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THE ROLE OF METHIONINE-192 IN THE ACTIVATION AND CATALYTIC MECHANISM OF CHYMOTRYPSIN

By

William J. Treadway Jr.

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

February 1976

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This dissertation is dedicated to my wife, Martha

LIFE

William J. Treadway Jr. was born in Johnson City, Tennessee on February 22, 1949. He attended North Chicago Community High School in North Chicago, Illinois and graduated in June of 1967.

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He is the co-author of the following abstracts and publication:

Treadway, W. J., Jr., Schultz, R. M., (1973), "Conformations of the Specificity Site in Chymotrypsin: Effect of the N-Terminal Ile16 Residue on the Met192 Residue of the Chymotrypsin Active Site." Fall Meeting of the American Chemical Society, Biological Chemistry Abstract 10.

Schultz, R. M., Konovessi-Panayotatos, A., Treadway, W. J., Jr., (1974), "The Existence of Multiple Conformational Forms in Anhydrochymotrypsin." Biochem. Biophys. Res. Comm. 60, 535-541.

Schultz, R. M., Peters, J. R., Treadway, W. J., Jr., Konovessi-Panayotatos, A., (1975), "Changes in the Active Site Conformation on Methylation of Histidine-57 in α-Chymotrypsin (α-Cht)." Fall Meeting of the American Chemical Society, Biological Chemistry Abstract 47.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
DEDICATION	iii
LIFE	iv
TABLE OF CONTENTS	vi
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xii
SECTION I. INTRODUCTION	1
A. General Information on Chymotrypsin	1
B. Mechanism of Action of Chymotrypsin	9
1. Explanation of Mechanism	9
2. Essential Residues	11
3. Non-Essential Residues	18
4. Topology of the Active Site	23
C. The Activation Process	25
1. The Nature of Activation Mechanisms in Biological Processes	25
2. Interesting Features of Chymotrypsinogen and its Activation	26
3. A Structural Comparison of Chymotrypsinogen and Chymotrypsin	27
D. The Participation of Methionine-192 in Chymotrypsin	30
1. In the Activation of Chymotrypsinogen	30
2. In the Mechanism of Chymotrypsin Catalysis	35
E. The Goals of This Thesis	46

Page

2. Preparation of Anhydro-α-Chymotrypsin 62
3. Preparation of 3-Methyl-Histidine-57-α-Chymotrypsin 62
4. Preparation of Monosulfoxide-δ-Chymotrypsin (MS-δ-Cht) 65
5. Alkylation of Chymotrypsin with α-Bromo-4-Nitroacetophenone 65
6. Alkylation of N-Acetyl-L-Methionine Amide with α-Bromo-4-Nitroacetophenone 68
7. Synthesis of N-Acetyl-L-Methionine Amide-4-Nitroacetophenone 68
8. Performic Acid Oxidation of Modified Chymotrypsin 70
9. Amino Acid Analysis 71
10. N-Acetyl-L-Tyrosine Ethyl Ester Activity Assays 71
11. *p*-Nitrophenyl Acetate Active Site Titrations 72
12. N-*trans*-Cinnamoylimidazole Active Site Titrations 73
13. Circular Dichroism Studies 74
14. Synthesis of Substrates for Peptide Hydrolyses 75
15. Hydrolyses of Peptide Substrates 81
16. Hydrolysis of α-Casein 84
17. Measurement of Rates of Methionine Alkylation with α-Bromo-4-Nitroacetophenone 85

SECTION III. RESULTS 88

A. Preparation and Characterization of Acetylated (Isoleucine-16)-Chymotrypsin 88

1. Activity Assays of Modified Chymotrypsin 88
2. Active Site Titrations of Acetylated Forms of Chymotrypsin 92
3. Circular Dichroism Patterns of Acetylated Chymotrypsins 94

B. Alkylation of Acetyl-L-Methionine Amide and Various Forms of Chymotrypsin with α-Bromo-4-Nitroacetophenone 97

1. Ac-Met-NH_2, Chtgen, DIP-α-Cht, α-Cht and δ-Cht . . . 98
2. Acetylated Chymotrypsins 100
3. GuCl Treated Chymotrypsinogen 105
4. Anhydro-α-Chymotrypsin 106

Page

5. 3-Methyl-Histidine-57-α-Chymotrypsin 110
6. Spectra of Alkylated Chymotrypsins 110
7. CD Spectra of Alkylated Chymotrypsins 110

C. Kinetic Parameters of Peptide Hydrolysis of Several Substrates by δ-Cht and MS-δ-Cht 116

SECTION IV. DISCUSSION 131

A. Relationship Between Methionine-192 and Isoleucine-16 131

B. Juxtaposition of Methionine-192, Serine-195 and Histidine-57 in the Active Site 137

C. The Role of Methionine-192 in the Catalytic Mechanism of Peptide Hydrolysis 143

D. Conclusions 148

SECTION V. BIBLIOGRAPHY 149

LIST OF TABLES

	Page
Table 1 - Major Conformational Differences of Active Site Residues in Chymotrypsinogen and α-Chymotrypsin	4
Table 2 - Overall Homologies Between Mammalian Serine Proteinases	8
Table 3 - Characterization of Various Intermediates in the Peptide Syntheses of Ac-Phe-Gly-NH_2 and Ac-Phe-Ala-NH_2	77
Table 4 - N-Acetyl-L-Tyrosine Ethyl Ester Activity Assays	90
Table 5 - ρ-Nitrophenyl Acetate Active Site Titrations	93
Table 6 - Alkylation with BrNAP	99
Table 7 - Alkylation of Acetylated Cht with BrNAP	101
Table 8 - Amino Acid Analysis	104
Table 9 - Alkylation of Anhydro-α-Cht with BrNAP	107
Table 10 - Alkylation of 3-Methyl-His57-α-Cht with BrNAP	111
Table 11 - Initial Rates of Hydrolysis of Peptide Substrates at 25°C, pH 7.8, 0.1 M $CaCl_2$, 0.1 M Carbonate	119
Table 12 - α-Casein Hydrolysis at 35°C, pH 7.8, 0.005 M $CaCl_2$, 0.1 M Borate	124
Table 13 - Kinetic Parameters for the Hydrolysis of Peptide Substrates	126
Table 14 - Comparison of the Kinetic Parameters of Amide Hydrolysis	127
Table 15 - Comparison of the Kinetic Parameters of Peptide Hydrolysis	128
Table 16 - Ratios of Kinetic Parameters for the Hydrolysis of Peptide Substrates	130
Table 17 - Ratios of Kinetic Parameters for the Hydrolysis of Peptide Substrates	146

LIST OF FIGURES

	Page
Figure 1 - Primary Amino Acid Sequence of α-Chymotrypsin	3
Figure 2 - An Equation of the Proposed Mechanism of Action of Chymotrypsin	5
Figure 3 - A Possible Mechanism for Acylation and Deacylation by Chymotrypsin	10
Figure 4 - A pH-Rate Profile Typical of Reactions Catalyzed by Chymotrypsin	15
Figure 5 - Topology of the Active Site of Chymotrypsin	24
Figure 6 - Scheme for the Activation of Chymotrypsin from Chymotrypsinogen	28
Figure 7 - The Active Site of α-Chymotrypsin with the Pseudo-Substrate Formyl-L-Tryptophan	43
Figure 8 - Schematic Diagram of the Acetylation of δ-Chymotrypsin	56
Figure 9 - Schematic Diagram of the Direct Acetylation of δ- and α-Chymotrypsin	59
Figure 10 - Schematic Diagram of the Specific Acetylation of δ-Chymotrypsin	63
Figure 11 - Preparation of Anhydro-α-Chymotrypsin	64
Figure 12 - Preparation of 3-Methyl-His57-α-Chymotrypsin	66
Figure 13 - Alkylation of the Met192 Residue of Chymotrypsin with BrNAP	69
Figure 14 - Flow Diagram of Peptide Hydrolysis Apparatus	82
Figure 15 - Kinetic Equations for Alkylation of Chymotrypsin with BrNAP	86
Figure 16 - Circular Dichroism Patterns of Various Forms of Chymotrypsin in the 220-240 nm Range	95

Page

Figure 17 - Circular Dichroism Patterns of δ-Chymotrypsin and A_{16}-δ-Chymotrypsin (Fersht) in the 220-240 nm Range 96

Figure 18 - Semilogarithmic Plot of Change in Absorbance at 350 nm due to Reaction of BrNAP with Native α-Chymotrypsin 108

Figure 19 - Semilogarithmic Plot of Change in Absorbance at 350 nm due to Reaction of BrNAP with Anhydro-α-Chymotrypsin 109

Figure 20 - Semilogarithmic Plot of Change in Absorbance at 350 nm due to Reaction of BrNAP with 3-Methyl-His57-α-Chymotrypsin 112

Figure 21 - Absorption Spectra Characteristic of Various Forms of Chymotrypsin 113

Figure 22 - Circular Dichroism Patterns of Various Forms of Chymotrypsin in the 300-400 nm Range 114

Figure 23 - Model for the Association of Bovine Pancreatic Trypsin Inhibitor and α-Chymotrypsin 117

Figure 24 - Lineweaver-Burke Plots for the Hydrolysis of Ac-Phe-Ala-NH_2 by δ-Cht and MS-δ-Cht 120

Figure 25 - Lineweaver-Burke Plots for the Hydrolysis of Ac-Phe-Gly-NH_2 by δ-Cht and MS-δ-Cht 121

Figure 26 - Lineweaver-Burke Plots for the Hydrolysis of Ac-Phe-NH_2 by δ-Cht and MS-δ-Cht 122

Figure 27 - Standard Activity Curves for the Hydrolysis of α-Casein by δ-Cht and MS-δ-Cht 125

Figure 28 - Structural Changes in the Vicinity of the Active Site of α-Chymotrypsin due to Changes in pH 134

LIST OF ABBREVIATIONS

A	-	absorbance
Å	-	angstrom unit (10^{-10} centimeter)
A-Cht	-	chymotrypsin acetylated with acetic anhydride as chymotrypsinogen prior to activation with trypsin
A_{16}A-Cht	-	chymotrypsin acetylated as chymotrypsinogen, activated and reacetylated with acetic anhydride
ReA_{16}A-Cht	-	A_{16}A-Cht acetylated once again with acetic anhydride
A_{16}-Cht	-	chymotrypsin directly acetylated with acetic anhydride
ReA_{16}-Cht	-	A_{16}-Cht acetylated once again with acetic anhydride
A_{16}-Cht(F)	-	chymotrypsin specifically acetylated on the N-terminal group of isoleucine-16 by the method of Fersht (108)
Ac-Met-NH_2	-	acetyl-L-methionine amide
Ac-Phe-NH_2	-	acetyl-L-phenylalanine amide
Ac-Phe-Ala-NH_2	-	acetyl-L-phenylalanyl-L-alanine amide
Ac-Phe-Gly-NH_2	-	acetyl-L-phenylalanyl-glycine amide
am	-	acylamido region of the active site of chymotrypsin
ar	-	aromatic binding region of the active site of chymotrypsin
ATEE	-	acetyl-L-tyrosine ethyl ester
BA	-	benzamidine
BrNAP	-	α-bromo-4-nitroacetophenone
CBZ	-	benzyloxycarbonyl

CM	-	carboxylmethyl
C-I	-	N-*trans*-cinnamoylimidazole
CD	-	circular dichroism
Cht	-	chymotrypsin
Chtgen	-	chymotrypsinogen
DFP	-	diisopropyl fluorophosphate
DIP-Cht	-	diisopropyl fluorophospho-chymotrypsin
DMF	-	dimethyl formamide
DMMA	-	dimethylmaleic anhydride
[E]	-	enzyme concentration
ESR	-	electron spin resonance
GuCl	-	guanidine hydrochloride
$\underline{h}$	-	region of the active site of chymotrypsin where the hydrogen atom of the α-carbon of substrates is located
k_{cat}	-	rate constant of catalysis of enzyme reactions
K_m	-	michaelis constant of enzyme reactions
LBTI	-	lima bean trypsin inhibitor
m p	-	melting point
MS-Cht	-	monosulfoxide-methionine-192 chymotrypsin
$\underline{n}$	-	nucleophillic region of the active site of chymotrypsin
NMM	-	N-methyl morpholine
P	-	product
PMSF	-	phenylmethylsulfonyl fluoride
PMS-Cht	-	phenylmethylsulfonyl-chymotrypsin
*p*NPA	-	*p*-nitrophenyl acetate

psi	-	pounds per square inch
R_f	-	$\frac{\text{migration distance of substance}}{\text{distance of solvent front from start}}$
rpm	-	revolutions per minute
[S]	-	substrate concentration
TLC	-	thin layer chromatography
TPCK	-	L-1-tosyl-amido-2-phenylethyl chloromethyl ketone
TAME	-	tosyl-L-arginine ethyl ester
v	-	initial velocity
V_{max}	-	maximum velocity
(v/v)	-	measurement by volume

I. INTRODUCTION

General Information on Chymotrypsin

Chymotrypsin (Cht) is a proteolytic enzyme found in the duodenum and small intestine of all mammals. Together with pepsin, trypsin, carboxypeptidase, elastase, collagenase, aminopeptidase and dipeptidase it breaks down ingested protein into the constituent amino acids so they can be absorbed and assimilated into the body.

Chymotrypsin is synthesized as an inactive zymogen, chymotrypsinogen (Chtgen), in the acinar cells of the pancreas and secreted into the duodenum via the pancreatic duct. It exists as a single polypeptide chain of 245 amino acids, held together in a globular structure by five intramolecular disulfide bonds. The amino acid sequence has been determined accurately by two separate groups of investigators (1, 2). Excision of two specific dipeptides from the main chain by the limited proteolytic attack of trypsin and chymotrypsin activates chymotrypsinogen. The crucial step in the activation involves the cleavage of the Arg15-Ile16 peptide bond (3). This bond lies on the periphery of the molecule where it is accessible to attack by trypsin. Once the cleavage takes place the molecule becomes active and capable of hydrolyzing certain peptide bonds of proteins at neutral pH. Subsequent autocatalytic action of Cht results in various forms of the active enzyme (π, δ, γ and α) which all possess approximately the same activity and specificity. The amino acid sequence of α-chymotrypsin, the predominant form in vivo, is shown

in Figure 1 (1).

One of the significant breakthroughs concerning Cht research was the elucidation of the x-ray crystallographic structures of Cht (4, 5) and Chtgen (6) to 2.0 and 2.5 Å resolution respectively. These studies have made it possible to discern changes in structure associated with the activation of Chtgen. The most pronounced changes, which are presumably responsible for catalytic activity in Cht, are listed on Table 1 (6) and discussed in detail later in this introduction.

Specifically, Cht hydrolyzes peptide bonds on the carboxyl side of aromatic and some hydrophobic amino acids (7, 8, 9, 10). The specificity of the enzyme has also been determined through the study of artificial ester and amide substrates (11). Chymotrypsin appears to affect hydrolysis by the nucleophilic attack of the γ oxygen of the Ser195 on the carbonyl carbon of ester and amide bonds (8, 12, 13). An acyl intermediate (ES') is formed between the enzyme and the substrate liberating an alcohol or amine first product (P_1). Deacylation of the intermediate by the nucleophilic action of H_2O in the active site then liberates the acid second product (P_2) completing the hydrolysis (Figure 2) (14, 15).

Ser195 and His57 were identified as essential catalytic amino acid residues in the proposed mechanism of Cht catalysis, the criteria for an essential catalytic residue being the complete loss of enzymic activity upon chemical modification of that residue. The variety of specific chemical modifications for Ser195 and His57 leading to complete inactivation of Cht is extensive (139). Two selective reagents, diisopropyl fluorophosphate (DFP) and L-1-tosyl-amido-2-phenylethyl chloromethyl ketone (TPCK) were used to chemically modify Ser195 and His57 respectively

Figure 1. - Primary amino acid sequence of α-chymotrypsin [revised from Hartley (1)].

