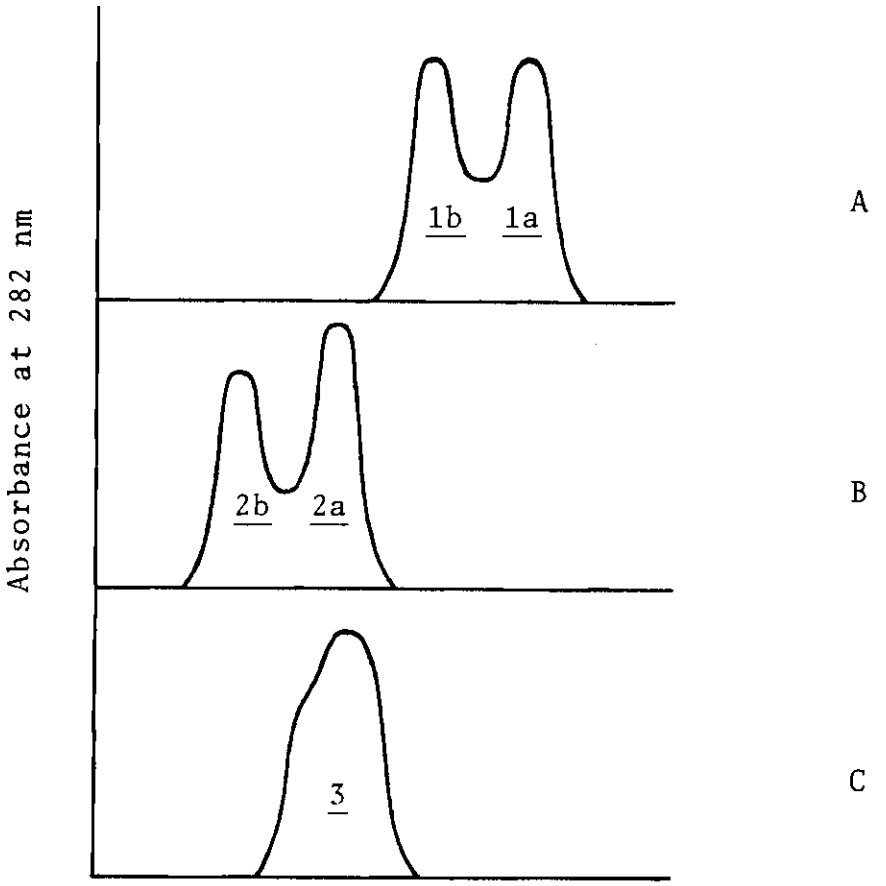


Figure 4. CM-Cellulose Chromatograms of Chymotrypsin A Derivatives. Note: (A) O-Carbamyl-Ser-195 chymotrypsin A_α (1a) and the disubstituted chymotrypsin A_α (1b) (Robillard et al, 1972), eluting at 0.105 M K^+ and 0.095 M K^+; (B) Derivatives 2a and 2b, from the reaction of chymotrypsin A_α with Ac-Phe(OCN)-OEt, eluting at 0.085 M K^+ and 0.074 M K^+; (C) Derivative 3, from the reaction of N-carbamyl-Cys-1 chymotrypsin A_δ with Ac-Phe(OCN)-OEt, eluting at 0.084 M K^+. The elution points refer to the center tube for each peak in the chromatograms. In each case 70-80% of the total protein applied to the column eluted in the major peaks.

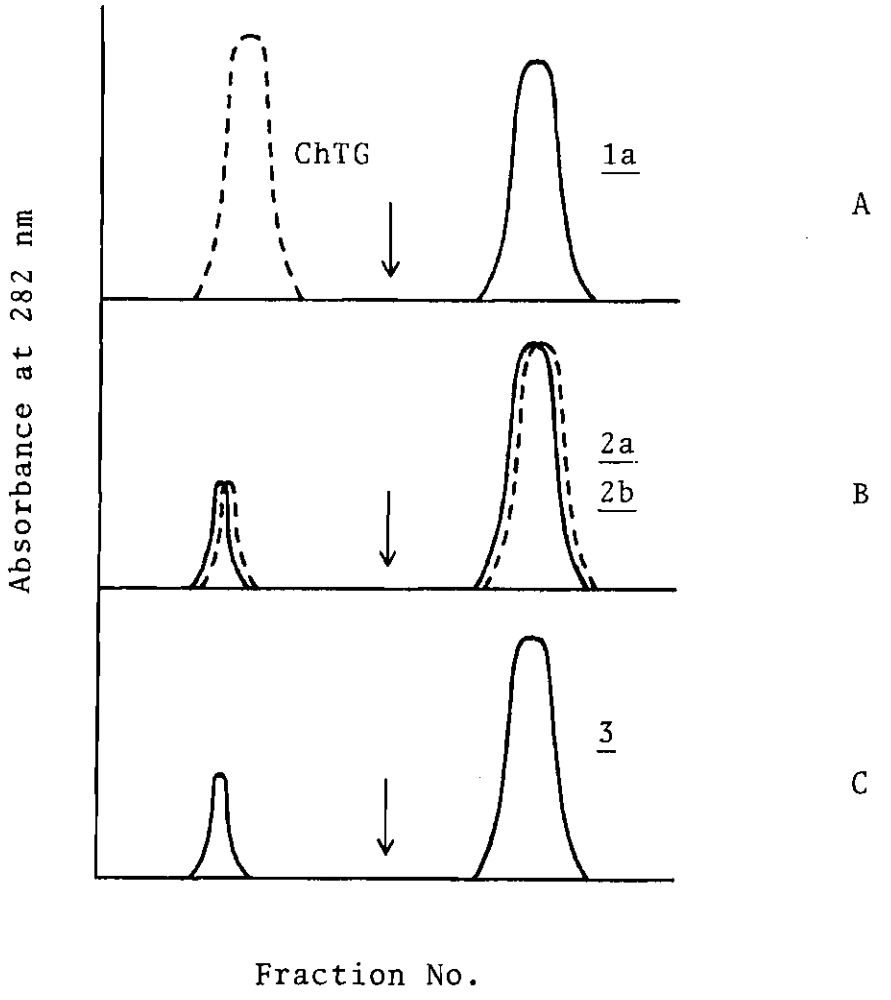


Figure 5. Affinity Column Chromatograms of Chymotrypsin A Derivatives. Note: The column consisted of Sepharose-4-phenylbutylamine. (A) O-Carbamyl-Ser-195 chymotrypsin A_α (1a) (—), (Robillard et al, 1972), mostly retained, and chymotrypsinogen A (ChTG) (--), unretained; (B) Derivatives 2a (—) and 2b (--), from the reaction of chymotrypsin A_α with Ac-Phe(OCN)-OEt, both mainly retained; (C) Derivative 3, from the reaction of N-carbamyl-Cys-1 chymotrypsin A_δ with Ac-Phe(OCN)-OEt, mainly retained. Arrows mark change to an eluent of 1 M TEAB. In each case 60-70% of the total protein applied to the column eluted in the major peak.