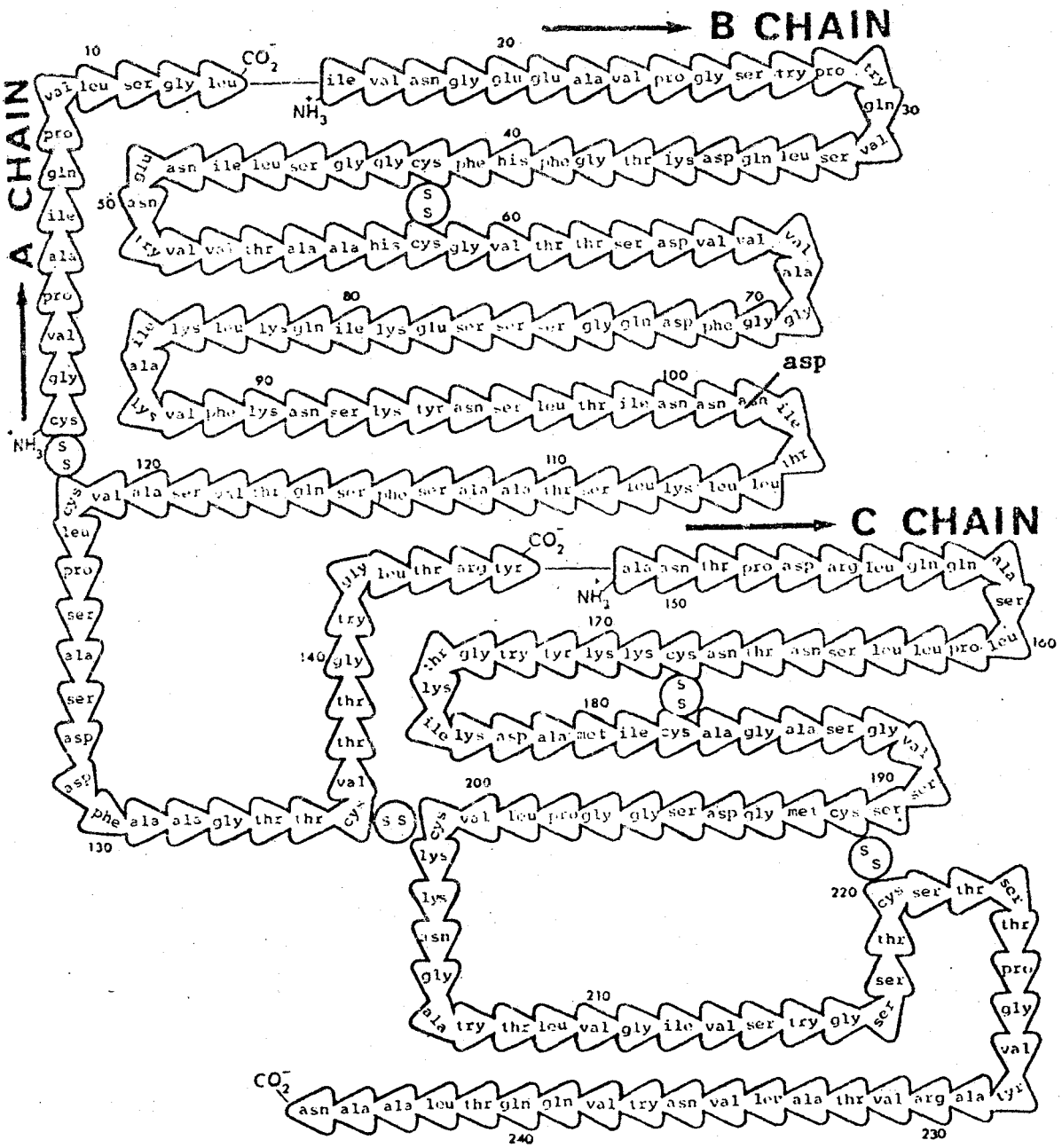


TABLE 1

MAJOR CONFORMATIONAL DIFFERENCES OF ACTIVE SITE RESIDUES IN CHYMOTRYPSINOGEN AND α-CHYMOTRYPSIN

Residue	Change from Chtgen to α-Cht
Asp194	Side chain moves 4 Å to form an ion-pair with the α-amino group of the N-terminal Ile16
Peptide segment residues 189-193	Forms a more extended conformation in α-Cht
Met192	Changes from a buried to a surface position in the active site. Forms lid of specificity cavity in α-Cht
Arg145	Movement of 9 Å from a surface position into the solvent
Ile16	Rotation of approximately 90° about its C_α-C_β bond away from the ring of His57

From x-ray crystallographic data of Freer and coworkers (6)

Figure 2 - An equation of the proposed mechanism of action of chymotrypsin (14, 15).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$$

and found to cause complete inactivation of Cht (16, 17, 18, 19). Later, the x-ray data for Cht offered more support for the participation of Ser195 and His57 in the catalytic mechanism (5). Although selective modification of Asp102 has not been possible, x-ray structures of Cht have indicated that it is part of the "charge transfer system" necessary for catalysis (20). For this reason, it is also referred to as an essential catalytic residue.

Other amino acid residues in the vicinity of the active site were designated non-essential residues because they do not participate directly in the bond-making or bond-breaking steps in Cht catalysis. Among these residues are the Asp194, Ile16, Met192 and Tyr146. A variety of experiments have shown that these residues are in some way necessary for either the stability of the active site conformation of Cht or for maximum efficiency in the catalysis of substrates by Cht. Ile16 and Asp194 fall in the former category because ion-pair formation between the positively charged α-amino group of Ile16 and the negatively charged carboxylate side chain of Asp194 has been implicated as a prerequisite for efficient catalytic activity (21). The x-ray structure of Cht has also revealed that the two functional groups involved are within ionic bonding distance of one another. Met192 and Tyr146 fall in the latter category because only partial loss of activity (utilizing small pseudo substrates) has been observed upon chemical modification of these two residues (22, 23).

Indirect evidence suggests that certain proteases all evolved from a common ancestral gene (24). This evidence is primarily based on the large number of homologous amino acid residues between Cht, trypsin,

elastase and thrombin. Table 2 shows the overall homology between the amino acid composition of these serine proteinases (25). The x-ray crystallographic data for Cht, trypsin, elastase and subtilisin also show great similarity in structure (4, 26, 27, 28). In addition, Cht, trypsin, elastase and thrombin all have practically the same mechanism of action which involves essential serine, histidine and either aspartic acid or glutamic acid residues in the active site (12).

The preceding information was included here to provide a framework upon which an understanding of this dissertation can be built. Most of the topics presented above will be explanined in much greater detail in the remaining sections of this introduction.

TABLE 2

OVERALL HOMOLOGIES BETWEEN MAMMALIAN SERINE PROTEINASES

	Percent identity (or chemical similarity[a]) with					Chain Length (No. of residue)
	Chymotrypsin B[b] (%)	Trypsin[b] (%)	Elastase[b] (%)	Thrombin[b] (%)	Any other of these five[c] (%)	
Chymotrypsin A[d]	78 (85)	43 (53)	39 (51)	32 (39)	63 (76)[e]	230
Chymotrypsin B[d]	-	38 (49)	38 (47)	41 (38)	58 (71)[f]	230
Trypsin		-	35 (48)	32 (38)	61 (74)	223
Elastase			-	27 (39)	55 (72)	240
Thrombin[d]				-	46 (56)	265

[a] Chemical similarities are defined as Arg = Lys, Asp = Asn, Glu = Gln, Asp = Glu, Asn = Gln, Ser = Thr, Val = Ile, Ile = Leu, Tyr = Phe = Trp, in addition to identities

[b] Calculated as percent of the minimum length required to accomodate the sequences being compared when aligned as in Figure 4 (see reference 25), discounting common deletions

[c] Calculated as percent of the individual chain length

[d] Residues 16-245 only (chymotrypsin A numbering)

[e] Excluding chymotrypsin B [f] Excluding chymotrypsin A

From B.S. Hartley and D.M. Shotton (25)

Mechanism of Action of Chymotrypsin

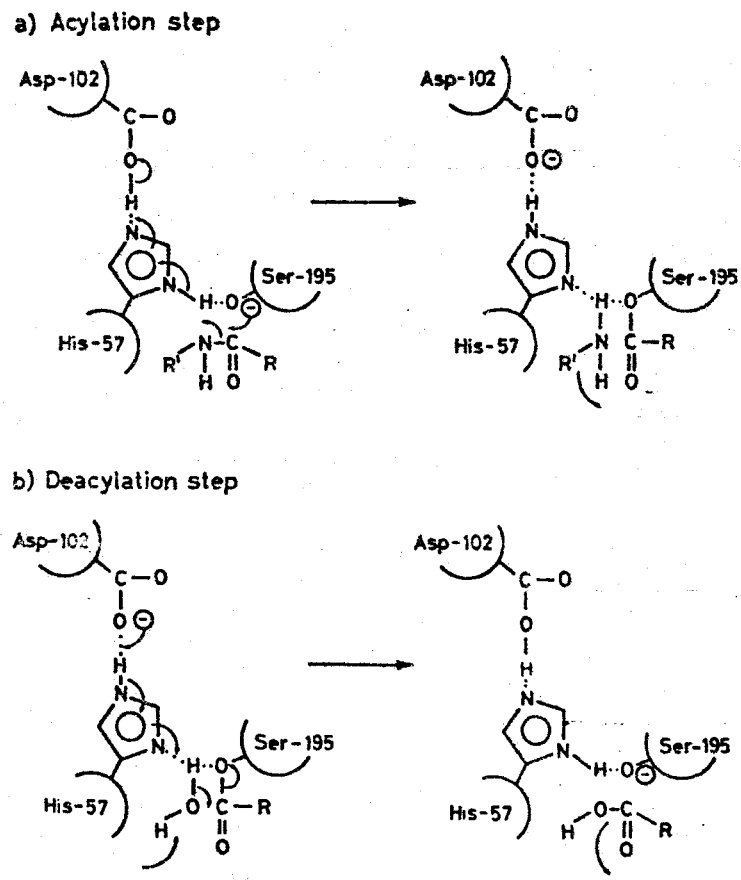
Explanation of Mechanism

Many features in the mechanism of action of Cht catalysis are known. However, there are gaps in our knowledge of this intricate process which must be understood if we are to fully comprehend it. This endeavor is complicated by the fantastic speed at which enzymic reactions take place. The participation or movement of a certain residue for only a fraction of a second can make it almost impossible to detect.

Investigators have used kinetic, spectroscopic, chemical modification and x-ray experiments to pinpoint the involvement of amino acid residues in the catalytic mechanism. These studies made it possible to formulate a hypothesis for the mechanism of Cht catalysis implicating the Ser195, His57 and Asp102 residues as participants.

Our present perception of the mechanism of Cht catalysis, refined from the x-ray structures of Chtgen, Cht and other derivatives of Cht is summarized in Figure 3 (20, 29). At neutral pH, a hydrogen bond network exists between Asp102, His57 and Ser195 which is referred to as the "charge transfer complex" (20). A hydrogen bond is formed between the negatively charged Asp102 carboxylate side chain and the N1 atom of the His57 imidazole side chain. This causes the N3 atom in the ring to attract and hydrogen bond the hydroxyl side chain of Ser195. The increased nucleophilic character of the γ oxygen of the Ser195 results in the formation of a covalent bond between it and the carbonyl carbon of a bound ester,

Figure 3 - A possible mechanism for a. acylation and b. deacylation by chymotrypsin. Each step may go through a tetrahedral intermediate (29).



amide or peptide substrate (k_2, acylation). It has been theorized that a tetrahedral high energy intermediate exists at this point in the reactions. The protonated N3 hydrogen on the imidazole ring of His57 is drawn to the oxygen or nitrogen of the ester, amide or peptide bond on the substrate donating a proton to the heteroatom and contributing to cleavage of the bond. This results in an acyl-enzyme intermediate (ES') and the alcohol or amine first product (P_1). Water enters the active site, replaces the alcohol or amine first product, and provides a nucleophilic oxygen to attack the carbonyl carbon of the substrate. Another tetrahedral high energy intermediate is probably formed prior to deacylation of the substrate (k_3). This deacylation step liberates the acid second product (P_2) and regenerates the original enzyme molecule. The whole reaction occurs extremely fast. Proteolytic hydrolysis of proteins by Cht takes place about 10^5 times faster than the non-catalyzed hydrolysis of proteins (7,8).

Essential Residues

Serine-195

A serine amino acid residue was first found to be essential in Cht when several investigators discovered that diisopropyl fluorophosphate (DFP) irreversibly inhibits Cht (DIP-Cht) (16,17). Analysis of peptides from protease digests of the DIP-Cht revealed that only a single serine was modified (later established as Ser-195 from the sequence studies). This fact was significant because there are a total of 26 serine residues in Cht and DFP reacts stoichiometrically with only one of them. The

investigators concluded that a reactive serine residue must be present in the active region of the enzyme. Similar experiments with trypsin, elastase and thrombin yielded identical results and for this reason all of these enzymes were designated as "serine proteinases" (30, 31, 32). The inhibition of acetylcholinesterase was also found to occur via a serine residue in the active site of this enzyme (33, 34).

Other chemical reagents were also found to inhibit chymotryptic activity. Diphenylcarbamyl chloride is one example of a carbamyl chloride that binds in the aromatic site of Cht and subsequently inhibits enzymic activity by reacting irreversibly with the Ser195 (35). The sulfonyl halides, phenylmethylsulfonyl fluoride (PMSF) and tosyl-fluoride, utilize the same principle in affecting inhibition of Cht (36). These are a few of the inhibition reactions which entail acylation of the reactive serine residue rendering Cht catalytically inert.

Another chemical modification procedure which results in an inactive species of Cht was reported in 1966 by Koshland and coworkers (37). This technique involved a water elimination reaction of the Ser195 residue converting it to dehydroalanine. Theoretically, this derivative of Cht should be inactive and experimental results have substantiated this. Dehydroalanine-Cht (also referred to as anhydro-Cht) is a particularly interesting form of Cht because the binding site should remain intact. Therefore, substrate binding studies can be carried out independent of the catalytic steps of a reaction. Another interesting feature of this derivative of Cht is the fact that the modification does not involve placing a large modifier molecule in the active site which may sterically inhibit catalysis.

After it was established that a particular serine residue was important in the activity of Cht, other investigators carried out kinetic experiments which offered indirect evidence for the currently accepted mechanism for Cht. Experiments utilizing *p*-nitrophenyl acetate (*p*NPA) resulted in the mechanism proposed by Hartley and Kilby (38). This explanation of Cht-catalysis was applied to the hydrolysis of specific ester and amide substrates by Gutfreund and Sturtevant (14, 15) and supported by the work of Bender and coworkers on different esters of N-acetyl phenylalanine (39). Additional evidence for this mechanism was also obtained by Balls and Alrich who were able to isolate the acyl-enzyme intermediate, acetyl-Cht, in the hydrolysis of *p*NPA at low pH (140). By labeling the acetyl group of *p*NPA with ^{14}C, hydrolyzing the stable acetyl-Cht intermediate with pepsin and pancreatine and isolating and identifying the labeled peptides Osterbaan and van Alrichem were able to strongly suggest that the acetyl group is bound to a serine residue, presumably the same serine which reacts with DFP (141). Furthermore, the rate of hydrolysis of denatured *trans*-cinnamoyl-α-Cht and the model compound O-cinnamoyl-N-acetylserinamide were found to be practically identical (142). These data indicate the involvement of a reactive serine residue in the mechanism of Cht catalysis.

Stopped-flow experiments have offered more indirect evidence for the proposed mechanism involving the Ser195 residue in Cht catalysis. Hess and coworkers followed the absorbancy change in Cht at 290 nm (caused by the formation of enzyme-substrate complex) during the hydrolysis of N-acetyl-L-phenylalainine methyl ester (40). Before steady state conditions were reached a gradual increase in absorbance occurred which was

interpreted as the quantitative acylation of the Ser195.

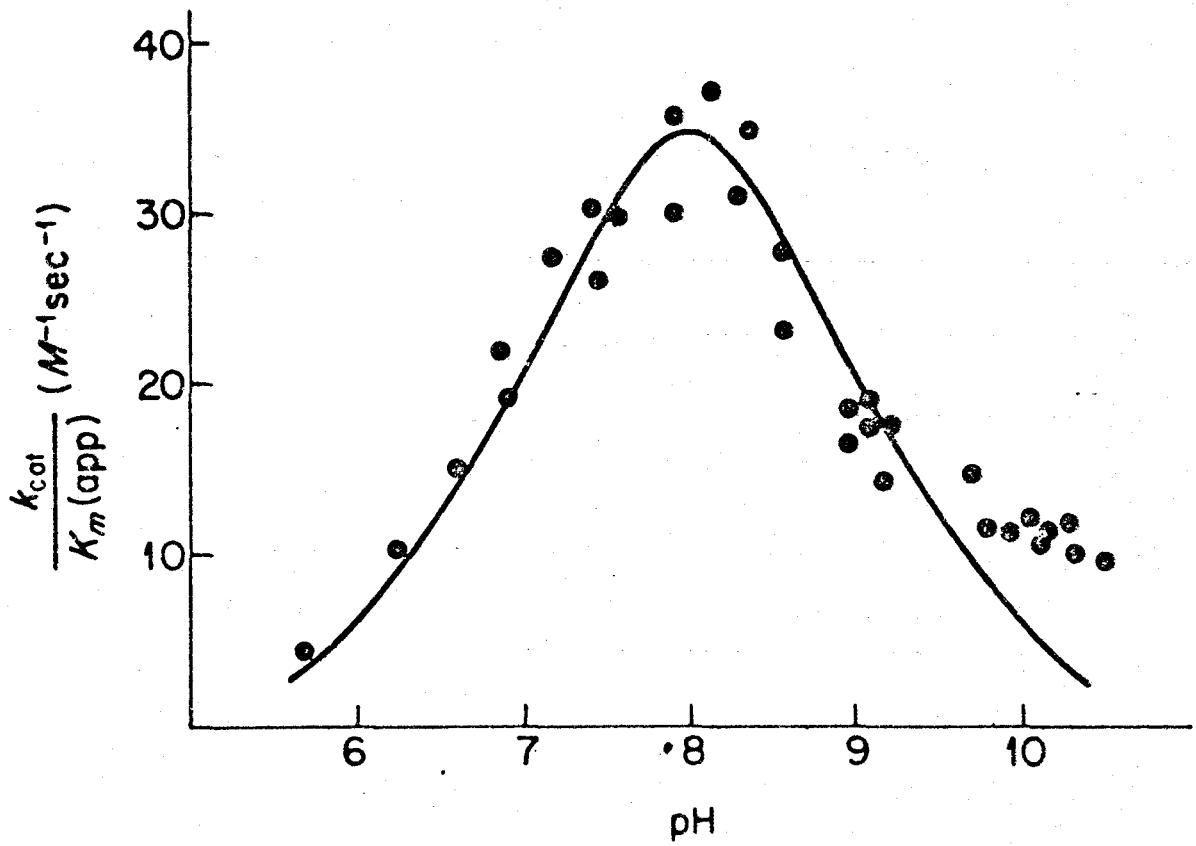
The x-ray work on Cht supports the active role of Ser195 in Cht catalysis. The results for tosyl-Cht definitely show a covalent bond between the γ oxygen of Ser195 and the substrate-like molecule at 2.0 Å resolution (5). Serious objections have been raised regarding the conclusions drawn from the x-ray work. These objections are based on the assumption that there are differences between the conformation of the enzyme molecules in solution and in crystalline form. However, recent experiments have shown that enzymic reactions actually take place between crystals of Cht and substrates infused in the crystals (41). These results, as well as x-ray structures of Cht with bound substrate analogs, dispute the objections and argue stongly in favor of the applicability of the x-ray crystallographic studies to the solution behavior of Cht.

In summary, there is direct chemical, indirect kinetic (steady-state and pre-steady-state) and x-ray crystallographic evidence for the participation of Ser195 in the catalytic action of Cht. The γ oxygen of this residue is thought to be the reactive part of the "charge transfer complex" which forms a covalent bond with the carbonyl carbon of substrates. This acyl-enzyme bond is then broken, regenerating the enzyme and completing the hydrolysis.

Histidine-57

In contrast to Ser195, His57 was first suspected as an essential catalytic residue by kinetic experiments rather than by chemical modification experiments (42). pH rate profiles, such as the one shown in Figure 4 for N-acetyl-L-tryptophan amide, revealed that the activity of Cht (expressed as k_{cat}/K_m) was dependent upon the ionization of amino

Figure 4 - A pH-rate profile from steady state kinetic data on the chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide at 25°C (43).



acids with pK_{app}s of 7.0 and 8.5 (43). Since the histidine side chain is known to have a pK_a of 7.0, it was automatically a candidate for participation in the mechanism of action of Cht. Stopped-flow experiments also showed that k_2 and k_3 were dependent on an ionizing group with a pK_{app} of 7.0 (44).

Chemical modification experiments established His57 as an essential catalytic residue when photooxidation and selective alkylation of this residue caused significant decreases in enzymic activity (18, 19, 45, 46). The photooxidation modifications were not specific for His57 since irradiation of the enzyme could affect other residues, particularly Met192. Amino acid analyses of the hydrolysates of the irradiated enzyme substantiated this (47). Shaw and coworkers carried out alkylation of a histidine residue in Cht with L-1-tosyl-amido-2-phenylethyl chloromethyl ketone (TPCK) (18, 19). This reaction caused complete loss of enzymic activity which demonstrated the importance of a histidine residue in the catalytic mechanism. The specificity of this reaction was verified by amino acid analyses. The analyses indicated that only one of the two histidines of Cht was modified and sequence studies later established the His57 residue as the one modified. Several groups have succeded in specifically methylating the His57 on the N3 atom of the imidazole ring (48, 49). Their observation of a significant decrease in enzymic activity in this form of the enzyme contributed to the evidence for His57 being designated an essential catalytic residue.

The chemical and kinetic studies on the active site His57 residue of Cht however, did not help explain the exact role of this residue in catalysis. Investigators had suggested the formation of N-acyl-imidazole

intermediates to explain His57's role in Cht catalysis (50, 51). This theory was contradicted by the fact that there was no kinetic and spectroscopic evidence for this intermediate and also by the x-ray crystallographic data (5). The results supported an earlier model proposed by Cunningham where one of the imidazole nitrogens acts as a hydrogen bond acceptor of the Ser195 hydroxyl group making the γ oxygen a better nucleophile (52).

In more specific terms His57 is thought to act as a general acid-base during catalysis. The N3 of the imidazole ring is believed to act as a general acid-base catalyst when it transfers a hydrogen to the leaving group of the substrate. A water molecule enters the active site and forms a bridge between the N3 of the imidazole ring of His57 and the carbonyl carbon of the substrate. The His57 then acts as a general acid-base catalyst in abstracting a hydrogen from water. At this time there is experimental evidence for His57 acting as a general base catalyst (53, 54). The evidence consists of a definite decrease in the Cht hydrolysis of N-acetyl-L-tryptophan ethyl ester in deuterium oxide (D_2O) as opposed to water (H_2O). If general base catalysis by His57 was not involved, there would either be no change or an increase in the rate of hydrolysis of substrate.

Aspartic Acid-102

Even though Asp102 does not qualify as an essential catalytic residue according to the definition previously stated it is generally referred to as an essential residue. No specific chemical modification of the Asp102 carboxylate side chain has been accomplished (55) and none of the

non-specific modifications have led to the complete loss of enzymic activity (56). Despite this, it cannot be unequivocally stated that modficiation of this residue will not result in complete loss of enzymic activity.

The x-ray structure of Cht offers the best evidence for the participation of Asp102 in the action of Cht (19). The carboxylate side chain of Asp102 is within hydrogen bonding distance of the N1 of the His57 imidazole ring. Presumably electrons are donated from the Asp102 carboxyl side chain to the hydroxyl group of Ser195 via the His57 imidazole ring. It has been proposed that this "charge transfer or proton shuttle complex" makes it possible for the γ oxygen of Ser195 to become a better nucleophile which is a prerequisite for catalysis (20).

Non-essential Residues

Isoleucine-16

Desnuelle and coworkers and Neurath and Gladner discovered that a free positively charged α-amino group of an isoleucine residue was necessary for activity in Cht (58, 59). They found two basic forms of active Cht, δ-Cht which contains only one extra α-amino group (Ile16) compared to Chtgen and α-Cht which contains two (Ile16 and Tyr146). From these results they concluded that the α-amino group of Ile16 was necessary for activity in Cht.

Other experiments dealing with kinetics of Cht-catalyzed reactions offered indirect support of this finding (12, 42). The pH-rate profile in Figure 4 shows that Cht activity is dependent on an ionizing group

with a pK_a of about 8.5. This pK_a lies very close to the pK_a of an N-terminal group. Therefore, it was suggested that deprotonation of the N-terminal Ile16 (formed in the activation of Cht from Chtgen) accounts for the loss of enzymic activity observed at high pH.

Specific chemical modification of the α-amino group of Ile16 supports the importance of this residue in chymotryptic activity (21, 60, 61). In the experiments of Oppenheimer et. al. and Ghélis et. al. all free amino groups of Chtgen were first acetylated with acetic anhydride (21, 60, 61). The modified Chtgen was activated to δ-Cht (A-δ-Cht) and found to be completely active. Active A-δ-Cht was then reacetylated in order to specifically acetylate the Ile16. Loss of Ile16 α-amino group was measured by N-terminal analysis which correlated well with loss of enzymic activity.

Due to this specific acetylation, the Ile16 cannot acquire a positive charge. The Cht molecule appears to be in a conformation similar or identical to the inactive, high pH (pH>9) conformation (where Ile16 is deprotonated) and the zymogen form of the enzyme (where Ile16 is in peptide linkage). Circular dichroism (CD) spectra, sensitive to conformational changes in proteins, revealed that the conformation of acetylated (Ile16)-δ-Cht was virtually identical to that of Chtgen and δ-Cht at high pH (62). The CD spectra for acetylated (Ile16)-δ-Cht were different from those of δ-Cht at neutral pH. The similarity between the three forms of the enzyme mentioned above is their inability to acquire a positive charge on the α-amino group of the Ile16 residue. Therefore, the necessity for a positively charged α-amino group of Ile16 in the catalytically active conformation of Cht was clearly indicated. The question remained however,

why this positive charge on Ile16 was so important.

The mystery concerning Ile16 was solved when the x-ray structures of Cht and Chtgen became available (5, 6). In the structure of active Cht an ion-pair exists between the positively charged group of Ile16 and the negatively charged carboxyl group of Asp194. On the contrary, the x-ray structure of Chtgen shows the Ile16 in peptide linkage on the surface of the molecule and Asp194 ion-paired to His40. Apparently, upon activation of Chtgen to Cht the newly formed α-amino group of Ile16 becomes postively charged, folds into the interior of the molecule, attracts the negatively charged carboxyl group of Asp194 and stabilizes the active conformation of the enzyme. This explains the necessity for the positive charge on the Ile16 in active Cht. These findings coupled with the chemical modification and CD results strongly suggested that the Ile16-Asp194 ion-pair is a prerequisite for the active conformation of Cht.

Aspartic Acid-194

Chemical modification of the carboxyl group of Asp194 would be expected to cause loss of enzymic activity in Cht just as modification of the Ile16 did. According to the x-ray structure the modification would prevent the ion-pair formation. Koshland and coworkers were successful in blocking the carboxylate group of Asp194 with glycine methyl ester causing complete loss of active sites (63). Therefore, chemical modification of both Asp194 and Ile16 substantiated the necessity for the ion-pair between these two residues and complemented the x-ray work done on Cht concerning them.

Alanine-149

The importance of the Ala-149 residue in Cht activity received attention when it was found that δ-Cht is not completely inactive at high pH (64, 65) as α-Cht is. The kinetic parameters of δ-Cht and α-Cht hydrolysis of specific ester and amide substrates at high pH indicated that the K_m values for the δ-Cht reactions were considerably lower than those of the α-Cht reactions at high pH (64). In addition, a new form of Cht, α_1-Cht, was isolated which possessed a Thr147 amino terminus (66, 67, 68, 69, 70). The K_m values for α_1-Cht catalyzed hydrolysis were also much lower than those for α-Cht at high pH (70). The results for α_1-, α- and δ-Cht were taken as strong evidence for the participation of the Ala-149 amino terminus in the control of enzymic activity in the alkaline pH region. It was further suggested that the deprotonation of the Ala-149 amino terminus is a major factor in the disruption of the substrate binding site in α-Cht in contrast to the deprotonation of the Ile16 amino terminus which is a minor factor (69). Further experiments on the amino terminal residues of α-Cht showed a dependence between the Ala-149 and Ile16 amino terminal groups (71). The author suggested that the additional cleavage of the Thr147-Asn148 dipeptide from δ-Cht by Cht weakens hydrophobic interactions in the Ile16 amino terminal region and makes the native structure of α-Cht more sensitive to deprotonation of the Ile16 amino group (71). Therefore, Ala149 must be considered an important nonessential residue just as Ile16 is.

Methionine-192

Methionine-192 (Met192) is referred to as a non-essential residue in Cht catalysis because modification of this residue results in only partial rather than complete loss of enzymic activity. The basic modifications that have been carried out are oxidation and alkylation. The results have varied from 55% to 80% loss of activity (72, 73, 74).

Even though the role of Met192 in Cht catalysis has been disregarded due to the results obtained, there is renewed interest in this residue. The x-ray structure of Cht and Chtgen made it possible to take a close look at the Met192 and speculate on its function. The movement of the Met192 residue in Cht upon binding dioxan was shown in an x-ray diffraction study of active Cht and Cht-dioxan crystals (75). This was the first indication that Met192 may play a more important role in catalysis than was previously believed. A more significant movement of the Met192 residue was detected when the x-ray structure of Chtgen was elucidated (6). This data suggested that the movement of the Met192 was of major significance in the activation process of Cht from the zymogen, Chtgen.

This has been a brief introduction to the Met192 residue and an overview concerning its role in the activation and catalytic mechanism of Cht. It will be treated in a more detailed manner in a later part of this introduction.

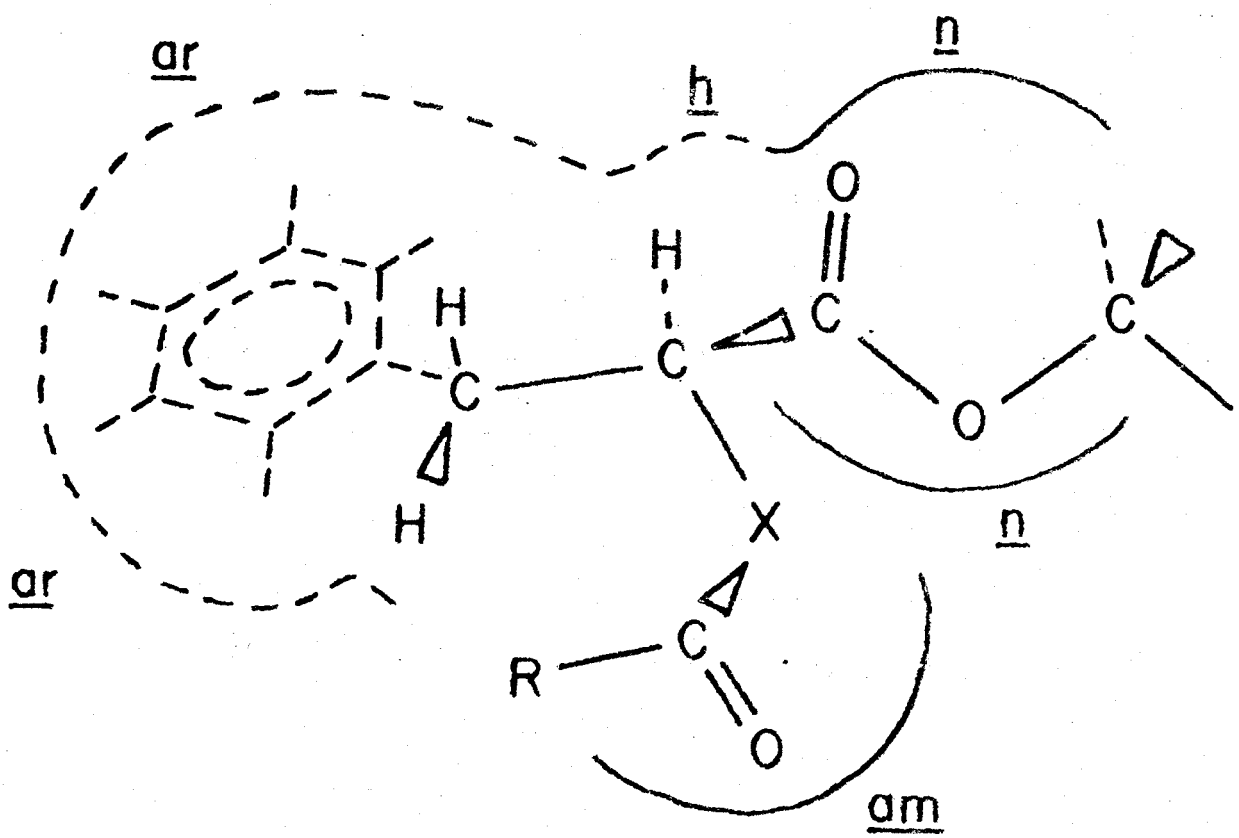
Topology of the Active Site

The extensive experimentation on Cht and its substrates has led to a topographical description of the active site (see Figure 5). Although many investigators have contributed to this model Niemann and coworkers and Cohen and coworkers were basically responsible for its construction (76, 77, 78, 79). It is based on the good substrate N-acetyl-L-phenylalanine methyl ester and is composed of four regions complementary to the different parts of the substrate. They are:

1. The *ar* site responsible for binding the phenyl substituent.
2. The *am* site responsible for hydrogen bonding the acylamido substituent.
3. The *h* site responsible for binding the hydrogen of the α-carbon.
4. The *n* site responsible for hydrolysis of the substrate.

The x-ray structure of Cht made it possible to identify the residues in each particular site. The *ar* site is the hydrophobic binding pocket and contains various hydrophobic residues. The *am* site contains the Ser214 residue which hydrogen bonds the amide portion of the substrate. The *h* site is composed of the Met192 residue which accomodates the hydrogen of the α-carbon of the substrate. The *n* site naturally contains the essential Ser195 and His57 residues which function to cleave the ester or amide bond of the substrate.

Figure 5 - Topology of the active site of chymotrypsin according to Niemann et. al. (76) and Cohen et. al. (77, 78, 79).



The Activation Process

The Nature of Activation Mechanisms in Biochemical Processes

The activation of inactive precursors (zymogens)of proteins by limited proteolysis is a common phenomenon in biochemical processes. In addition to the case of Cht which has already been mentioned, other examples include: the proteolytic enzymes trypsin (80), pepsin (80), carboxypeptidase (80), elastase (81) and streptococcal proteinase (80), the blood clotting proteins thrombin (80), fibrin (80) and plasmin (82), various proteins in the complement system (83) and the hormones insulin (84, 85), renin (80) and angiotensin (86). All the examples above exist as zymogens and are activated by the cleavage of certain peptide bonds within their structures. In some cases such as Chtgen, trypsinogen, prothrombin and fibrinogen only a few amino acids are excised from the molecule (80) while the activation of proinsulin to insulin liberates a long chain of 33 amino acids from the molecule (84, 85).

The activation processes for the examples stated above serve several diverse functions. In two of the cases, complementation and blood clotting, they function as a regulatory mechanism. Even though all of the necessary components for the reactions are present in the blood stream, the systems cannot be activated until a certain event takes place. In the complement system the fixation of antibody to the surface of bacteria is the signal which sets the cycle into action (83). Likewise, in the cascade mechanism of blood clotting chemical substances from the vicinity of the damaged tissue initiate the chain of activation

events ultimately leading to a blood clot (80). The activation process may also serve a structural role as in the case of insulin activation. It has been suggested that the entire proinsulin molecule is required for proper folding of the protein so that excision of the 33 amino acid chain results in a conformation of insulin which is biologically active (84, 85). A third function of the activation process concerns protection of the body from the action of proteolytic enzymes. These enzymes must remain inactive upon synthesis until they reach their particular site of action. The proteolytic enzymes, secreted as zymogens by the pancreas, are classic examples since premature activation could result in destruction of the pancreas.

In summary, the activation of biological molecules by limited proteolysis is ubiquitous in biochemical processes. This phenomenon probably evolved from a common mechanism which presently includes diverse functions in the body (i.e. blood clotting, complementation, hormone activation and proteolytic enzyme activation).

Interesting Features of Chymotrypsinogen and Its Activation

Certain similarities and differences exist between Chtgen and active Cht. Chtgen-A is composed of a single polypeptide chain of 245 amino acids whereas, active Cht is either composed of two polypeptide chains (i.e. δ-Cht) of 243 amino acids or three polypeptide chains (i.e. α- or γ-Cht) of 241 amino acids. The molecular weights of Cht and Chtgen vary between 24,200 and 25,000 respectively. These forms of the enzyme possess five disulfide bonds which help hold them together in a globular structure with little α-helical content and more β-structure.