Table 1. Inhibition Kinetics of Chymotrypsin A with Ac-Phe(OCN)-OEt.[a]

Kinetic Parameter	Run 1-1	Run 2-1	Run 2-2
[I], M	8.0×10^{-4}	6.0×10^{-4}	6.0×10^{-4}
[E], M	8.0×10^{-8}	8.0×10^{-8}	8.0×10^{-8}
[S][b], M	1.0×10^{-2}	1.0×10^{-2}	1.0×10^{-2}
A[c]	10.95	14.46	14.26
B	10.89	14.36	14.16
R	0.758	0.802	0.808
V_c, units min^{-1}	6.89	6.89	6.89
V_o, units min^{-1}	6.62	6.75	6.74
V_c/V_o	1.04	1.02	1.02
$k_{obsd} \times 10^1$, min^{-1}	5.54	4.42	4.26
Half-life, min	1.25	1.57	1.63
$k_3/K_I \times 10^{-3}$, M^{-1} min^{-1}	4.33	4.50	4.34
K_I[d], M	-	-	-

[a]Reaction in 5% (v/v) methanol at 25° and pH 7.5. [b] S is Ac-Tyr-OEt, K_M = 2.0 mM, α = 0.833. [c]A,B,R are coefficients in the equation of the curve. [d]K_I is indeterminate since V_c/V_o < 1.05, meaning that K_I > 2 mM.

Table 2. Amino Acid Analysis of Enzyme Derivatives Modified by Aryl Cyanate Reagents.

Residue	theory[a]	ChT[b]	1a[c]	2a	2b	3
Tyr	4	3.89	3.86	4.98	5.42	3.98
Phe	6	6.0	6.0	6.0	6.0	6.0

[a]Values predicted from the amino acid sequence of bovine chymotrypsin A. [b]Control run. [c]Values reported as μmoles of amino acid per μmole of protein; calculation based on Phe = 6.0 μmoles per μmole of protein.

Table 3. ^{14}C Incorporation into Enzyme Derivatives Reacted with Ac-Phe(OCN)-OEt and DFP.

Reagent	DIP-ChTG[a]	2a[b]	2b	3
[^{14}C] Ac-Phe(OCN)-OEt	--	2.08	2.39	1.13
[^{14}C] DFP	0.92	0.20	--	0.18

[a]Control run for [^{14}C] DFP. [b]Values for all products reported as the molar ratio of ^{14}C to protein, based on the specific activity of the reagent.

CHAPTER IV

DISCUSSION

The direction of our efforts in enzyme engineering was pointed towards the development of a new species of enzyme with the only difference being a modified primary binding site. The specific goal was the modification of the binding pocket of chymotrypsin *via* a carbamylation reaction using an aryl cyanate reagent. Our rationale in designing Ac-Phe(OCN)-OEt to be both a reactive and specific reagent for this task was to equip it with a labile cyanate group plus a substrate-like structure. It was expected to carbamylate a Ser-189 or Ser-190 group inside the binding pocket and to change the stereoelectronic environment. The anticipated result was the formation of an active enzyme, a chymotrypsin derivative with an altered substrate specificity. Since the serine proteases exhibit a high degree of homology and differ mainly in their specificities, it was hoped that this modification would easily lead to an extra member of the enzyme family. If such an engineered enzyme had been successfully obtained, it would have been characterized to define its specificity and activity. The overall value of this work lies not only in the techniques for creating this kind of enzyme but also in the potential cat-

alytic utility of the product.

The experimental final outcome is that the desired product was plainly not obtained, namely a chymotrypsin derivative modified at the primary binding site. Although this situation may be classified as a failure to achieve a positive result, we feel that the idea of enzyme engineering may be viable in better circumstances. This term "enzyme engineering" has acquired a different meaning in the recent literature which should not be confused with the definition herein. There it describes an interdisciplinary field dealing with the application of immobilized enzyme technology to industrial reactions, commercial processes, analytical and medicinal chemistry, etc.

Proposed Chymotrypsin Reaction Sites. It was recognized at the outset that the reaction of chymotrypsin A with Ac-Phe(OCN)-OEt could yield a variety of possible products and not just one derivative modified at the binding site. The justification for proposing certain structures is based on the previous results of the reaction of chymotrypsin A with p-nitrophenyl cyanate (Robillard et al, 1972). We obtained similar results in repeating a few of these experiments for analytical purposes. Hence, chymotrypsin A_α reacts with p-nitrophenyl cyanate to yield predominantly O-carbamyl-Ser-195 chymotrypsin A_α, modified by carbamylation at Ser-195 (although our experiments gave a mixture of this primary product la and a disubstituted chymotrypsin A_α lb).

Chymotrypsin A_∂ reacts differently with p-nitrophenyl cyanate to yield exclusively N-p-nitrophenoxyiminomethyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_∂, modified by isourea formation at Cys-1 in addition to carbamylation at Ser-195. So, the α-form of enzyme reacts with a small excess of reagent more rapidly at Ser-195 than at Cys-1 while the ∂-form reacts equally rapidly at these two sites. Furthermore, the disubstituted chymotrypsin A_∂ could be hydrolyzed to the N-carbamyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_∂ upon treatment at pH 10, modified by carbamylation at both Cys-1 and Ser-195. The chief effect of these modifications is the irreversible inhibition of chymotryptic activity by carbamylation of the catalytic residue.

Returning to the reaction of chymotrypsin A with Ac-Phe(OCN)-OEt, the modified enzyme derivatives may be characterized in terms of their site, type, and extent of reaction with inhibitor. Several likely enzyme structures are proposed based on the foregoing results and are pictured in Figure 7. The modifications may obtain at the following three sites: Ser-195 hydroxyl group at the active site (A and AC); Cys-1 α-amino group at the N-terminal site (T and TC); and Ser-189 or Ser-190 hydroxyl group in the binding pocket (B and BC). In A, T, and B, the entire inhibitor is attached to the enzyme thru the cyanate group via an iminocarbonate, -OC(=NH)O-, or isoureido, -NHC(=NH)O-, linkage. In AC, TC, and BC (C=carbamyl), this linkage is subsequently hydrolyzed leaving a carbamyl group attached to

the enzyme and releasing a tyrosine. These modifications may occur individually or in combination, and there are also many other enzymatic sites, exposed and nucleophilic, at which reaction could possibly occur. The majority of the experimental work was undertaken to identify the actual reaction sites.