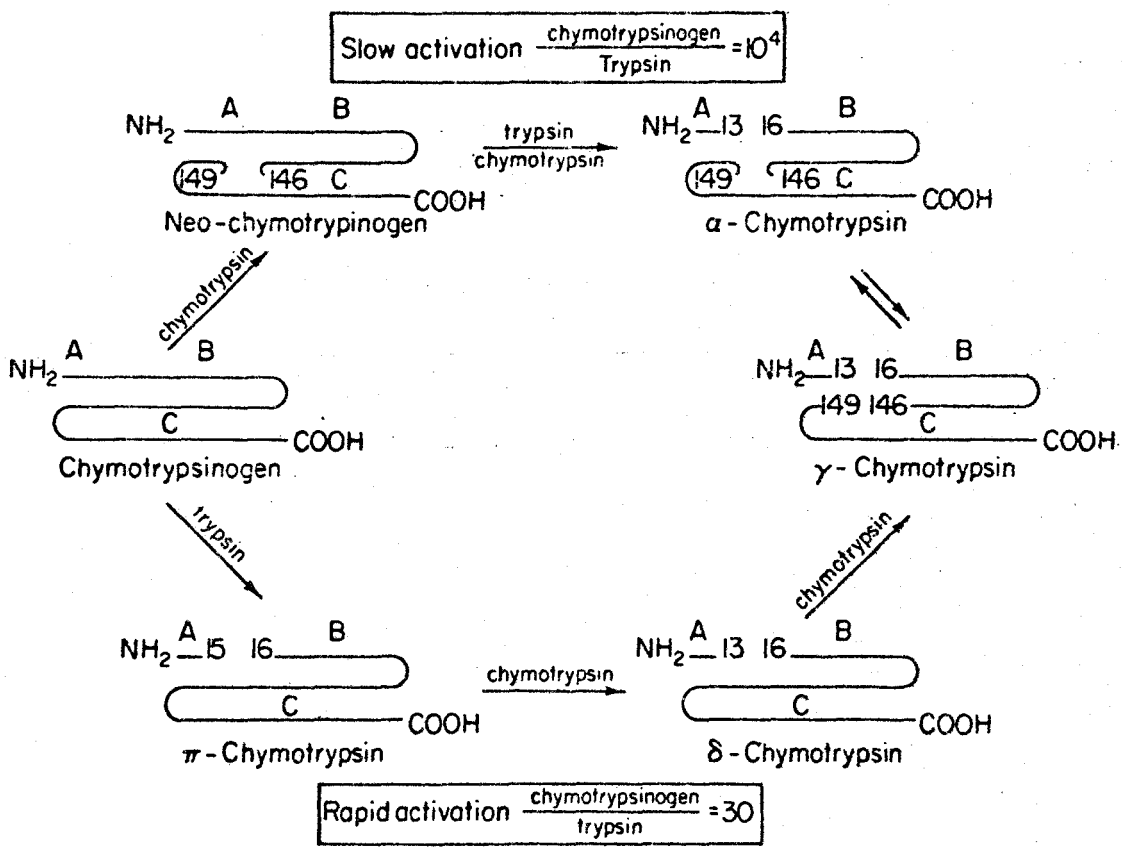
The activation scheme in Figure 6 summarizes the different forms of Cht and presents the two possible routes of activation (3, 87). In the slow activation process, substantial quantities of neo-Chtgen are generated due to the high ratio of Cht to trypsin. Neo-Chtgen, as well as Chtgen, remains inactive since the Arg15-Ile16 bond is intact. The Tyr146-Thr147 and Asn148-Ala149 bonds, susceptible to chymotryptic attack, are cleaved before trypsin can cleave the Arg15-Ile16 bond. Ultimately α-Cht is generated upon hydrolysis of this bond. In the rapid activation the Arg15-Ile16 bond is hydrolyzed quickly due to the lower Cht/trypsin ratio. This results in π-Cht and finally δ and γ-Cht. Although γ and α-Cht have identical amino acid sequences conformational differences have been found which distinguish them from one another. All of the active forms, π, δ, α and γ, have relatively the same activity toward substrates investigated to date (87).

A Structural Comparison of Chymotrypsinogen and Chymotrypsin

The x-ray structures of Cht and Chtgen are very similar with the exception of a comparatively small number of amino acid residues (about 21) (5, 6). It is obvious that the movement of these residues upon activation accounts for biological activity and is, for this reason, of utmost importance in understanding the activation process.

Table 1 lists some of the significant changes between the x-ray structures of Chtgen and Cht (6). A major difference is the existence of seven amino acid chain 187-193 in a more extended conformation in Cht compared to Chtgen. The x-ray structures of Chtgen and active Cht indicate that this movement helps form the specificity pocket of Cht for

Figure 6 - Scheme for the activation of chymotrypsin from chymotrypsinogen (86).



substrates. The Ile16 residue previously in peptide linkage on the exterior of the Chtgen molecule folds into a buried position where it is within ionic bonding distance of the carboxylate group of Asp194. Asp194 moves 4 Å from an ion-pair with His40 to its new position in Cht. Previous experiments on these two residues support the hypothesis that this ion-pair is required to maintain a functional active site. Gly193 moves 6.6 Å to replace Asp194 by hydrogen bonding His40. The Arg145 side chain moves 9 Å from a possible association with His40, Asp194, and Ser195 on the surface of the zymogen to a more extended position in active Cht. Upon activation of Chtgen the Ile99 side chain rotates 90° from van der Waals contact with the imidazole side chain of His57 to a more open conformation. Some investigators have suggested from the x-ray structures that Ile99 blocks access of solvent to the His57-Asp102 region of the active site in Chtgen and in this way contributes to the inactivity of Chtgen. Finally, Met192 undergoes an alteration in the environment of its side chain from a deeply buried position in Chtgen to a surface position in Cht.

The Participation of Methionine-192 in Chymotrypsin In The Activation of Chymotrypsinogen

Several chemical modification experiments using different modifying reagents established that one of the two methionines in Cht is accessible to solvent (Met192) while the other is buried and inaccessible (Met180). Photooxidation experiments first indicated this situation (47). During the course of the photooxidation reaction of Cht aliquots were removed, hydrolyzed and assayed on an amino acid analyzer. In this way rate constants of oxidation could be determined for the residues affected. The results revealed one methionine residue which is easily oxidized with a rate constant of 0.36 min^{-1} and another which is more difficult to oxidize with a rate constant of 0.037 min^{-1}. Specific alkylation of one of the methionines with *p*-nitrophenyl bromo-acetyl-α-aminoisobutyrate also pointed to an accessible methionine residue (22, 73). This bifunctional reagent first acylates Cht on the Ser195 and then alkylates a methionine in the vicinity of the active site. Amino acid analysis of the alkylated Cht confirmed that only one methionine was modified. Tryptic digestion and chromatography of Cht oxidized with hydrogen peroxide (H_2O_2) showed that the susceptible methionine is three residues from the essential serine residue (Ser195) in the active site (78). The use of 8 M urea to denature Cht made it possible to easily oxidize the other methionine residue with H_2O_2. This experiment supports the hypothesis of a buried methionine (Met180) in Cht (88). In a more recent study Chtgen and Cht were reacted

with H_2O_2 for varying periods of time over a wide temperature range (4-60°C). The analysis of these forms of oxidized Cht confirmed the previous conclusions concerning the two methionines in Cht (89). The chemical evidence for the existence of a buried (Met180) and exposed methionine (Met192) in Cht is therefore, quite convincing.

The x-ray crystallographic data on the structure of Cht placed one of the methionines in the locus of the active site and the other in a buried position (4). The side chain of the active site methionine sits on the surface of the molecule where it would be accessible to solvent. The other methionine is surrounded by residues in the inner hydrophobic environment. The correlation between chemical studies and the x-ray diffraction studies clearly established the positions of the two methionine residues in Cht.

X-ray studies of Chtgen made it possible to compare the zymogen and active structures of Cht. One of the most striking features to emerge from this comparison is the movement of the Met192 from a buried position in Chtgen to an exposed or surface position in active Cht (6). The movement of Met192 is only part of an extensive rearrangement of the 187-193 peptide segment. This peptide segment assumes a more extended conformation upon activation and apparently forms part of the specificity cavity (6). It has been suggested that rotation of the main chain about the carbonyl-carbon-α-carbon bond of Asp194 is responsible for this movement. The rotation appears to be caused by the formation of the Asp194-Ile16 ion-pair which occurs upon tryptic cleavage of the Arg15-Ile16 bond and protonation of the newly formed α-amino group. It is obvious from the structural studies that the major movement of Asp194 profoundly affects the position of the Met192 in Cht.

A theory on the activation of Chtgen has been proposed from the x-ray structures of Chtgen and Cht by Freer and coworkers (6). It states that upon activation the peptide segment 187-194 and the Ile16 residue rearrange in a "single unified event". This type of all-or-none mechanism predicts that the inactive high pH form of Cht is in a *zymogen-like* conformation. An x-ray study of the crystalline forms of α-Cht in the pH range from 1 to 10 has recently been completed (90). The Met192 occupies a different position in the alkaline structure of α-Cht than it does in the structure of α-Cht at lower pH. However, the Met192 remains on the surface of the protein throughout the movement and never reburies into the interior of the molecule as the "single unified event" theory would have it. Blocking the Ile16-α-amino group by acetylation would also generate an inactive conformation of Cht similar to the high pH form (61). Theoretically, the Met192 thio-ether side chain would rebury into the interior of the molecule and therefore be inaccessible. A perfect test of the theory could be easily carried out by attempting to alkylate or oxidize the Met192 residue of acetylated (Ile16)-Cht. This type of experiment would be helpful in either confirming or contradicting the "single unified event" theory.

Electron spin resonance (ESR) experiments by Kosman have also indicated differences in the environment of the Met192 in Cht compared to Chtgen (91). Cht and Chtgen were specifically labeled with an ESR reagent which is a nitroxide analog of bromoacetamide. ESR spectra were then taken during the activation of Chtgen to Cht and upon increasing the pH environment of Cht. Activation of Chtgen decreased the ESR peak sensitive to the spin label. This change was interpreted as a loss of rotational freedom by the spin label. Conversely, an increase in the

ESR peak of the spin label upon increasing the pH environment of α-Cht indicated greater rotational freedom of the label. A greater degree of rotational freedom can also be looked on as a lessening of protein-label interaction. In this respect, the thio-ether side chain of Met192 seems to be further away from the surface of the protein in the high pH form of Cht. This is in contrast to what is predicted from the Freer hypothesis where the Met192 would rebury into the interior of the protein molecule at high pH. Questions also arise concerning the ESR pattern obtained for Chtgen (similar to that of the high pH form of Cht). The lesser degree of protein-label interaction for Chtgen compared to Cht is again at odds with the x-ray and chemical modification studies of Chtgen and Cht. Therefore, the interpretation of the ESR data in this particular set of experiments does not agree with the movement of the Met192 residue predicted from the currently accepted hypothesis.

One chemical modification experiment has been done recently by Neurath and coworkers which directly tests the "single unified event" hypothesis concerning Met192 (92). The Met192 side chain of Chtgen was selectively oxidized to the sulfoxide form (MS-Chtgen) by a procedure developed by Wasi and Hofmann (89). The activities of Chtgen and MS-Chtgen were determined for the hydrolysis of certain *p*-nitrophenyl esters. The rate of reaction for MS-Chtgen increased 7 to 8-fold compared to that of Chtgen alone. The increased rate of reaction of MS-Chtgen may be due to an increased rate of acylation of the Ser195. This is implied from the finding that the rate of deacylation and pH dependence remained unchanged. The investigators concluded that one possible explanation for the increased reactivity of modified Chtgen was a shift toward a

more active conformation caused by the increased size and hydrophilic nature of the Met192 side chain upon oxidation. Due to its size the Met192-sulfoxide side chain cannot occupy the same position in Chtgen that the Met192 thio-ether side chain occupies in Chtgen. The hydrophilic nature of the Met192-sulfoxide group would also tend to prevent its association with the interior hydrophobic residues of Chtgen. These two considerations point to the probable outward movement of the Met192 side chain. The "single unified event" hypothesis predicts that this type of a movement automatically affects the other residues, partially forms the specificity cavity and induces enzymatic activity. The experimental results qualitatively support the hypothesis.

In summary, there are three studies which directly address the question of the participation of the Met192 residue in Chtgen activation:

1. X-ray crystallographic comparison of the structures of Chtgen and Cht. A dramatic movement of the Met192 side chain occurs from a buried interior position to an exposed surface position. The movement is just part of a rearrangement that affects a certain peptide segment 187-194 and is hypothesized to occur in a "single unified event" upon activation.

2. An electron spin resonance study involving the labeling of the Met192 side chain. The results are not definitive and do not agree with the x-ray studies.

3. A specific oxidation of the Met192 side chain of Chtgen. The reactivity of Met192-sulfoxide-Chtgen increased toward *p*-nitrophenyl esters by a factor of 7-8 compared to Chtgen. These findings support the "single unified event" hypothesis based on the x-ray crystallographic structures of Chtgen and Cht.

In the Mechanism of Chymotrypsin Catalysis

Chemical modification studies of the methionines in Cht have been concerned with effects on the catalytic activity of the enzyme from the outset. As previously mentioned the effects have varied from a loss of 55% activity upon oxidation to a loss of 80% activity upon alkylation with a specific reagent (72, 73, 74). A close examination of the results obtained from the chemical modification experiments performed over the past 15 years provides a basis for understanding the role of the Met192 in Cht catalysis and indicates the right direction in which further experimentation should be directed. Thus, a review of the literature on chemical modification of the Met192 residue follows.

Oxidation of the Met192 residue of Cht has undoubtedly been the most popular chemical modification used to date. The photooxidation experiment done by Koshland and coworkers in 1960 was the forerunner of all oxidations of this residue (47). However, conclusions regarding the rate constant of photooxidation of the accessible methionine (Met192), 0.36 min^{-1}, and the rate constant of the loss of enzymic activity upon photooxidation, 0.67 min^{-1}, were limited due to the fact that a histidine residue (His57) also displayed a rate constant of photooxidation, 0.32 min^{-1}, similar to that of Met192. The authors pointed out the obvious fact that the sum of photooxidation rate constants approximately equaled the rate constant of inactivation of enzyme and therefore, suggested analogous involvement of a methionine residue and a histidine residue in Cht catalysis. This implication of a major role for a methionine residue in Cht catalysis generated a great deal of interest toward the accessible methionine (Met192).

Specific alkylation of a methionine in the vicinity of the active site of Cht was reported by Lawson and Schramm in 1962 (22). This alkylation involved the bifunctional reagent, *p*-nitrophenyl-bromoacetyl-α-aminoisobutyrate, which was found to initially acylate the essential serine (Ser195) and subsequently alkylate an accessible methionine residue. The reaction, at pH 5, resulted in a 78% loss of enzyme activity using N-acetyl-L-tyrosine ethyl ester (ATEE) as substrate. The investigators further determined that there was an 11 fold increase in K_m upon alkylation indicating a distorted substrate binding site.

Later in 1962, Koshland and coworkers demonstrated significant decreases in Cht activity by specific oxidation and alkylation of the accessible methionine (74). Only 37% of the original ATEE enzyme activity remained after one of the two methionines was completely oxidized with H_2O_2 under mild conditions. A Lineweaver-Burkeplot for this enzyme derivative revealed a 4-fold increase in K_m for ATEE hydrolysis. Qualitatively, this finding agreed with the alkylation results alluded to above concerning distortion of the substrate binding site. The V_{max} for the reactions decreased only 10% indicating little effect on the catalytic steps of hydrolysis. In addition, these investigators specifically alkylated a methionine residue in Cht with iodoacetate. The decrease in ATEE enzyme activity (70%) was the only parameter measured in this case and was similar to that for the H_2O_2 oxidation (64%). Koshland's group reached the conclusion that the accessible methionine in Cht is not an essential residue since activity remained after it was completely modified, either by oxidation or alkylation.

Schachter and Dixon substantiated the H_2O_2 oxidation results reported above and perfected techniques for the quantitation of oxidized methionine in Cht (87, 93). Their procedure entailed a secondary alkylation with ^{14}C labeled iodoacetate or iodoacetamide. Under denaturating conditions, these reagents reacted with non-oxidized methionines after oxidation had been carried out. The main advantage to this technique was the direct measurement of alkylated methionine by liquid scintillation counting of the oxidized-alkylated samples of protein. These investigators also concluded that modification of the active site methionine (Met192) decreased the affinity of Cht for substrates.

In 1965 Knowles used sodium periodate to selectively oxidize one methionine in Cht (72). He observed a 55% decrease in enzyme activity to L-tyrosine ethyl ester hydrolysis which was 9% less than Koshland's result (64%). More specific information concerning enzymic activity was obtained for four different substrates. The acylation and deacylation rate constants for the monosulfoxide(Met192)-α-Cht(MS-α-Cht) hydrolysis of three specific substrates (N-acetyl-L-tryptophan ethyl ester, N-acetyl-L-tryptophan amide and N-acetyl-L-valine ethyl ester) and one non-specific substrate (*p*-nitrophenyl acetate) were unchanged. The K_m for reactions with the two aromatic substrates mentioned above doubled while it remained the same for reactions with the two non-aromatic substrates. The results supported the previous observations linking a methionine residue to the binding site of Cht. It also offered evidence that methionine does not affect the catalytic steps in Cht reactions.

In 1966, Hartley revealed the full amino acid sequence of Chtgen and positively identified the active site methionine 3 residues away from the reactive serine (Ser195) as Met192 (1).

The kinetic parameters of Cht alkylated with Lawson and Schramm's bifunctional reagent, *p*-nitrophenyl-bromoacetyl-α-aminoisobutyrate, (73) were determined by Kézdy and coworkers in 1967 (94). In contrast to the observations reported by Knowles (72) the k_3 increased 3-8 fold for various substrates studied. Alkylation had no effect on the binding of two aromatic substances, benzamide and hippuramide, but did affect the binding of indole and N-acetyl-L-tryptophanamide by increasing the K_m by a factor of 3-5. Although these experimental results appeared to strengthen the growing hypothesis that the accessible Met192 is located in the specificity cavity of the active site, Kézdy and coworkers proposed an alternative explanation. They reasoned that the bulky alkyl group on the modified enzyme decreased binding of substrate by interaction at the acylamido site (*am* site) instead of the aromatic site (*ar* site). They said that the k_3 increased due to an interaction of the alkyl group with substrate at this site.

Reaction of Met192 with two aromatic alkylating reagents was also carried out in 1967. Hille and Koshland used 2-bromoacetamido-4-nitrophenol to alkylate α-Cht (95). The modified enzyme was found to stoichiometrically react with ^{14}C labeled phenylmethylsulfonyl fluoride (PMSF), an active site titrant for the Ser195 residue. On the other hand, the activity to ATEE hydrolysis decreased by a factor of 10. This great loss of activity (90%) to a specific ester substrate was attributed to the aromatic group of the alkylating reagent decreasing the binding of ATEE to the enzyme. No specific data was presented to support this speculation. Sigman and coworkers also alkylated α-Cht with another aromatic alkylating reagent, α-bromo-4-nitroacetophenone (BrNAP) (96). The specificity of this reagent for the Met192 was

demonstrated by amino acid analysis as well as the appearance of a new long-wavelength absorption band centered at 350 nm for Cht-NAP. The simple statement that the enzyme derivative was inactive toward ATEE was not accompanied by any experimental data. As with Hille and Koshland's reagent, the results would lead one to believe intramolecular competitive inhibition at the substrate binding site was responsible for the decreased enzymic activity.

In 1971, Biltonen and Lumry studied the thermal unfolding of MS-α-Cht and disulfoxide(Met192, Met180)-α-Cht(DS-α-Cht) by following spectral changes in the enzyme at 293 nm (97). They found MS-α-Cht and α-Cht both exist in a folded state whereas, DS-α-Cht and Chtgen exist in a partially unfolded state. This result was significant because it demonstrated that modification of the Met192 by oxidation did not adversely affect the total conformation of the molecule. Previously, Koshland had suggested that the effect of modification of Met192 on enzymic activity was due either to a direct interaction with substrate or an indirect effect on the tertiary structure of the enzyme (74). The thermodynamic data presented above argues against the latter possibility. Kosman and Piette, in 1972, reported the ESR spectrum of α-Cht spin-labeled on Met192 was pH dependent (98). There was a notable increase in the ESR peak for the Met192 derivative of α-Cht indicating a greater degree of rotational freedom of the spin-label as the pH was raised from 3 to 11. The pKa in the acidic region was 3.8, corresponding to a carboxylic acid group, and the pKa in the basic region was 9.0, corresponding to an α-amino group. The residues which are most likely to be responsible for these changes are the Asp194 and Ile16 residues

which compose the ion-pair found in the x-ray structure of Cht. When taken as a whole, Kosman and Piette's research drew two basic conclusions: 1. alkylation of Met192 causes a loss of "structure" of the active site and 2. this loss of structure has little effect on the catalytically functional groups of Cht, but diminishes productive binding of substrates. These conclusions are consistent with the explanations proposed previously for modification of Met192.

Further studies were performed on α-Cht with a new oxidizing reagent, trichloromethanesulfonyl chloride, by Taylor and coworkers in 1973 (99). The reagent proved specific for Met192 and the properties of the resulting MS-α-Cht were found to be identical to those of H_2O_2oxidized Cht. The K_m increased by a factor of 3-4 for ATEE hydorlysis just as for the other oxidized samples. The authors proceeded to test the material for proflavin binding, circular dichroism changes with pH and susceptibility to autolysis. They found this derivative of the enzyme binds proflavin more effectively than does α-Cht, undergoes autolysis to a lesser degree than does α-Cht and has a circular dichroism pattern at 231 nm which is pH independent between 5 and 9. Fersht and coworkers had reported earlier that at neutral pH 15-20% of α-Cht exists in an inactive conformation due to the equilibrium between protonated and unprotonated states of the enzyme (100, 101). The molar ellipticity in the circular dichroism experiment for MS-α-Cht turned out to be 10-15% greater than the value for native α-Cht. The results all led to the conclusion that oxidation of the Met192 locks the protein in the active conformation. Theoretically, this could be caused by the inability of the Met192 sulfoxide side chain to revert to a *zymogen-like* conformation. The hydro-

philic nature as well as the size of this side chain could prevent the transition to an inactive form of the molecule.

Collectively, the results for the chemical modification of Met192 implicate its participation in substrate binding. The losses in Cht activity upon selective oxidation of Met192 were attributed to a 2 to 5-fold increase in K_m which represents decreased affinity for substrates. Decreases in enzymic activity upon specific alkylation of Met192 were also accompanied by increases in K_m up to 11-fold. These data led the primary investigators to believe the Met192 residue was located in the vicinity of the aromatic binding site (*ar* site) of Cht.

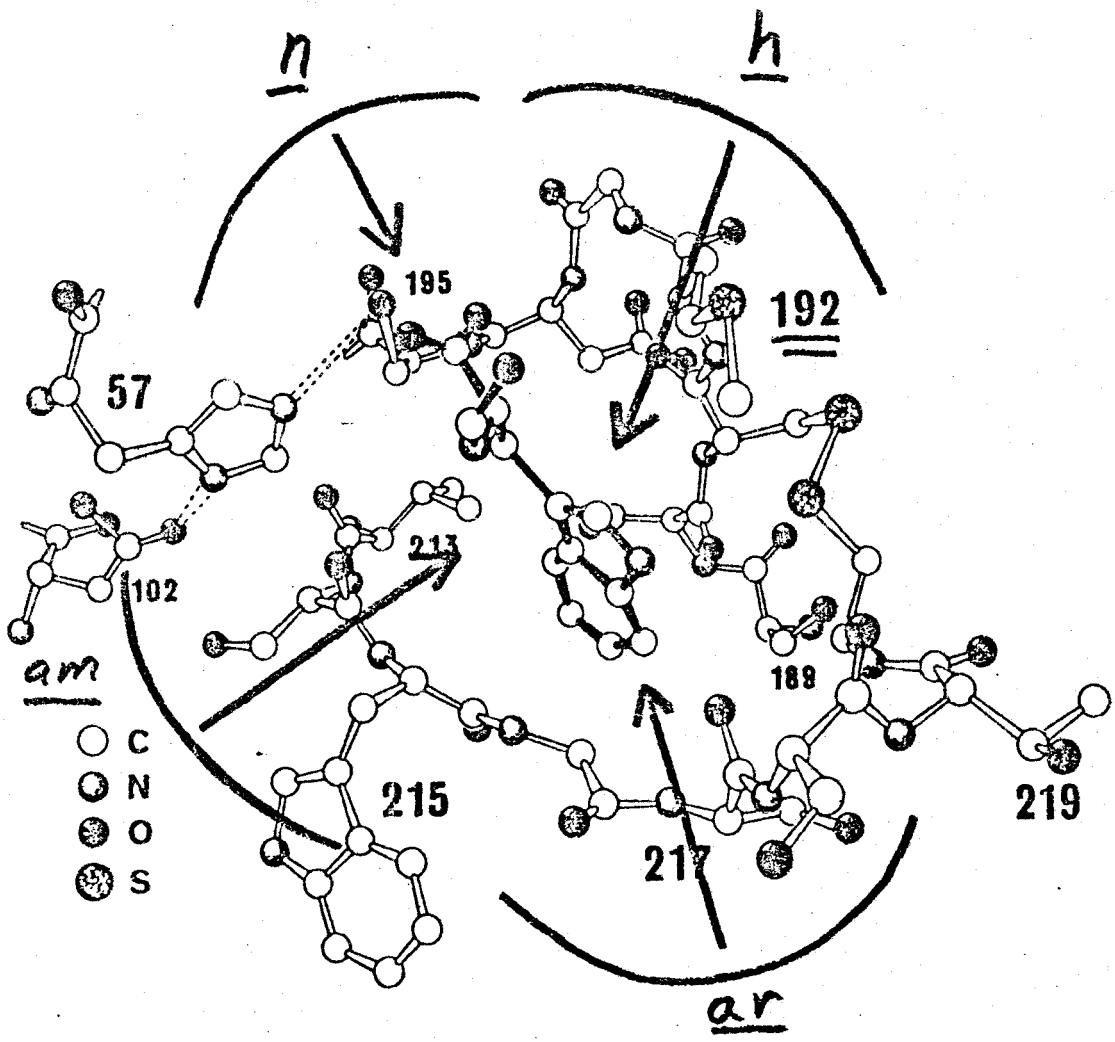
In most of the studies cited, modification of Met192 had little effect on the catalytic rate constant (k_{cat}) or the maximum velocity (V_{max}) of enzymic hydrolysis with one exception. A 3 to 8-fold increase in the deacylation rate constant (k_3) for various substrates was observed by Kézdy and coworkers for an alkylated (Met192)-α-Cht (94). Their explanation for this significant increase in k_3 was an interaction of the bulky alkyl group of Met192 with the acylamido site (*am* site) in the active site. This evaluation of the situation demonstrates the flexibility of the long alkyl group attached to the Met192 but does not help pinpoint the location of the Met192 in the action of Cht.

X-ray crystallographic structures of Cht and Cht derivatives shed new light on the question at hand. The x-ray structure of α-Cht was published in 1966 by Sigler and coworkers revealing the Met192 in a position exposed to solvent in the active site of the enzyme (4). This position was consistent with the existing chemical modification data on the Met192 residue (72, 73, 74). The valuble structural information

made it possible to envision the location of Met192 much more accurately than before. In relation to the topology of the active site of Cht the x-ray structure placed the Met192 primarily in the *h* site and bordering the *ar* site (see Figure 7).

Further x-ray crystallographic work on Cht with bound substrate-like molecules provided even more information on active site residues. A significant change in the position of Met192 was apparent when the structure of tosyl-α-Cht was determined and a similar change was denoted between the structures of α-Cht and dioxan-α-Cht (4, 75). Essentially, the change involved a 180° rotation of the C_α-C_β bond of Met192 bringing the thioether side chain into contact with the bound substrate-like molecule. The Met192 side chain also becomes less exposed to solvent in these enzyme derivative crystals. Eventually, a theory emerged based on these observed changes. It states that the Met192 side chain acts as a "flexible hydrophobic lid" on the specificity pocket (75, 102). A similar movement in the analogous Gln192 residue has been shown in the latest x-ray structures of inhibited derivatives of trypsin (103). The x-ray structures were obtained for DFP inhibited trypsin (DIP-trypsin) and benzamidine inhibited trypsin (BA-trypsin). Benzamidine is an aromatic molecule possessing a substituent similar to the positively charged side chains of arginine and lysine which occupy the specificity pocket of trypsin during proteolysis. The Gln192 undergoes a reorientation in the BA-trypsin structure compared to the DIP-trypsin structure. It appears to act as a polar flap covering the entrance to the binding pocket. The polar nature of the Gln192 side chain is the major difference between it and the Met192 side chain which hydrophobic. Otherwise, the

Figure 7 - The active site of α-chymotrypsin with the pseudo-substrate formyl-L-tryptophan in the position deduced from a difference electron density map (75).



two residues seem to have the same function as hypothesized from the x-ray work done on them.

Yet another hypothesis for the participation of Met192 in the catalytic mechanism of Cht was recently proposed by Fersht and coworkers (104). A stereochemical analysis of the interaction between α-Cht and pancreatic trypsin inhibitor, based on the x-ray structure of these respective proteins, was the basis for this theory. It suggests that the α, β and γ carbons of Met192 maintain close van der Waals repulsive contacts with the leaving group of substrates. In this way, strain is exerted on the ground state of the substrate in the Michaelis complex resulting in the high observed rates of enzyme catalysis. One of the major objections to this approach is that it involves model building and analysis with little experimental evidence to back it up.

Summarizing, there are three theories on the participation of Met192 in the catalytic mechanism of Cht:

1. Interaction of Met192 at the aromatic site of Cht. Experimental results on the chemical modification of Met192 (increased K_m values in the hydrolysis of monoamino acid ester and amide substrates) are basically responsible for this hypothesis. Lack of significant changes in the catalytic rate constant and maximum velocity of Cht reactions (with one exception) upon modification of Met192 also support this viewpoint.

2. Location of the Met192 in the *h* site of Cht. The x-ray crystallographic structure of Cht is the basis for this theory. Even though application of the x-ray structure data to catalysis is limited due to the static nature of the molecules, some speculation can arise from studying crystals of Cht with bound substrate-like molecules. This

type of study led to the theory that the thio-ether side chain of Met192 might act as a "flexible hydrophobic lid" on the specificity cavity during catalysis.

3. Application of strain on bound substrate molecules by the α, β and γ carbons of the Met192. A stereochemical analysis of a proteinase inhibitor-Cht complex led to this possible explanation of Met192 involvement in enzyme catalysis.

The Goals of This Thesis

The goals of this thesis are the following:

1. To compare the x-ray crystallographic data on chymotrypsin and chymotrypsinogen structure to the solution conformation of these proteins by chemical modification studies on the active site amino acid residue methionine-192.

2. To demonstrate the usefulness of α-bromo-4-nitroacetophenone (BrNAP) as an active site reporter group in forms of Cht devoid of catalytic activity.

3. To help establish the possible roles of inert active site residues such as methionine-192 of Cht in the mechanism of enzyme catalysis.

4. To study the stereochemical mechanism of proteolytic activation during the elaboration of active chymotrypsins from chymotrypsinogen.

In order to achieve these goals the following experiments were performed:

Alkylation of various chemically modified forms of Cht were attempted with BrNAP. In these forms of Cht, essential and non-essential catalytic residues of the enzyme were chemically modified.

i. The Ile16 residue of δ-Cht was specifically acetylated to generate a form of Cht, acetylated(Ile16)-δ-Cht, similar to the high pH form of the enzyme.

ii. The Ser195 residue of α-Cht was converted to dehydroalanine by an elimination reaction resulting in anhydro-α-Cht.

iii. The His57 residue of α-Cht was methylated on the N3 atom of imidazole ring resulting in 3-methyl-His57-α-Cht.

The studies concerning alkylation of acetylated(Ile16)-δ-Cht relate to the role of Met192 in the activation process. The alkylation studies of ii. and iii. relate to the role of Met192 in the catalytic mechanism and changes in the active site conformation on modification of catalytically essential residues in the enzyme. Circular dichroism and ultraviolet spectra of the different forms of Cht-NAP were also carried out.

The kinetic parameters, k_{cat} and K_m, of the peptide hydrolysis of Ac-L-Phe-L-Ala-NH_2, Ac-L-Phe-Gly-NH_2 and Ac-L-Phe-NH_2, as assayed by a continuous automatic ninhydrin development method, were determined for native δ-Cht and MS-δ-Cht for comparison. Estimates of the rate constants of catalysis for the hydrolysis of α-casein by δ-Cht and MS-δ-Cht were also determined. The results of these peptide hydrolyses are useful in determining the role of Met192 in catalysis.

Due to the extensive characterization of its structure and mechanism, Cht is one of the few enzymes in which careful and systematic studies of the role of active site residues in the mechanism of enzymic catalysis can be studied. Information gained on the role of active site residues in the catalytic mechanism of Cht will be useful for the elucidation of the mechanisms of less-well characterized enzymes. This dissertation concentrates on the role of the active site methionine residue (Met192) in the activation and catalytic mechanism of chymotrypsin.

II. MATERIALS AND METHODS

Biological Molecules

All protein molecules (Chtgen-A, DIP-α-Cht, α-Cht, δ-Cht, trypsin, lima bean trypsin inhibitor and α-casein) were obtained from Worthington Biochemical Corporation and used without further purification. All forms of Cht, trypsin and α-casein were bovine in origin.

Chemical Reagents

α-Bromo-4-nitroacetophenone (BrNAP) was obtained from Alfred Bader Chemical Company and recrystallized from chloroform-hexanes (m p 95-98°C, literature m p 95-96°C (105)). N-acetyl-L-tyrosine ethyl ester (ATEE) (m p 79-81°C, literature m p 79-80°C (106)), dimethyl maleic anhydride, N-hydroxysuccinimide, N-acetyl-L-tryptophan amide, CBZ-L-phenylalanine, L-alanine ethyl ester hydrochloride and L-methionine sulfone were purchased from Sigma Chemical Company. Methyl-*p*-nitrobenzene sulfonate (m p 89.5-90.0°C, literature m p 91-92°C (48)), phenylmethylsulfonyl fluoride, *p*-nitrophenyl acetate and glycine ethyl ester hydrochloride were secured from Pierce Chemical Company. N-acetyl-L-phenylalanine amide and N-acetyl-L-methionine amide were from Fox Chemical Company. Ultrapure ninhydrin and ultrapure guanidine hydrochloride were products of Mann Research Laboratories. Acetic anhydride and *p*-nitrophenol were obtained from Fisher Chemical Company. N-*trans*-cinnamoylimidazole was prepared according to Bender and coworkers (107).* The 99% formic acid used was secured from Matheson, Coleman, Bell Chemical Company. All other chemical reagents used were purchased through Scientific Products Company from Mallinckrodt Chemical Works.

G-25 Sephadex (fine), CM-50 Sephadex and Sepharose 4B were supplied by Pharmacia Fine Chemicals.