Modification of Chymotrypsin A by Ac-Phe(OCN)-OEt. The evidence now gathered is sufficient to elucidate the detailed interactions between the enzyme and inhibitor and to characterize the two important enzyme derivatives 2a and 3. Diagrams of structures for the identified sites and types of reaction are pictured in Figure 8. Chymotrypsin A_α reacts with Ac-Phe(OCN)-OEt to give derivatives 2a and 2b, and 2a is found to be a N-tyrosyliminomethyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_α (structural features T and AC). An entire inhibitor moiety is linked to N-terminal Cys-1 while a carbamyl group is attached to active-site Ser-195. This assignment for 2a is consistent with the observations of the incorporation of two equivs of ^{14}C label and one extra tyrosine residue, and the nonreactivity towards DFP. Next, N-carbamyl-Cys-1 chymotrypsin A_∂ reacts with Ac-Phe(OCN)-OEt to give one derivative, 3, which is found to be N-carbamyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_∂ (structural features TC and AC). A carbamyl group is attached to both Cys-1 and Ser-195 but in this case only the latter one is derived from the inhibitor. This assignment for 3 is consis-

tent with the observations of the incorporation of one equiv of ^{14}C label and no extra tyrosine residue, and the nonreactivity towards DFP. No information was obtainable about the relative reactivities of the α- and ∂-forms of enzyme at Ser-195 and Cys-1 with the reagent. The combination of all the results leads to an unambiguous structural identification of the enzyme derivatives 2a and 3 (Figure 8).

Identified Chymotrypsin Reaction Sites. It has been demonstrated clearly that active-site Ser-195 underwent some kind of modification because neither 2a nor 3 could be made to react with [^{14}C] DFP. Normally, DFP inhibits chymotrypsin at Ser-195 alone stoichiometrically and extremely rapidly. It is conceivable that Ac-Phe(OCN)-OEt could modify a reactive residue in the active-site region other than Ser-195, e.g. His-57, Met-192, or Tyr-228, and sterically block access of substrates to the binding region or the catalytic residue. This is the situation in chymotrypsinogen, where the active site is catalytically fully operational but the binding site is undeveloped, which prevents the normal binding of substrates required before hydrolysis (Gertler et al, 1974). Chymotrypsinogen A does tend to react with DFP though very slowly, ca. 10^4 times more slowly than chymotrypsin A (Morgan et al, 1972). Thus 2a and 3 could probably be made to react with DFP at least as fast as the zymogen, if Ser-195 were intact and even if the approach to this residue were sterically hindered. However, neither 2a nor 3 was

observed to react significantly with DFP meaning that Ser-195 itself must have been modified by Ac-Phe(OCN)-OEt. So the ultimate conclusion is that the abolition of chymotryptic activity is unequivocably due to the carbamylation of the catalytic residue by the inhibitor. This inhibition is irreversible because the carbamyl acyl-enzyme is very stable to hydrolysis and cannot undergo a regenerating deacylation.

In connection with this, the binding pocket has evidently not been modified because derivatives 2a, 2b, and 3 were all mainly retained on an affinity chromatography column. This Sepharose 4-phenylbutylamine column is known to bind chymotrypsin-like proteases but not trypsin-like proteases, zymogens, or chymotrypsin inhibited by Tos-Phe-CH_2Cl or nisyl fluoride (Stevenson and Landman, 1971). The observation that the column does bind 2a, 2b, and 3 means that their binding sites are unobstructed and free as in the parent enzyme. Hence the loss of chymotryptic activity cannot be attributed to the inhibitor causing an alteration of the enzyme's binding ability.

It has also been demonstrated that N-terminal Cys-1 was modified by comparing the reactions of native and N-carbamyl-Cys-1 chymotrypsins A with the inhibitor. The observation is that 3, derived from the N-terminal-blocked chymotrypsin A, incorporated 1.0 less equiv of Ac-Phe(OCN)-OEt than 2a, derived from unblocked chymotrypsin A, according to ^{14}C labeling results (Table 3). This identifies

Cys-1 as the second reaction site on 2a besides Ser-195 above. The information is also sufficient to classify the types of reaction at these two sites (Figure 8). Apparently in both 2a and 3 the initially formed iminocarbonate intermediate at Ser-195 is spontaneously hydrolyzed at pH 6.5 to the carbamate since an amino acid analysis of 3 revealed no extra tyrosine present (Table 2). In contrast, in 2a the initially formed isoureido intermediate at Cys-1 is stable at pH 6.5 and resistant to hydrolysis since in 2a there was found to be one extra tyrosine present. The disubstituted chymotrypsin A_α 1b was shown to be converted to N-carbamyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_α by hydrolysis on treatment at pH 10 (as in Figure 6). Likewise, 2a could probably be easily converted to this product, N-carbamyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_α, the α-form of 3, by similar hydrolysis on treatment at pH 10. Also, since 5-6 equiv of inhibitor was required for complete inhibition, it is likely that moderate hydrolysis of the cyanate group occurs at pH 6.5.

Derivative 2b. Although 2b was not characterized as thoroughly as 2a or 3, it is regarded to be a N-tyrosyl-iminomethyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_α, the same as 2a. The CM-cellulose chromatography results suggest that 2b is more highly substituted than 2a and contains another separate reaction site. In agreement with this idea, as compared with 2a, 2b exhibited the incorporation of ca.

0.3 extra equiv of inhibitor by ^{14}C analysis and of ca. 0.4 extra equiv of tyrosine by amino acid analysis. These results are consistent with the existence in 2b of the identical modified sites Ser-195 and Cys-1, plus a third undetermined reaction site which interacts at a slower rate with the inhibitor.

A compelling basis for justifying the structures of the chymotrypsin derivatives is the analogy between the reactions of enzyme with Ac-Phe(OCN)-OEt and p-nitrophenyl cyanate (Robillard et al, 1972). The modified enzyme derivatives from the reactions with both aryl cyanates are extremely similar in terms of the site, type, and extent of reaction. A comparison of the two reactions and their products is illustrated in Figure 9. Thus, chymotrypsin A_α is proven to react with Ac-Phe(OCN)-OEt to yield predominantly the disubstituted chymotrypsin A_α 2a plus another product which is more extensively substituted 2b. The derivatives are modified by carbamylation at Ser-195 and isourea formation at Cys-1, analogous to the results with p-nitrophenyl cyanate. Remarkably, it is possible for chymotrypsin A to react with both inhibitors to yield, after alkaline hydrolysis of the intermediates, an identical product: N-carbamyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A (3). It turns out that although Ac-Phe(OCN)-OEt is a more complex reagent, it has essentially the same inhibitory effect on this enzyme.

Modification with Aryl Cyanate Reagents. The modifi-

cation results provide some interesting revelations about the chemistry of aryl cyanate compounds and their potential utility and value as protein modification reagents. First, no improvement in the selectivity of reaction sites in the modification of chymotrypsin is produced by placing the cyanate group on a substrate-like molecule, Ac-Phe(OCN)-OEt, rather than on a non-specific molecule, NO_2-Ph-OCN. The amino-acid inhibitor reacts at Ser-195 and Cys-1 in almost the same way as the non-specific inhibitor, instead of reacting within the binding pocket. This is most likely a consequence of the failure of inhibitor to bind to enzyme, with a concurrent bimolecular reaction. In this instance at least, the various factors affecting enzymatic reactivity must dominate those affecting selectivity. Second, the reactivity of the cyanate functional group may be controlled by directing the electronic nature of the substituents present on the aromatic aryl ring. The electron-withdrawing ability of a substituent, which activates the cyanate group by increasing its susceptibility to nucleophilic attack, is smaller for a p-alkyl group than a p-nitro group. Thus, Ac-Phe(OCN)-OEt is observed to inhibit the enzyme at a slower rate than p-nitrophenyl cyanate under comparable conditions. The cyanate group may also be externally stabilized by a low solution pH to reduce its rate of spontaneous hydrolysis.