* Obtained from Dr. Allen Frankfater

Instrumentation

Spectrophotometric readings were taken on either a Cary 15, Heath 707 double beam or Heath 701 single beam spectrophotometer in a thermostated cell compartment with 1 cm silica cuvettes (Beckman Instruments Inc., Pyrocell Manufacturing Company Inc.). Circular dichroism spectra were recorded on a Durrum-Jasco ORD/CD/UV-5 spectropolarimeter modified to a maximum sensitivity of 2×10^{-3} deg/cm. Acetylations were performed on a Radiometer pH Stat (TTT2).

All centrifugations were carried out in a refrigerated International Centrifuge (model PR-1).

The apparatus used for peptide hydrolysis assays was composed of components from a Technicon amino acid analyzer and Technicon auto analyzer (Technicon Corporation). Teflon spaghetti tubing, 0.095 inch diameter, 0.012 inch in thickness, 100 feet long, (Cadillac Plastic and Chemical Company) was used for the ninhydrin color development system in a boiling water bath (100°C).

Amino acid analyses were performed on a Beckman Model 120C Amino Acid Analyzer (Beckman Instruments Inc.).

Miscellaneous

An extinction coefficient of 5×10^{4} $\underline{M}^{-1}$ cm^{-1} was used for the determination of enzyme concentration at 280 nm (21, 61). Enzyme solutions were filtered through Millipore prefilters and filters (0.22 μm and 0.8 μm pore size) with a Swinnex filter holder. Enzyme solutions were dialyzed in dialysis tubing from Union Carbide Corporation and A.A. Daigger Company (1 1/4 and 8/32 inch in diameter respectively) and in cellulose hollow fiber devices (Bio-Fiber 50 beakers and minibeakers, Bio-Rad Laboratories) with a nominal molecular weight cutoff of 5,000. Lyophilization was performed on a VirTis lyophilizer and proteins were then stored, desiccated at -20°C. A Forma circulating temperature bath (Forma Scientific Company) was used to thermostat acetylation and alkylation reactions. It was also used for cooling purposes in the peptide hydrolysis apparatus. A Lauda circulating temperature bath (Brinkman Scientific Company) was employed to thermostat the peptide hydrolysis reactions.

Experimental Procedures

Acetylation of the α-Amino Group of Isoleucine-16 of Chymotrypsin

Acetylated (isoleucine-16)-δ-Cht (A_{16}-δ-Cht) was prepared by three different procedures. In each case, acetylation was carried out in a jacketed sample cup of a Radiometer pH Stat in 0.01 M $CaCl_2$ at 4°C. The pH was maintained automatically with 5 N NaOH.

Procedure One: Preparation of Acetylated (Ile16)-acetylated-δ-chymotrypsin

A. Acetylation of Chymotrypsinogen-A with Acetic Anhydride

δ-Cht acetylated on the α-amino group of Ile16 and all other free amino groups (primarily ε-amino groups of lysine) was first prepared by the procedure of Oppenheimer and coworkers (21). One gm of Chtgen was dissolved in 95 ml 0.01 M $CaCl_2$ (approximately 10 mg/ml) and placed in a 200 ml Radiometer pH Stat flask. An ice bath was used to maintain the temperature at 4°C. To this solution 0.13 ml 1 M diisopropyl fluorophosphate (DFP) in isopropanol was added. The enzyme solution was then adjusted to pH 8.0. After 30 minutes, a 0.1 ml aliquot was removed and diluted 1:100 with mM HCl for an ATEE assay. The end point control was set to maintain the pH of the acetylation reaction at 6.7. The reaction was initiated by the addition of 0.25 ml acetic anhydride and sustained by addition of 0.25 ml after 20 minutes and 0.50 ml after 35 minutes. Base consumption leveled off after 80 minutes with the total addition of 4.169 ml of base. A 0.1 ml aliquot was removed from the reaction

solution for an ATEE assay, but it precipitated upon 1:100 dilution with mM HCl making a determination of activity impossible. At this point the ATEE activity of Chtgen which had been reacted with DFP prior to the acetylation reaction was calculated and found to be 2.0%. It was therefore decided to subject the Chtgen to another DFP reaction. This was done in the same way as previously described. An ATEE assay of a 1:10 dilution of this solution with 0.01 M $CaCl_2$ revealed only 0.09% activity. The acetylated-Chtgen (A-Chtgen) solution was extensively dialyzed in two large dialysis sacs against 0.01 M sodium borate buffer at pH 8.0.

Dialyzed A-Chtgen solution (7.5 ml) was placed in each of four 100 ml centrifuge tubes and diluted to 75 ml with 0.01 M sodium borate buffer (pH 8.0). The solutions were equilibrated in an ice water bath at which time 27 ml of saturated ammonium sulfate (ice-cold) was added to each tube. The precipitate, Cut I (0-27% ammonium sulfate precipitation), was obtained by centrifugation at 3400 rpms (2500 g) for 30 minutes at 2-3°C. The resulting supernatants were divided into two classes, two 100 ml tubes and two 95 ml tubes. Solutions of ice-cold saturated ammonium sulfate (14.19 ml and 13.45 ml respectively) were added to the two classes of tubes. This resulted in a precipitate designated Cut II (27-36% ammonium sulfate precipitation). Centrifugation was carried out as mentioned above to obtain Cut II.

B. Generation of Acetylated-δ-chymotrypsin from Chymotrypsinogen-A

Cut II pellets were dissolved in 0.02 M, pH 8.0 sodium borate buffer, combined (40 ml) and placed in dialysis tubing. A 1.32 ml aliquot of a trypsin solution (4 mg/ml in mM HCl) was added and the reaciton solution

dialyzed against two 4 L changes of pH 8.0, 0.02 M sodium borate buffer, 0.01 M in $CaCl_2$ for 20 hours. Oppenheimer and coworkers reported that under these conditions A-Chtgen is converted to acetylated-δ-Cht (A-δ-Cht) in 15 hours (21). The additional time of exposure of the sample to trypsin (5 hours) may have converted some of the preparation to α-Cht. The enzyme solution was dialyzed extensively against distilled deionized water prior to application to a 0.8 x 12 cm column of CM-50 Sephadex equilibrated with distilled deionized water at pH 5.5. The A-δ-Cht-trypsin solution was passed through the column and collected in a lyophilizing flask. A tosyl-L-arginine methyl ester (TAME) assay showed no measureable trypsin activity indicating the trypsin had indeed been retained by the resin. The A-δ-Cht was lyophilized to dryness and stored disiccated at -20°C. Only part of the initial preparation was treated in this manner (yield 201 mg). ATEE activity of this form of the enzyme was 91% that of native δ-Cht.

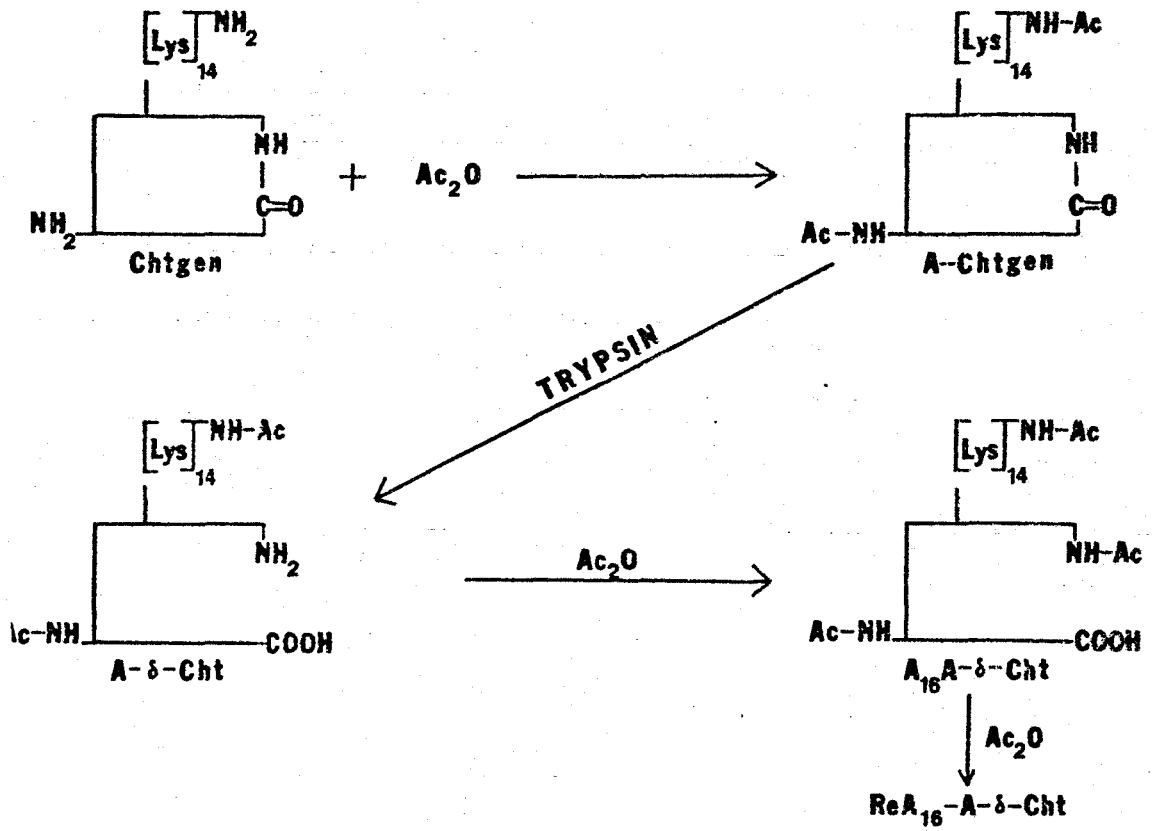
C. Generation of Acetylated (Ile16)-acetylated-δ-chymotrypsin from Acetylated-δ-Chymotrypsin

A-δ-Cht was reacetylated in order to specifically acetylate the α-amino group of Ile16 which had been generated upon tryptic cleavage and activation of A-Chtgen. A-δ-Cht (50 mg) was dissolved in 4.5 ml 0.01 M $CaCl_2$, the pH adjusted to 6.8 and the solution centrifuged for 30 minutes at 3000 rpms. The solution was placed in a small pH Stat vessel thermostated at 4°C. The end point control was set to maintain the pH at 6.7. The acetylation reaction commenced with the addition of 0.025 ml acetic anhydride. Another 0.025 ml acetic anhydride was added

after 20 minutes of reaction. Base consumption leveled off at 180 minutes. A 1:1000 dilution of a 0.1 ml aliquot of the reaction solution (with 0.01 M $CaCl_2$) was used to determine ATEE activity. A 55% decrease in activity was observed after this first acetylation of A-δ-Cht. Extensive dialysis against distilled deionized water, lyophilization and desiccated storage at -20°C followed. The yield of acetylated (Ile16)-acetylated-δ-Cht (A_{16}A-δ-Cht) was 35 mg (70%).

A_{16}A-δ-Cht was reacetylated again in an attempt to lower the enzymic activity even further. A_{16}A-δ-Cht (30 mg) was dissolved in 2.9 ml of 0.01 M $CaCl_2$. This solution was centrifuged at 3000 rpms for 30 minutes, transferred to a Radiometer pH Stat vessel thermostated at 4°C and the pH adusted to 6.9. The end point control was again set to maintain the pH at 6.7. Addition of 0.01 ml acetic anhydride started the reaction and 0.01 ml acetic anhydride was added at 10 minute intervals for 30 minutes. Therefore, the solution was 1% (v/v) in acetic anhydride at the end of the reaction. Base consumption leveled off after 180 minutes of reaction and equaled 0.19 ml. ATEE assays of a 1:1000 dilution of the reaction solution with 0.01 M $CaCl_2$ were 12% that of native δ-Cht. These results were consistent with the value obtained by Oppenheimer and coworkers (21). Extensive dialysis against distilled deionized water was followed by lyophilization and storage under desiccated conditions at -20°C. A schematic diagram of the procedure is shown in Figure 8.

Figure 8 - Schematic diagram of the acetylation of δ-chymotrypsin by the procedure of Oppenheimer and coworkers (21).



Procedure Two: Preparation of Acetylated (Ile16)-chymotrypsin

A. Direct Acetylation of Native δ-Chymotrypsin

Another procedure for the acetylation of the Ile16 residue of δ-Cht involved direct acetylation of native δ-Cht. δ-Cht (200 mg) was dissolved in 19 ml 0.01 M $CaCl_2$ in a thermostated pH Stat vessel. The pH of the solution was adjusted to 7.9, instead of 6.7. Addition of 0.05 ml acetic anhydride initiated the reaction and 0.05 ml of reagent was added every 10 minutes for 30 minutes. This resulted in a solution 1% (v/v) in acetic anhydride. Base consumption leveled off after 120 minutes with the addition of 0.788 ml of 5 N NaOH. After 40 ml of a 0.375 M hydroxylamine solution was added, the reaction solution was adusted to a pH of 7.5 and a temperature of 25°C. The solution was transferred to a Bio-Fiber beaker 30 minutes later and dialyzed with 4 L of pH 7.0, mM phosphate buffer and 13 L of distilled deionized water. Lyophilization yielded 161 mg of acetylated (Ile16)-δ-Cht (A_{16}-δ-Cht) which was stored desiccated at -20°C.

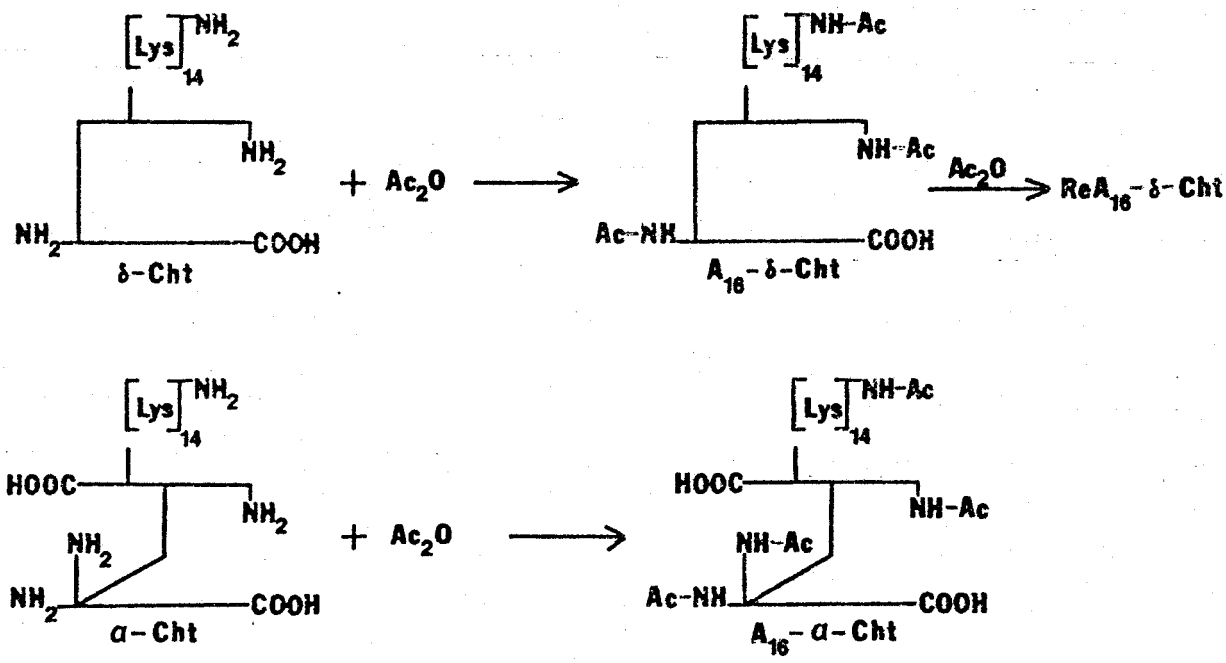
The A_{16}-δ-Cht was reacetylated under conditions identical to the aforementioned procedure - pH 7.9, 10 mg/ml, 4°C etc. A_{16}-δ-Cht (125 mg) was dissolved in 15 ml 0.01 M $CaCl_2$. A 0.025 ml volume of acetic anhydride was added to begin the reaction and every 10 minutes thereafter for 50 minutes yielding a solution 1% (v/v) in acetic anhydride. Base consumption leveled off after 150 minutes and the addition of 0.585 ml of base. A 30 ml solution of 0.375 M hydroxylamine was added and the solution brought to 25°C and pH 7.5 for 30 minutes. This resulted in a solution 0.25 M in hydroxylamine. Dialysis against two changes of 4 L

of pH 7.0, m$\underline{M}$ phosphate buffer and two changes of 4 L of distilled deionized water followed. The material (designated ReA_{16}-δ-Cht) was lyophilized and stored desiccated at -20°C (yield 109 mg). ATEE assays revealed 1.8% activity compared to native δ-Cht.

B. Direct Acetylation of Native α-Chymotrypsin

α-Cht was directly acetylated with acetic anhydride in an analogous manner to the acetylation of δ-Cht described above. The enzyme (350 mg) was dissolved in 33 ml 0.01 $\underline{M}$ $CaCl_2$ in a thermostated pH Stat vessel at 4°C and the pH adjusted to 7.9. The pH of the reaction was maintained at 7.9 with 5 $\underline{N}$ NaOH. Acetylation commenced with the vigorous mixing of 0.05 ml acetic anhydride in the solution and the reaction sustained by addition of 0.05 ml of reagent every 7 minutes for 42 minutes. The resulting solution was 1% (v/v) in acetic anhydride. Base consumption leveled off after 140 minutes and titration with 1.35 ml of base. A solution of 70 ml of 0.375 $\underline{M}$ hydroxylamine was added resulting in a solution 0.25 $\underline{M}$ in hydroxylamine. The pH was then adjusted to 7.5 and the temperature to 25°C for 30 minutes. The solution was dialyzed in a Bio-Fiber beaker with 4 L of pH 7.0, m$\underline{M}$ phosphate buffer and 15 L of distilled deionized water. The lyophilized protein was weighed (323 mg) and stored desiccated at -20°C. ATEE assays revealed 6.0% activity compared to native α-Cht. The direct acetylations of δ-Cht and α-Cht are represented in Figure 9.