Finally, the surprising selectivity of aryl cyanates for certain reaction sites on chymotrypsin A is a notable

feature with respect to their utility and value as protein reagents. The interactions between the enzyme and Ac-Phe-(OCN)-OEt are confined mostly to two reaction sites: active-site Ser-195 and N-terminal Cys-1 only. It is credible that the reagent would react with enzyme at Ser-195 since this key residue is highly nucleophilic from being specially activated by the charge relay system. In fact this residue is so reactive that it can interfere with modification at secondary reaction sites. However, it is harder to explain why the reagent would react with enzyme at Cys-1 so specifically and not elsewhere, e.g. at lysine ε-amino groups or tyrosine phenolic groups. Remarkably, the α-amino group of Cys-1 is found to be positioned not close to the active-site region but away on the other side of the molecule (Blow, 1971). Perhaps the orientation of Cys-1 or its surrounding environment or product stability renders this site unexpectedly more reactive. By virtue of their limited selectivity combined with their moderate reactivity, aryl cyanate reagents are now beginning to gain more importance as enzyme modifiers and inhibitors. Recently, p-nitrophenyl cyanate was used to inhibit chymotrypsin A specifically at Ser-195 to obtain a derivative for x-ray structural studies on the properties of acyl-enzymes (Robillard et al, 1972). Our new reagent Ac-Phe(OCN)-OEt may be employed similarly, e.g. to selectively inhibit a N-blocked chymotrypsin A at Ser-195 or to selectively modify chymotrypsinogen A at Cys-1.

Non-Modification of the Binding Pocket. Since chymotrypsin A undergoes modification by Ac-Phe(OCN)-OEt not within the binding pocket but rather at active-site Ser-195, the question remaining is why the reaction does not proceed according to theoretical plan. The reason seems to lie in a combination of over-reactivity of the enzyme active site and under-selectivity of the aryl cyanate reagent. The inhibition kinetics for the reaction are incapable of demonstrating the existence of an enzyme-inhibitor complex usually formed in an initial binding step for substrate-like inhibitors. In addition, the discovery that the ester group of Ac-Phe-(OCN)-OEt does not undergo hydrolysis implies that the inhibitor does not bind normally like the substrate Ac-Tyr-OEt. If inhibitor binding to enzyme is not observable it is fairly weak (K_I > 2 mM) or non-existent which would preclude reaction within the binding pocket. One likely reason for the lack of binding is that the catalytic residue and inhibitor cyanate group could interact directly via a bimolecular reaction. Thus, the reactive Ser-195 may immediately attack the inhibitor as it diffuses toward the binding site and form an irreversible adduct before any true binding has an opportunity to occur. A bimolecular reaction of this kind best rationalizes the appearance of active-site modification and the absence of binding-site alteration.

The alternate situation is that Ac-Phe(OCN)-OEt does bind to chymotrypsin but that binding-site modification can-

not take place for special reasons. For example, the inhibitor binding may be very weak, K_I >> 2 mM, such that the equilibrium is not favorable for the formation of a significant amount of E·I complex to sustain a reaction. The occurrence of non-productive binding modes could also contribute to a similar outcome. In contrast, the inhibitor binding may be moderate, K_I = 2-10 mM, yet this condition may be necessary but not sufficient for binding-site alteration to take place. As the inhibitor diffuses in and out of the binding site, the cyanate group might not remain in the binding pocket a long enough time for reaction to occur there. Even if the equilibrium is favorable towards an E·I complex, a relative rate phenomenon could operate in the enzyme. Thus, an inherent rate difference whereby the reaction rate with inhibitor is much faster at Ser-195 than at a binding pocket residue may explain the preference for inhibitor reaction at the former site. While Ser-195 is a super-reactive residue, Ser-189 or Ser-190 in the binding pocket might be too unreactive to compete for attack on the cyanate group. Also it is possible that upon inhibitor binding the cyanate group may be held into a position not close enough to either Ser-189 or Ser-190 to be within the proper reacting distance. The opposite effect is less likely, i.e. the bulk of the aryl cyanate group might be slightly oversized and create steric interference at the bottom of the binding pocket. The hydrophobic environment of the pocket may also tend to destabilize

the postulated intermediate with its separated charge.

One expedient not yet employed is a model-building study to ensure the correct placing and fit of the reagent within the primary binding site of chymotrypsin to allow reaction inside the binding pocket. However, a model-building study could have predicted non-reaction within the pocket, but not the reactions actually observed. In an inhibition study of the related protease trypsin with lysine chloromethyl ketones, it was shown that the affinity of the inhibitor for enzyme is not necessarily related to the rapidity of the next step, inactivation (Shaw and Glover, 1970). Hence, whether adequate binding exists between chymotrypsin and the inhibitor still does not clarify the whole story.

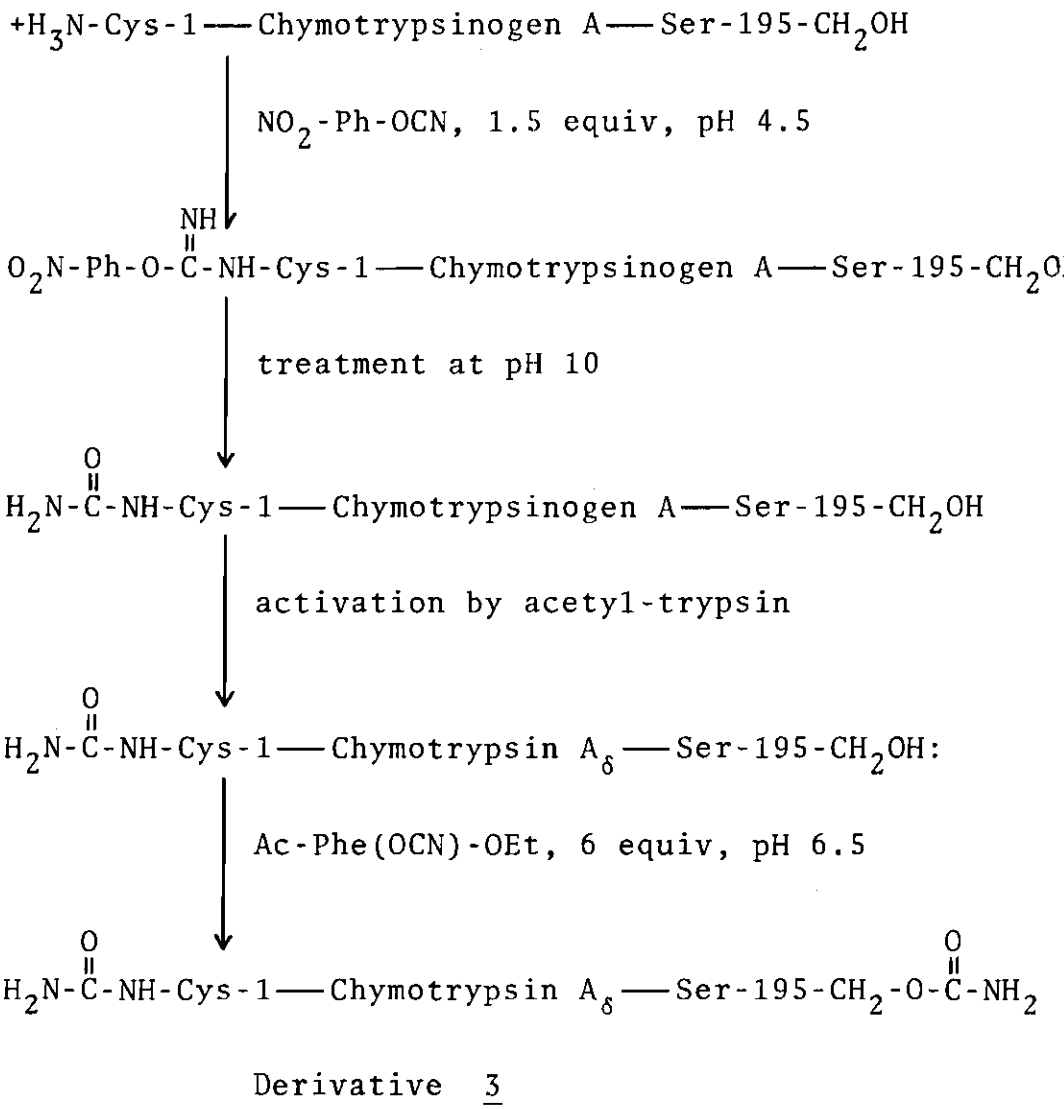
Protection of Active-Site Residues. The principal finding from this study is that it is necessary to protect the reactive active-site residues during selective enzyme modification procedures in order to suppress their reactivity. Specifically, this protection is required before the intended alteration of secondary enzymatic sites, i.e. residues at locations other than within the active-site region. This serves the dual purpose of preserving enzymatic activity by preventing inhibition, and allowing a chance for modification to ensue at less reactive sites. In the case of chymotrypsin A and Ac-Phe(OCN)-OEt, the reagent was found to inhibit the catalytic residue despite efforts to make it specific for interaction only in the binding pocket. One very effective

counter-action would be to decrease or block the activity of the active-site residues and thereby prevent them from attacking the reagent. Otherwise, the mere development of a more powerful or selective reagent would not alleviate the problem of potential reaction with these activated residues.

If the enzyme's active-site residues can be reversibly protected by a small-sized blocking group then in theory a specifically modified yet active enzyme may be regenerated. In practice this could be achieved by the use of a suitably designed bifunctional protein reagent or by the formation of a reasonably stable long-lived acyl-enzyme intermediate. The enzyme active site in each case would be temporarily inhabited while a second reaction at a neighboring site would be allowed to proceed. The blocking group must be small-sized to reduce steric problems and also reversible so the enzyme may then be reactivated. To illustrate, the bifunctional reagent p-nitrophenyl bromoacetyl-α-aminoisobutyrate was used to modify chymotrypsin A specifically at Met-192 via Ser-195 (Lawson and Schramm, 1965). Also, a fairly stable acyl-enzyme was demonstrated to be formed in the reaction of chymotrypsin A with a specific tryptophan p-nitrophenyl ester at low pH, 3-4 (Kezdy et al, 1964). A specific related reagent like Ac-Phe(OCN)-ONp might be more successful than Ac-Phe-(OCN)-OEt in modifying the binding pocket of chymotrypsin. If a fairly stable acyl-enzyme is formed, the active site would be reversibly protected, and the inhibitor cyanate

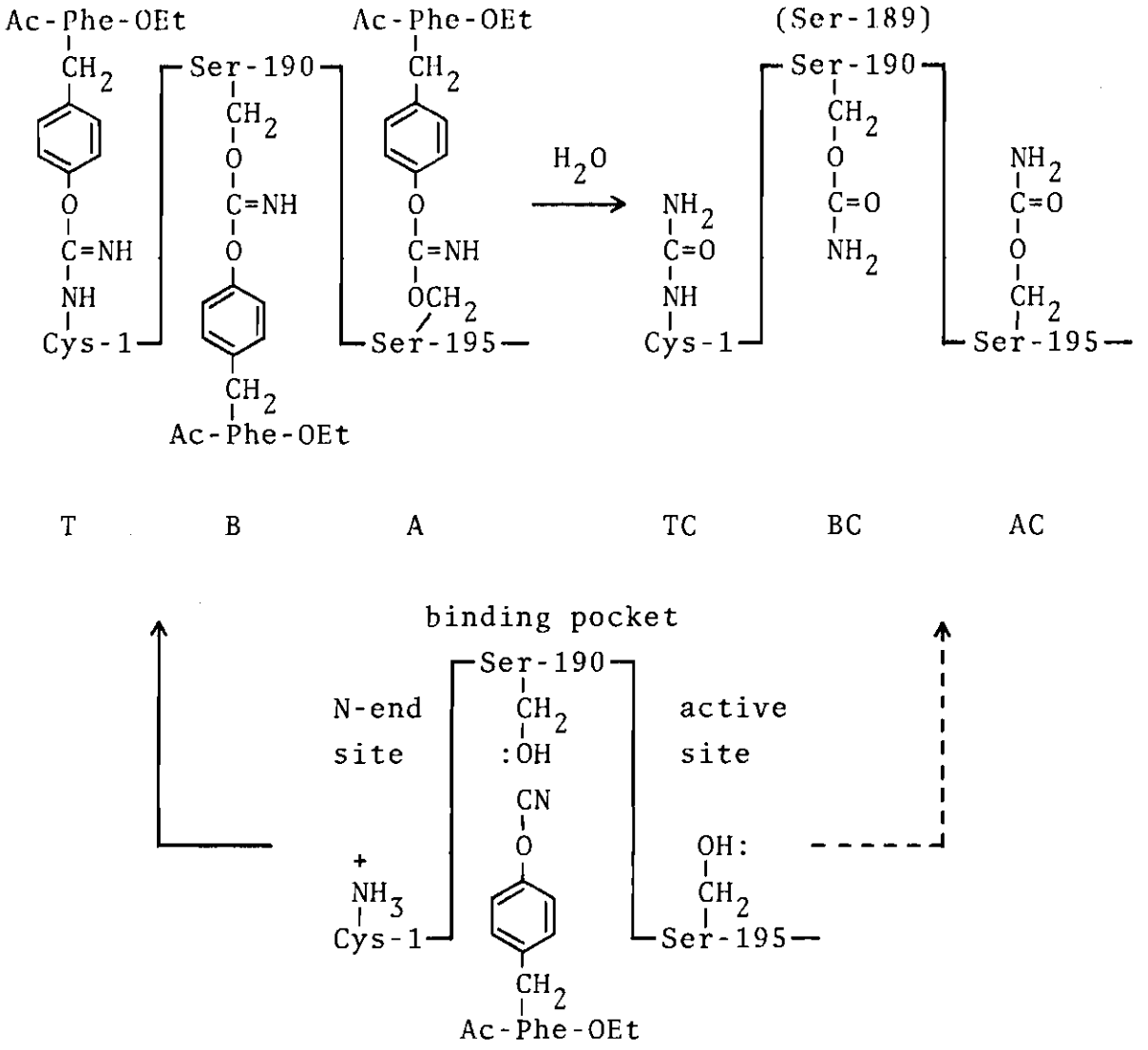
group would be held inside the binding pocket to react for a much longer time. The proximity effect should tend to override the relatively slow reaction rate and help lead to a more specific enzyme alteration process.