Figure 9 - Schematic diagram of the direct acetylation of δ- and α-chymotrypsin.



Procedure Three: Preparation of Acetylated (Ile16)-δ-chymotrypsin by the Method of Fersht

A third procedure, that of Fersht (108), was used to specifically acetylate the α-amino group of Ile16 of δ-Cht. Chtgen (125 mg) was dissolved in 12.5 ml of pH 9.0 carbonate buffer and the solution placed in a pH Stat vessel thermostated at 0-1°C. Dimethylmaleic anhydride (DMMA) (0.5 gm) was dissolved in 1.5 ml acetonitrile at room temperature. Addition of 0.2 ml of this solution to the Chtgen solution began the dimethylmaleyation reaction. Every 10 minutes for 60 minutes 0.25 ml of reagent was added making the solution 0.26 M in DMMA. Base consumption leveled off after 125 minutes with the total addition of 1.415 ml of 5 N NaOH. A small amount of insoluble material (presumably DMMA) was filtered out of the solution with a prefilter.

Activation of this modified Chtgen solution (15.6 ml) was achieved by dissolving 3 mg of trypsin into the solution and dialyzing against 3.5 L of pH 8.0, mM sodium borate buffer, 0.01 M in $CaCl_2$, for 130 minutes. A 1:100 dilution of the solution with borate buffer indicated 112% ATEE activity compared to native δ-Cht. The reaction solution was combined with 10 ml of CM-50 Sephadex which had been equilibrated with pH 8.0, mM sodium borate buffer and mixed vigorously for 30 minutes at room temperature. The resin was filtered off with rinsing and TAME assays of this solution proved negative, demonstrating the removal of trypsin from the solution.

Acetylation of the enzyme solution (40 ml) with acetic anhydride followed immediately at pH 8.0 and 0-1°C. A 0.05 ml volume of acetic anhydride was vigorously stirred into the solution every 5 minutes for

40 minutes. The resulting solution was 1% (v/v) in acetic anhydride. Base consumption leveled off after 110 minutes and titration with 1.745 ml of 5 N NaOH. A 1:10 dilution with borate buffer and subsequent ATEE hydrolysis assay revealed 4% activity compared to native δ-Cht.

The temperature of the solution was increased to 25°C and the pH adjusted to 6.0 with 6 N HCl for 10 minutes. The pH was further lowered to 5.0 with 6 N HCl and the solution maintained under these conditions for 30 minutes. The purpose of this procedure was to remove the DMM groups from the molecule. At this point 4.85 ml of pH 7.8, 0.5 M Tris buffer was mixed with the solution resulting in a pH 7.8, 0.05 M Tris buffer solution of the modified enzyme. The resulting milky white solution (50 ml) was prefiltered twice before its application to an agarose-lima bean trypsin inhibitor affinity column at 4°C. Fractions of 7-7.5 ml were collected every 10 minutes. Spectrophotometric readings of the fractions at 280 nm designated a protein peak in fractions 11-35. At the beginning of fraction 25 a pH 2.0, 0.2 M KCl solution was applied to elute the column and a second protein peak collected in fractions 36-50. An ATEE assay showed 15% activity in peak 1 (combined fractions 11-21). There was 64 mg of material in peak 1 and 20 mg in peak 2. At this point, it was believed that the capacity of the column had been exceeded. This would explain the activity observed in peak 1. A second affinity column run of peak 1 supported the suspicion stated above. Two peaks were obtained again as in the previous run. Peak 1 of the second affinity column run contained 39 mg of the modified enzyme (A_{16}-δ-Cht(F)) with 4.25% ATEE activity while peak 2 contained only 3 mg of δ-Cht with activity comparable to the native enzyme. A_{16}-δ-Cht was obtained by extensive dialysis against distilled deionized water and lyophilization to

dryness. The resulting material was stored under desiccated conditions at -20°C. Figure 10 contains the protocol for this experiment.

Preparation of Anhydro-α-Chymotrypsin

Anhydro-α-Cht was prepared according to the procedure of Ako and coworkers (109).* PMS-α-Cht was dissolved in 0.1 N KOH and incubated for 1 hour at 3°C. The crude anhydro-α-Cht was chromatographed over an agarose-lima bean trypsin inhibitor (LBTI) affinity column equilibrated with pH 7.8, 0.05 M Tris buffer. A non-binding species of anhydro-α-Cht (11 mg) was eluted directly from the column upon application of the crude material. A binding species of anhydro-α-Cht (29 mg) was eluted with a pH 2.0, 0.2 M KCl, 0.01 M HCl solution. Ako has previously shown that binding and non-binding anhydro-α-Cht contain one equivalent of dehydroalanine per mole of enzyme (109). A diagram of this chemical reaction can be found in Figure 11.

Preparation of 3-Methyl-Histidine-57-α-Chymotrypsin

3-Methyl-His57-α-Cht was prepared by a modified procedure combining the methods of Bender and Nakagawa (48) and Henderson (49).** α-Cht (100 mg) was dissolved in 7.0 ml of 0.1 M sodium phosphate buffer (pH titrated to 7.8 with 0.1 N NaOH). Methyl-*p*-nitrobenzene sulfonate (6 mg) was dissolved in 0.7 ml acetonitrile and this solution was added to the enzyme solution at room temperature in a hood. ATEE assays were run initially and at 10, 30, 60, 90 and 150 minutes after the reaction was

* Prepared by A. Konovessi-Panayotatos

** Prepared by John R. Peters

Figure 10 - Schematic diagram of the specific acetylation of δ-chymotrypsin by the procedure of Fersht (108).

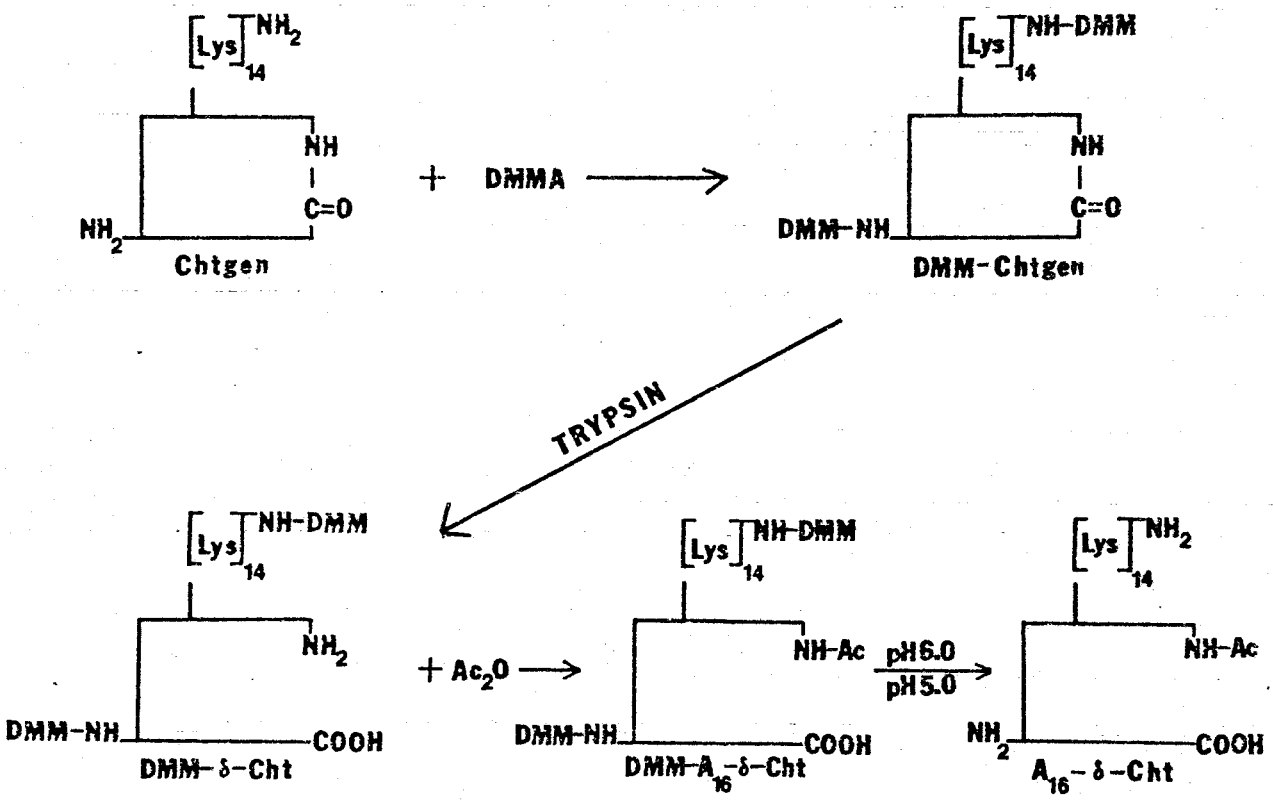
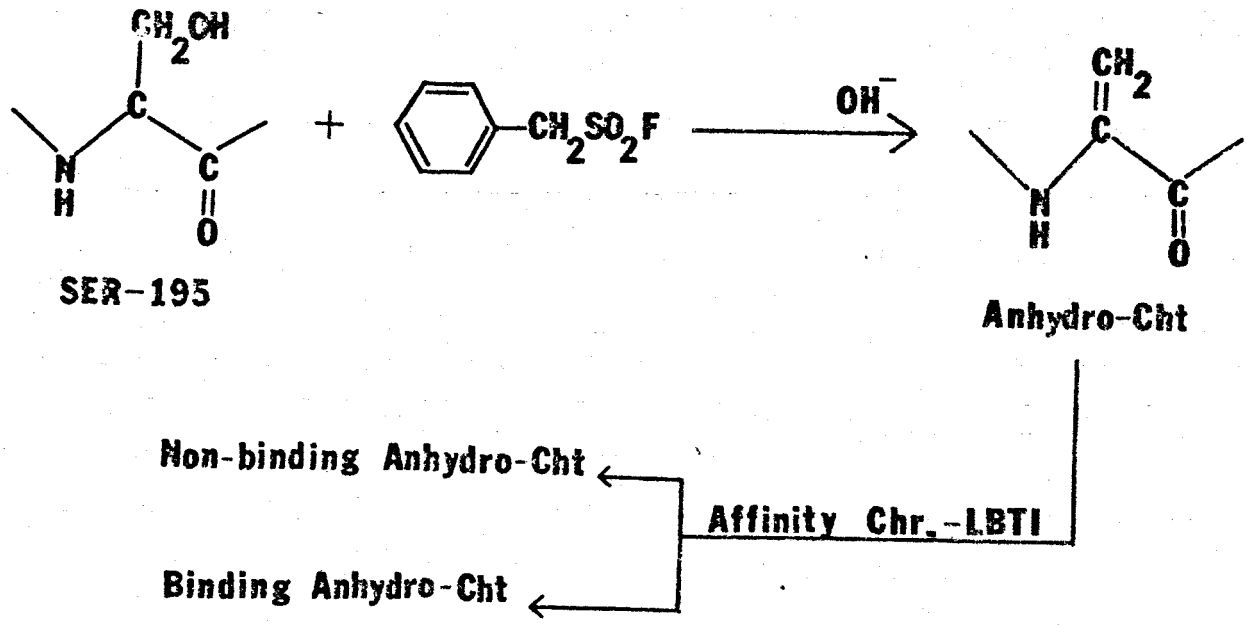


Figure 11 - Preparation of anhydro-α-chymotrypsin by the procedure of Ako and coworkers (109).



begun. The activity of the enzyme was approximately zero after 150 minutes of reaction. The reaction solution was dialyzed extensively against 10 L of mM HCl at 5°C and the protein lyophilized. A diagram of the chemical reaction is shown in Figure 12.

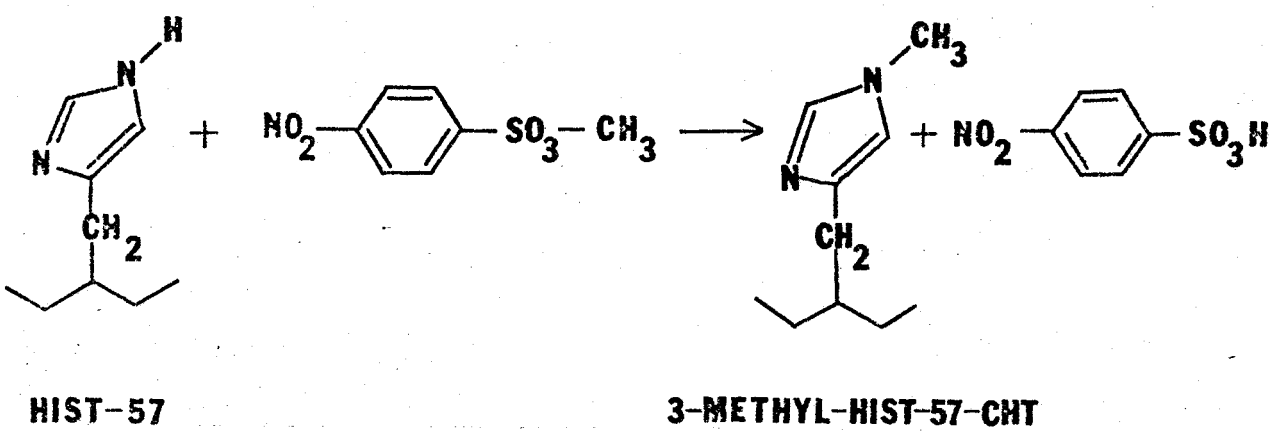
Preparation of Monosulfoxide-δ-Chymotrypsin

Monosulfoxide-δ-Cht (MS-δ-Cht) was prepared according to Weiner et.al. (110). This procedure, however, was a modification of a previous procedure of Koshland et.al. (74). δ-Cht (200 mg) was dissolved in 200 ml of mM HCl (1 mg/ml). The enzyme was oxidized at room temperature by the addition of 0.1 ml of 30% H_2O_2. The reaction was carried out in the dark with rapid stirring for 22 hours at which time it was assayed for ATEE activity. The solution was dialyzed in 1 1/4 inch dialysis tubing against mM HCl and then distilled deionized water. Lyophilization of the protein and storage at -20°C followed.

Alkylation of Chymotrypsin with α-Bromo-4-Nitroacetophenone

All alkylations were done at an α-bromo-4-nitroacetophenone (BrNAP) concentration of 2×10^{-4} M, an enzyme concentration of 2×10^{-5} M, in a pH 5.6, 0.05 M acetate buffer, 5% acetonitrile, at 25°C (96, 105). In most cases reactions were done in pyrocells (1 cm path length, 4 ml capacity) with ground glass stoppers to prevent loss of the volatile acetonitrile. Alkylation reactions were followed at 350 nm (visible range - tungsten lamp), the maximum wavelength of absorption of the Cht-NAP complex. The extinction coefficient for this complex was calculated by dividing the observed absorbance at 350 nm

Figure 12 - Preparation of 3-methyl-His57-α-chymotrypsin by the combined procedures of Bender and Nakagawa (48) and Henderson (49).



(approximately 0.115) at the end of the reaction by the concentration of enzyme active sites in solution (approximately 1.5×10^{-5} M as determined by N-*trans*-cinnamoylimidazole active site titration). It was 7.42×10^{3} M^{-1} cm^{-1} for δ-Cht and 7.36×10^{3} M^{-1} cm^{-1} for α-Cht. The α-Cht value correlated with that found previously (7.55×10^{3} M^{-1} cm^{-1}) (96). The solutions in the sample cuvette were thermostated at 25°C while the rest of the reaction solutions (in volumetric flasks) were equilibrated in a temperature bath at 25°C.

After completion most reaction solutions were dialyzed against pH 5.6 acetate buffer at 4°C. A spectrum (240-600 nm) was then taken of the solution after filtration through a millipore filter. The solutions were dialyzed against distilled deionized water followed by lyophilization of the alkylated proteins.

Alkylation reactions were also attempted on Chtgen using various concentrations of the denaturation reagent, guanidine hydrochloride (GuCl). Different concentrations of GuCl (3.6 N, 4.2 N, 4.8 N, and 5.7 N) were prepared from a 6 N stock solution of the reagent. All other conditions for these alkylation reactions were the same as mentioned above.

In some of the experiments (i.e. the alkylation reactions of the A_{16}-Chts) it was necessary to separate the protein from the modifying reagent, buffer and acetonitrile. A G-25 Sephadex (fine) column (2 x 16 cm) equilibrated with distilled deionized water was used for this purpose. The column effectively separated protein from the other constituents of the reaction solution. Distilled deionized water was

used to elute the column so the protein solution could immediately be lyophilized after separation.

A diagram of the BrNAP-Cht reaction is shown in Figure 13.