A functionalized acyl-enzyme like this is an ideal sort of reagent since its selectivity is enhanced at the expense of the enzyme's reactivity to make it more controllable. Another project would have been undertaken involving the reaction of chymotrypsin A and Ac-Phe(OCN)-ONp if it had not involved an inordinate amount of extra work.



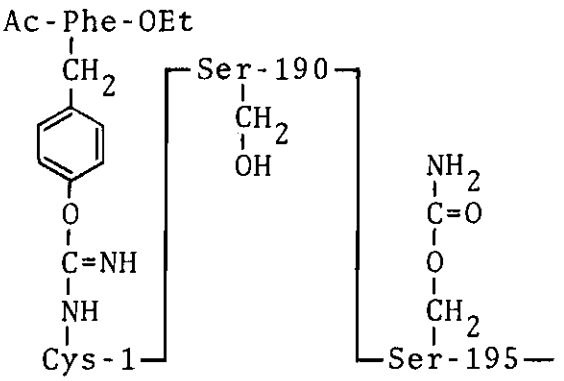
Derivative 3

Figure 6. Reaction Scheme for the Preparation of Derivative 3 from Chymotrypsin A. Note: 3 is a N-carbamyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_δ. Also, in Figures 6-9, the amino acid residues are shown with their reactive groups or side chains, e.g. Ser-CH_2OH.

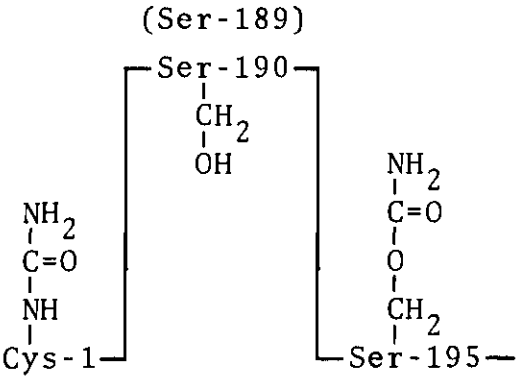


Chymotrypsin A + Ac-Phe(OCN)-OEt

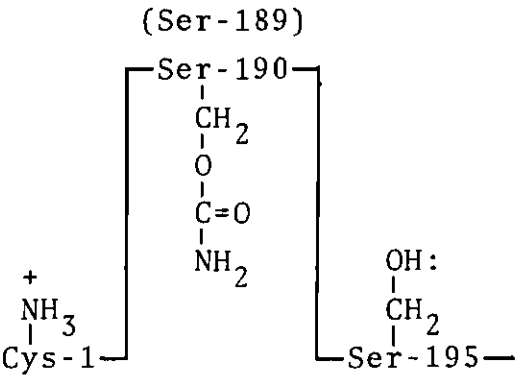
Figure 7. Diagram of Proposed Structures of Chymotrypsin A Derivatives Modified by Ac-Phe(OCN)-OEt. Note: The three sites and two types of reaction illustrated are: A, at the active-site Ser-195; B, in the binding pocket, Ser-189 or -190; T, at the N-terminal-site Cys-1; and A, B, T give AC, BC, TC upon hydrolysis to the carbamate (C=carbamate).



Derivative 2a, 2b



Derivative 3



Derivative BC

Figure 8. Diagram of Identified Structures of Chymotrypsin A Derivatives Modified by Ac-Phe(OCN)-OEt. Note: 2a, a N-tyrosyliminomethyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_α; 3, N-carbamyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_δ; 2b is analogous to 2a; and BC, O-carbamyl-Ser-189 or -190 chymotrypsin A_α (desired but not obtained).

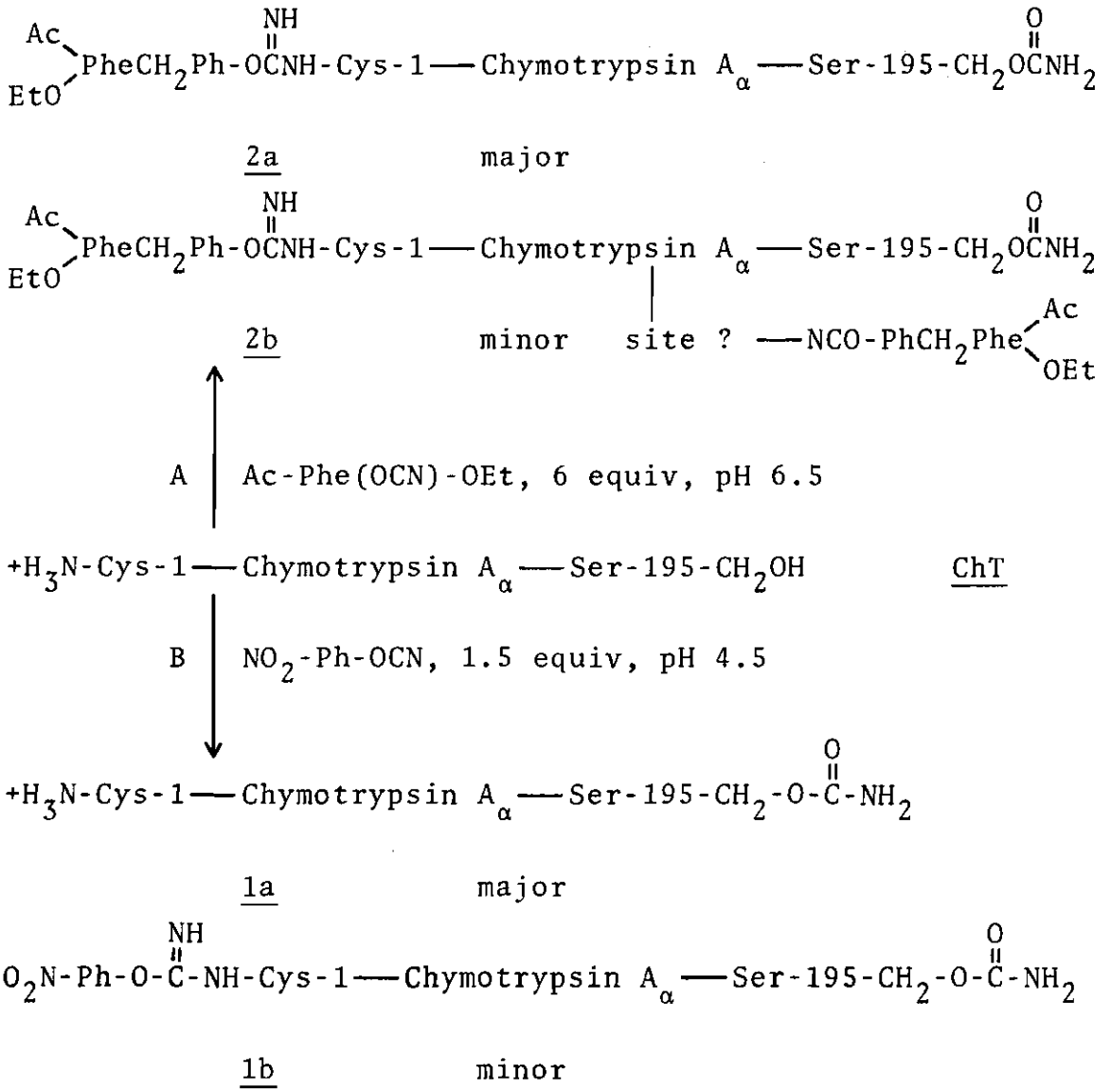


Figure 9. Comparison of the Reactions of Chymotrypsin A with Different Aryl Cyanate Reagents. Note: (A) The new Ac-Phe(OCN)-OEt, and (B) NO_2-Ph-OCN (Robillard *et al*, 1972). The identity of the proposed third reaction site on 2b is not yet determined.

Table 4. Chymotrypsin A Derivatives as Modified by Aryl Cyanate Reagents.[a]

Deriv.	Enzyme	Reagent	Site	Type
1a	ChT	NO_2-Ph-OCN[b]	Ser-195	O-carbamyl
1b	ChT	NO_2-Ph-OCN[b]	Ser-195 Cys-1	O-carbamyl N-inhibitor[c]
TC	ChTG	NO_2-Ph-OCN[b,d]	Cys-1	N-carbamyl
2a	ChT	Ac-Phe(OCN)-OEt	Ser-195 Cys-1	O-carbamyl N-inhibitor
2b	ChT	Ac-Phe(OCN)-OEt	Ser-195 Cys-1 unknown	O-carbamyl N-inhibitor ?-inhibitor
3	TC	Ac-Phe(OCN)-OEt	Ser-195 Cys-1	O-carbamyl N-carbamyl

[a]All derivatives are α-ChT except TC and 3 which are ∂-ChT; all are irreversibly inhibited except TC. [b]Prepared by Robillard et al, 1972. [c]A N-aryloxyiminomethyl-Cys-1 group. [d]After hydrolysis of the N-inhibitor to a N-carbamyl group, and activation of ChTG to the ∂-ChT.

CHAPTER V

CONCLUSIONS

In conclusion, the general results demonstrate that the new aryl cyanate reagent Ac-Phe(OCN)-OEt is moderately reactive and unusually specific in its inhibition reaction with chymotrypsin. It appears that the class of aryl cyanate reagents are reactive, selective, and versatile enough to establish them as important reagents in protein chemistry. The reaction of chymotrypsin A_α with Ac-Phe(OCN)-OEt yields two derivatives, a N-tyrosyliminomethyl-Cys-1 O-carbamyl-Ser-195 chymotrypsin A_α 2a, and a lesser amount of a partially trisubstituted chymotrypsin A_α 2b. The active-site Ser-195 is modified by carbamylation resulting in total inhibition of enzymatic activity, and the N-terminal Cys-1 is modified by isourea formation with inhibitor, but the primary binding site remains unaltered. This enzyme modification reaction is reasonably reactive and surprisingly selective in that it is largely limited to only the two reaction sites. A remarkable occurrence is that the reaction products of chymotrypsin A with Ac-Phe(OCN)-OEt and NO_2-Ph-OCN are virtually identical in their character. Nevertheless, the initial attempt at enzyme engineering has failed to generate a new and different species of active enzyme with a modified

binding pocket and an altered substrate specificity. Some reasons for this result may include a lack of enzyme-reagent binding with a concurrent bimolecular reaction, or an exaggerated reactivity difference in active-site and binding-site residues. The principal lesson arising from this work is the necessity to protect the reactive active-site residues during selective enzyme modification procedures intended elsewhere, e.g. with the aid of a reversible blocking technique like an appropriate acyl-enzyme species. A reaction of chymotrypsin with Ac-Phe(OCN)-ONp is a logical next step. The creation of a modified protease with an altered specificity is expected to be valuable for a variety of biochemical and functional investigations.

CHAPTER VI

RECOMMENDATIONS

As mentioned above it would be worthwhile to synthesize a closely related type of reagent, Ac-Phe(OCN)-ONp, and investigate its reaction with chymotrypsin at ca. pH 3-4. The expected formation of a long-lived acyl-enzyme could lead more readily to reaction in the binding pocket and an alteration of specificity. A positive result would substantiate the philosophy of enzyme engineering while a negative result would probably doom at least this aspect. Another interesting area for further work is the reaction of aryl cyanate reagents toward other related serine proteases compared with the reaction toward chymotrypsin. For example, Ac-Phe(OCN)-OEt and NO_2-Ph-OCN could be reacted with both trypsin and elastase to determine the relative rates of inhibition. It is surmised that NO_2-Ph-OCN, but maybe not Ac-Phe(OCN)-OEt, would be equally as effective an inhibitor against trypsin and elastase as demonstrated against chymotrypsin. In addition, the reaction of aryl cyanate reagents with acid proteases (pepsin), thiol proteases (papain), and metallo-proteases (carboxypeptidase) would be informative. If enzymatic inhibition is observed for all these other proteases then it might be profitable to again study the enzyme-

reagent interactions in detail. This includes the characterization of modified enzyme derivatives as before.

APPENDIX

Preparation of Affinity Chromatography Column. An affinity column for chymotrypsin A, composed of Sepharose-4-phenylbutylamine (PBA), is prepared according to the general method of Stevenson and Landman (1971). This procedure gives enough packing to fill a 50 x 1.2 cm Chromatronix column. Two solutions are initially prepared and cooled to 0°: 1. 7.5 g CNBr + 7.5 ml CH_3CN, and 2. 7.5 g 4-phenylbutylamine + 10 ml EtOH. In a 200 ml beaker is placed 75 ml decanted Sepharose 4B and 75 ml 1 M Na_2CO_3, and the slurry is cooled to 0° while stirring gently. Next the CNBr solution is added and the slurry is stirred rapidly for 2-3 min at 0°. The following sequence is performed as quickly as possible on the Sepharose: it is washed with 2 x 350 ml cold 0.1 M $NaHCO_3$ (pH 9) on a filter funnel, 50 ml 0.1 M $NaHCO_3$ is added, the slurry is poured into a 200 ml beaker, the PBA solution + 15 ml extra EtOH is added, and gentle stirring is begun at once. The mixture contains ca. 75 ml activated Sepharose, 7.5 g PBA, 50 ml 0.1 M $NaHCO_3$, and 25 ml EtOH, at pH > 10. The slurry is stirred gently for 16 hr at 4°. Next the Sepharose is washed with 2 x 350 ml 0.1 M $NaHCO_3$ + EtOH (2:1), and to it is added 100 ml 1 M 2-aminoethanol. The slurry is stirred gently for 3 hr at 4°. Then the Sepharose is washed with 500 ml 0.1 M $NaHCO_3$ + 1 M NaCl,

500 ml 0.1 M $NaHCO_3$, 1000 ml H_2O, and 200 ml 0.05 M tris, and to it is added 100 ml 0.05 M tris. A slurry of 120% wet settled volume is used for column packing. Finally the Sepharose is packed into a 50 x 1.2 cm column with an eluent of 0.05 M tris (pH 8) (containing 1 mM $CaCl_2$) at a pump flow rate of ca. 10 ml/15 min. The affinity column is checked as follows, operating at 4° and below 2/3 ml/min. Two protein samples are run separately thru the column: 1. chymotrypsinogen A, and 2. chymotrypsin A_α (both 12 mg in 1 ml 0.2 M KCl + 0.1 M $CaCl_2$). They are eluted with 400 ml 0.05 M tris, 200 ml 0.05 M tris + 1 M tetraethylammonium bromide (TEAB), and 200 ml regular 0.05 M tris again. The column is regenerated after each run by washing with 0.05 M tris, 200 ml backwards and 800 ml forwards. If the column is operating properly, the zymogen will come off as one major peak ($\geq$ 90%) upon regular elution (at ca. 150 ml), while the enzyme will come off as one major peak ($\geq$ 90%) only upon high-salt elution (at ca. 500 ml). This pair of chromatograms represents the column binding ability and must be re-established every 30 days. The affinity column binding capacity is ca. 1.5 g of chymotrypsin per 1 ml of packed Sepharose when freshly made.

References:

Stevenson, K. J., and Landman, A. (1971), Can. J. Biochem. 49, 119.

Cuatrecasas, P., and Anfinsen, C. B. (1971), Methods Enzymol.

22, 345.

March, S., Parikh, I., and Cuatrecasas, P. (1974), Anal. Biochem. 60, 149.

REFERENCES

Alazard, R., Bechet, J.J., Dupaix, A., and Yon, J. (1973), Biochim. Biophys. Acta 309, 379.

Birktoft, J.J., and Blow, D.M. (1972), J. Mol. Biol. 68, 187.

Blow, D.M. (1971), Enzymes, 3rd Ed., 3, 185.

Blow, D.M., Birktoft, J.J., and Hartley, B.S. (1969), Nature (London) 221, 337.

Bodländer, P., and Shaw, E. (1971), Arch. Biochem. Biophys. 147, 810.

Brown, W.E., and Wold, F. (1973), Biochemistry 12, 828.

Cohen, L.A. (1970), Enzymes, 3rd Ed., 1, 147.

Eyl, A.W. Jr., and Inagami, T. (1971), J. Biol. Chem. 246, 738.

Feinstein, G., Bodländer, P., and Shaw, E. (1969), Biochemistry 8, 4949.

Gertler, A., Walsh, K.A., and Neurath, H. (1974), Biochemistry 13, 1302.

Grigat, E. (1972), Angew. Chem. Int. Ed. Engl. 11, 949.

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

Hart, G.J., and O'Brien, R.D. (1973), Biochemistry 12, 2940.

Hartley, B.S. (1960), Ann. Rev. Biochem. 29, 45.

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

Henderson, R., Wright, C.S., Hess, G.P., and Blow, D.M. (1971), Cold Spring Harbor Symp. Quant. Biol. 36, 63.

Hess, G.P. (1971), Enzymes, 3rd Ed., 3, 213.

Hexter, C.S., and Westheimer, F.H. (1971), J. Biol. Chem. 246, 3928.

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

Kezdy, F.J., Feder, J., and Bender, M.L. (1967), J. Amer. Chem. Soc. 89, 1009.

Knowles, J.R. (1972), Accts. Chem. Res. 5, 155.

Kurachi, K., Powers, J.C., and Wilcox, P.E. (1973), Biochemistry 12, 771.

Lawson, W.B., and Schramm, H.J. (1965), Biochemistry 4, 377.

Main, A.R., and Dauterman, W.C. (1963), Nature (London) 198, 551.

Miller, D.D., Horbett, T.A., and Teller, D.C. (1971), Biochemistry 10, 4641.

Morgan, P.H., Robinson, N.C., Walsh, K.A., and Neurath, H. (1972), Proc. Nat. Acad. Sci. U.S. 69, 3312.

Nakaya, H., Tanizawa, K., and Kanaoka, Y. (1970), Biochem. Biophys. Res. Commun. 40, 537.

Neet, K.A., Nanci, A., and Koshland, D.E. Jr. (1968), J. Biol. Chem. 243, 6392.

Powers, J.C., Tuhy, P.M., and Witter, F.R. (1975), article in preparation.

Robertus, J.D., Alden, R.A., Birktoft, J.J., Kraut, J., Powers, J.C., and Wilcox, P.E. (1972), Biochemistry 11, 2439.

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

Schechter, I., and Berger, A. (1967), Biochem. Biophys. Res. Commun. 27, 157.

Segal, D.M., Cohen, G.H., Davies, D.R., Powers, J.C., and Wilcox, P.E. (1971b), Cold Spring Harbor Symp. Quant. Biol. 36, 85.

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

Shaw, E., and Glover, G. (1970), Arch. Biochem. Biophys. 145, 484.

Shotton, D.M., White, N.J., and Watson, H.C. (1971), Cold

Spring Harbor Symp. Quant. Biol. 36, 91.

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

Stevenson, K.J., and Landman, A. (1971), Can. J. Biochem. 49, 119.

Stroud, R.M., Kay, L.M., and Dickerson, R.E. (1971), Cold Spring Harbor Symp. Quant. Biol. 36, 125.

Weiner, H., Batt, C.W., and Koshland, D.E. Jr. (1966), J. Biol. Chem. 241, 2687.

Wilcox, P.E. (1970), Methods Enzymol. 19, 73.

VITA

Peter Mirko Tuhy, the eldest son of Mirko Joshua Tuhy and Judith Ludmilla Kuchárik Tuhy, was born on May 20, 1949, in Plainfield, New Jersey. His parents moved to Rocky Hill, New Jersey in 1962, and then to Overland Park, Kansas in 1965, where they now live. He attended Princeton High School in Princeton, New Jersey for three years, and graduated from Shawnee Mission West High School in Overland Park, Kansas in June, 1966. That September he entered the Georgia Institute of Technology and received a B.S. in Chemistry, with Highest Honor, in June, 1970. He remained at the Georgia Institute of Technology to earn a Ph.D. in Chemistry in March, 1975, specializing in organic biochemistry. During this time he was a NSF Trainee, a NDEA Trainee, and a Graduate Research Assistant. In January, 1975 he accepted a post-doctoral position in enzyme chemical research, with Dr. Elliott Shaw at Brookhaven National Laboratory, New York. He is also an officer in the U. S. Air Force Reserve.