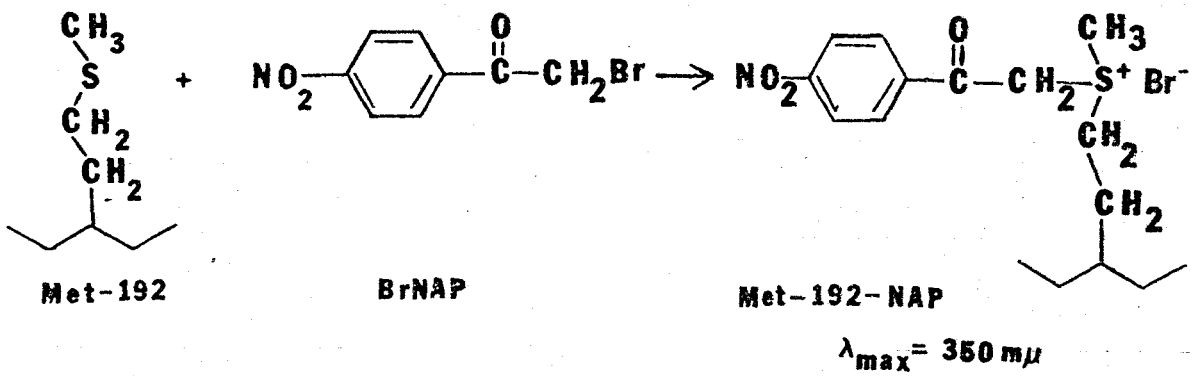
Alkylation of N-Acetyl-L-Methionine Amide with α-Bromo-4-Nitroacetophenone

The alkylation of N-acetyl-L-methionine amide (Ac-Met-NH_2) was also carried out for comparison with the alkylation of Met192 of Cht. It was necessary to increase the concentrations of BrNAP and Ac-Met-NH_2 5 and 10 times respectively in order to observe the alkylation reaction. A solution of 2×10^{-4} M Ac-Met-NH_2 was alkylated with 1×10^{-3} M BrNAP in 5% acetonitrile at 25°C. Due to the slow rate of reaction the initial increase in absorbance at 350 nm with time was used to calculate the alkylation rate constant. The extinction coefficient for Ac-Met-NH_2 was necessary in the calculation of the alkylation rate constant and was determined from a synthesis of Ac-Met-NH_2-NAP.

Synthesis of N-Acetyl-L-Methionine Amide-4-Nitroacetophenone

The procedure for the synthesis of Ac-Met-NH_2-NAP was adapted from that of Sigman and coworkers for dimethyl sulfonium salts (105). Instead of dimethyl sulfide, Ac-Met-NH_2 was used as the reactant. The reaction was scaled down by 1/3 due to a limited amount of starting material. Ac-Met-NH_2 (0.63 gm, 3.3 mmoles) was dissolved in 6.5 ml of an acetone: water (12:1) solution. BrNAP (0.8 gm, 3.3 mmoles) was then added and the reaction solution stirred vigorously at room temperature for 9 hours. The acetone phase was rotary evaporated and the aqueous phase frozen and lyophilized overnight. After several attempts crystallization of the resulting yellow oily material was accomplished from methanol-ether.

Figure 13 - Alkylation of the Met192 residue of chymotrypsin with BrNAP by the procedure of Blout and coworkers (105).



Thin layer chromatography (butanol:acetic acid:water, 4:1:1) indicated a homogeneous product (R_f-0.76). Subsequent quantitative organic microanalysis (% C 41.08, % H 5.67, % N 9.42) (Galbraith Laboratories, Knoxville, Tennessee), agreed with the theoretical expected values (% C 40.91, % H 5.95, % N 9.52) for Ac-Met-NH_2-NAP. A spectrum of the compound was run in pH 5.6, 0.05 M acetate buffer. The extinction coefficient at 350 nm was found to be 2.0×10^3 M^{-1} cm^{-1} (27% that of Cht-NAP). The significant difference between the two extinction coefficients is probably due to the altered environment of the chromophore. This alteration could have an effect on the pKa of the sulfonium to ylide equilibrium of this compound (105).

Performic Acid Oxidation of Modified Chymotrypsin

Performic acid oxidation by the method of Hirs (111) was used to determine whether certain samples of Cht-NAP had been truly alkylated with BrNAP. Samples (5 mg) of δ-Cht and modified Cht (δ-Cht-NAP, ReA_{16}-δ-Cht-NAP and Chtgen-NAP (4.8 N GuCl treatment)) were dissolved in 0.5 ml 99% formic acid at 0°C in 5 ml glass-stoppered test tubes. The oxidation reactions were then initiated by the addition of 1 ml performic acid solution to the enzyme solution(performic acid was prepared by mixing 9.5 ml 99% formic acid and 0.5 ml 30% H_2O_2 in a 10 ml stoppered flask for 2.5 hours at room temperature). The tubes were stoppered and the solutions maintained at 0°C in an ice water bath. The reactions were terminated after 25 minutes by the addition of 35 ml of cold distilled deionized water. The solutions were lyophilized and the oxidized proteins subjected to acid hydrolysis and amino acid analysis.

Amino Acid Analysis

Samples of enzymes for amino acid analysis were hydrolyzed at 110°C for 20 hours in 1-2 ml 6 N HCl (112). The hydrolysis tubes (7.5 x 1 cm) were evacuated by completely freezing the contents and placing the tube under a good vacuum for 5-10 minutes. The vacuum was preserved by the use of a stopcock device attached to the tube. The contents of the tube were then brought to room temperature for 5-10 minutes and the whole procedure repeated before sealing the tube with a flame and placing in an oven. The hydrolyzed samples were desiccated to dryness in the presence of sodium hydroxide pellets and dissolved in 0.02 M, pH 2.2 citrate buffer (0.5 mg/ml) prior to application to the amino acid analysis columns.

N-Acetyl-L-Tyrosine Ethyl Ester Activity Assays

Activity assays for Cht were carried out with N-acetyl-L-tyrosine ethyl ester (ATEE) according to a modified procedure of Schwert and Takanaka (106). Decreases in absorbance at 237 nm were measured with time. Cuvettes were thermostated at 25°C. ATEE (25.2 mg) was dissolved in a small volume of buffer (0.067 M, pH 7.0 phosphate) in a 100 ml volumetric flask. Buffer was then added and the whole solution rapidly cooled to 20°C. This resulted in a 100 ml solution 1×10^{-3} M in ATEE. For activity assays, the reference cell contained 3.0 ml of ATEE solution and 0.1 ml of the particular buffer the enzyme was dissolved in while the sample cell contained just 3.0 ml of ATEE solution. Straight baselines were obtained with the reference and sample cells in the instrument before any assays were run. Enzyme solution (0.1 ml,

4×10^{-7} M) was then added to the sample cell and the contents mixed manually. Difference extinction coefficients of 292 M^{-1} cm^{-1} for the Cary 15 and 260 M^{-1} cm^{-1} for the Heath 707 spectrophotometer (calculated from recordings of the total hydrolysis of substrate by Cht) were used to calculate the catalytic rate constants.

p-Nitrophenyl Acetate Active Site Titrations

p-Nitrophenyl acetate (*p*NPA) assays were done in a 0.067 M, pH 7.8 phosphate buffer at 25°C on a Heath 701 single beam spectrophotometer at 400 nm. This procedure is a modification of the original procedure of Bender and coworkers (113). To run an assay 3.9 ml of Cht solution (usually 2×10^{-5} M) in buffer is placed in a cuvette, thermostated at 25°C and a baseline obtained at a slow chart speed. A 0.1 ml volume of *p*NPA (4×10^{-3} M) in acetonitrile is then added and the contents of the cuvette mixed manually. The increase in absorbance at 400 nm due to hydrolysis of the substrate is then recorded with time. After steady state conditions are reached, extrapolation of the recording to zero time gives the absorbance (400 nm) due to the burst of *p*-nitrophenol liberated upon mixing the enzyme and substrate. Dividing this absorbance value by the extinction coefficient of *p*-nitrophenol at this pH (7.8) results in the molarity of active site present in the preparation. Comparison of these values with the concentration of the enzyme determined by 280 nm spectrophotometric readings will give per cent values.

The extinction coefficient for *p*-nitrophenol in the pH 7.8 buffer was determined by recording the absorbance of a 1×10^{-4} M solution of *p*-nitrophenol. The absorbance reading was 1.72 and therefore, the extinction coefficient equalled 1.72×10^{4} M^{-1} cm^{-1}.

All titrations and hydrolyses were calculated with subtraction of the contribution made when the *p*NPA was added to buffer alone. When the molarity of the active sites decreased substantially upon modification of the enzyme, the concentration of the enzyme solution used for the *p*NPA titration was increased so that an accurate value could be calculated.

N-*trans*-Cinnamoylimidazole Active Site Titrations

N-*trans*-Cinnamoylimidazole (C-I) active site titrations were performed on native α- and δ-Chts by the method of Schonbaum and coworkers (107). They were run essentially to correct the ATEE assay values of the modified enzymes for active sites. The assay required a 0.01 M solution of C-I in acetonitrile, a 7.0-7.5 x 10^{-4} M Cht solution in a 0.1 M, pH 5.0 acetate buffer. A baseline was first obtained at 335 nm for 3 ml of buffer solution in a sample cuvette (3 ml buffer solution in reference cuvette) on a Heath 707 double beam spectrophotometer. A 0.01 ml aliquot of the C-I solution was added to the cuvette with a λ pipette, the solution mixed and the absorbance recorded with time until a straight line could be extrapolated back to the point of addition (this value was designated A_2 accordingly). A 0.1 ml aliquot of enzyme solution was then added with a λ pipette, the solution mixed and the absorbance again recorded with time. Extrapolation back to the time of enzyme addition gives the absorbance due to the formation of the stable *trans*-cinnamoyl-Cht complex (absorbance value designated A_3). The normality of active sites in the enzyme stock solution can then easily be calculated with the following equation: $N = \frac{0.969\ (A_2)-A_3}{279.7}$ (107) where the dilution due to the addition

of enzyme is taken into account (0.969) and where the enzyme dilution and extinction coefficient of C-I are taken into account with a single conversion factor (279.7).

Circular Dichroism Studies

CD studies for the acetylated (Ile16)-Chts were performed by procedures of Fasman and coworkers (62, 114) with several minor modifications. Enzyme was dissolved in a 0.1 M sodium sulfate, 0.2 ionic strength solution in order to get a concentration of 4×10^{-5} M. A small volume of this solution was mixed with an equal volume of a pH 6.7, 0.33 M phosphate buffer at the same ionic strength. The final conditions were an enzyme concentration of 2×10^{-5} M, ionic strength of 0.2 and pH 6.7. The instrument was standardized at room temperature with a 1.0 mg/ml camphosulfonic acid solution (supplied by Durrum Instrument Company). A 15.65 cm deflection was obtained on the 2×10^{-2} deg/cm scale with this standard before all runs were made. Baselines were run prior to the spectra of each sample. CD patterns were obtained in a wavelength range of 220-240 nm on the 2×10^{-3} deg/cm sensitivity scale and in a 1 mm path length cell at room temperature. There was some noise between 220-225 nm due to a higher photomultiplier voltage but this level of noise did not affect the critical region of the spectrum (225-235 nm). CD spectra for Cht-NAP derivatives were carried out in the 300-400 nm range under the conditions stated above.

Synthesis of Substrates for Peptide Hydrolyses

The syntheses of CBZ-L-phenylalanine-L-alanine ethyl ester (CBZ-L-Phe-L-Ala-O-Et) and CBZ-L-phenylalanine-glycine ethyl ester (CBZ-L-Phe-Gly-O-Et) were accomplished by the mixed anhydride method according to Vaughn and Osato (115) with some modifications. Dimethylformamide (DMF) was used as the solvent rather than toluene, N-methyl morpholine (NMM) was used instead of triethylamine and all steps were carried out under anhydrous conditions. The product was crystallized from ethyl acetate-hexanes and characterized by thin layer chromatography (TLC). Melting point determinations were also carried out.

CBZ-L-Phe-L-Ala-O-Et. CBZ-L-Phe (2.5 gm, 8.35 mmole) was dissolved in 40 ml DMF. NMM (0.97 ml, 8.65 mmoles) was added with stirring and the solution lowered to -15°C in a carbon tetrachloride-dry ice bath. Isobutyl chloroformate (1.15 ml, 8.65 mmoles) was added with vigorous stirring. The temperature was maintained at -15°C for about 30 minutes at which time a solution of DMF containing HCl-L-Ala-O-Et (1.3 gm, 8.46 mmoles) and NMM (0.97 ml, 8.65 mmoles) was added. The reaction vessel was fitted with a U-tube filled with desiccant to prevent any accumulation of moisture. The reaction was allowed to slowly equilibrate to room temperature and left overnight with vigorous stirring. The white material filtered from the yellow reaction solution was presumed to be the hydrochloride salt of NMM. The solution was rotary evaporated to a solid and dissolved in 100 ml ethyl acetate. A yellow material was filtered off this solution. The filtrate was washed three times with equal amounts of 0.1 N HCl, 4% sodium bicarbonate and brine. The aqueous washes were all rewashed with a separate

ethyl acetate phase which was combined with the original ethyl acetate phase. The combined ethyl acetate phases were dried over magnesium sulfate and the volume reduced to about 60 ml at which time crystals formed. The solution was maintained at 4°C overnight to maximize the crystallization process. The 1st crop of the product (1.85 gm) was filtered from the solution and a second crystallization begun by the addition of hexanes to the filtrate (2nd crop - 0.36 gm). Both crops had a melting point of 124.0-125.5°C. The total yield of the reaction was 2.21 gm (61%). TLC of the product indicated homogeneous material with an R_f = 0.88 (97:3, $CHCl_3$:MeOH). The melting points (m p s) and TLC values for the intermediates and products of both syntheses can be found on Table 3.

-CBZ-L-Phe-Gly-O-Et. The same procedure was employed for the synthesis of CBZ-L-Phe-Gly-O-Et as was used for the synthesis of CBZ-L-Phe-L-Ala-O-Et described above with the exception of the quantities of reactants: CBZ-L-Phe (20 gm, 67 mmoles), NMM (7.5 ml, 67 mmoles), isobutyl chloroformate (8.9 ml, 67 mmoles), HCl-Gly-O-Et (9.35 gm, 67 mmoles) and another equivalent of NMM all in 300 ml DMF. The four crops had m p s within the range of 107.0-108.0°C (literature m p - 108-110°C (116)), TLC R_fs of 0.94 (97:3, $CHCl_3$:MeOH) and gave a yield of 18.9 gm (67%).

The two peptides were converted to the N-acetyl amide form by the procedures of Baumann and coworkers (117) for the synthesis of N-acetyl-L-tyrosine-glycine amide with several modifications. These were the use of t-butyl alcohol instead of ethanol in the hydrogenation step, the use of DMF instead of water in the acetylation step, crystallization from

TABLE 3

CHARACTERIZATION OF VARIOUS INTERMEDIATES IN

THE PEPTIDE SYNTHESIS OF Ac-Phe-Gly-NH_2 and Ac-Phe-Ala-NH_2

	m p °C	Literature m p °C	TLC (R_f)
CBZ-Phe-Gly-O-Et	107.0-108.0	108-110 (116)	0.88[a]
Ac-Phe-Gly-O-Et	140.0-142.0	133-135 (117)	0.52[a]
Ac-Phe-Gly-NH_2	182.0-184.0	183-184 (117)	0.70[b]
CBZ-Phe-Ala-O-Et	124.0-125.5	-	0.94[a]
Ac-Phe-Ala-O-Et	153.5-155.0	-	0.88[a]
Ac-Phe-Ala-NH_2	246.0-248.0	242-244 (118)	0.85[a]

[a] solvent system chloroform:methanol (97:3)

[b] solvent system chloroform:methanol (4:1)

ethyl acetate-hexanes instead of water in the acetylation step and crystallization of Ac-L-Phe-Gly-NH_2 from ethyl methyl ketone-ethanol.

HCl-L-Phe-L-Ala-O-Et. CBZ-L-Phe-L-Ala-O-Et 1.85 gm, 4.25 mmoles) was dissolved in 250 ml of t-butyl alcohol in a Parr hydrogenator flask. A small volume of 6.44 N HCl (0.66 ml, 4.25 mmoles) was added to the solution along with about 1 gm of 5% palladium on charcoal catalyst. The flask was placed on a Parr hydrogenator, filled with hydrogen gas and evacuated three times before applying 20 psi of hydrogen gas and shaking overnight (>12 hours). The palladium charcoal catalyst was filtered from the reaction solution before rotary evaporation. TLC established that the CBZ group had been removed (R_f = 0.215 with 97:3, $CHCl_3$:MeOH). The solution was further rotary evaporated to an oily-crystalline residue. Upon addition of cold hexanes a crystalline solid formed. This material was dried in a desiccator with a vacuum pump equipped with a dry ice-acetone trap. Due to the apparent homogeneous nature of the material it was used as starting material in the next reaction.

HCl-L-Phe-Gly-O-Et. The same procedure was used for the removal of the CBZ group for CBZ-L-Phe-Gly-O-Et as is stated above for the first substrate. CBZ-L-Phe-Gly-O-Et (13.7 gm, 32.5 mmoles) was hydrogenated in two batches. TLC of the resulting material indicated removal of the CBZ group (R_f = 0.37 with 97:3, $CHCl_3$:MeOH). The crystalline solid product was used as starting material for the next reaction.

N-acetoxysuccinimide. N-hydroxysuccinimide (5 gm, 43.5 mmoles) was dissolved in 150 ml of acetone. Acetic anhydride (4.1 ml, 43.5 mmoles) was added dropwise at room temperature with vigorous stirring.

The reaction was allowed to proceed for 160 minutes. Rotary evaporation gave a crystalline residue which was dissolved in 150 ml absolute ethanol at 45°C. The solution was equilibrated to room temperature and cooled at 4°C overnight to obtain the crystalline product. A yield of 5.39 gm (88%) was achieved and a melting point of 131-133°C (literature m p 132-133°C (117) obtained for the product.

Ac-L-Phe-L-Ala-O-Et. The HCl-L-Phe-L-Ala-O-Et residue from the hydrogenolysis reaction (4.25 mmoles) was dissolved in 43 ml DMF and NMM (0.48 ml, 4.25 mmoles) added with vigorous stirring. N-acetoxysuccinimide (0.6 gm, 4.25 mmoles) was added directly into the solution and the reaction allowed to proceed for 4 hours at room temperature with stirring. The material was rotary evaporated to dryness using a vacuum pump equipped with a dry ice-acetone trap and then treated in the same manner as the CBZ ethyl ester peptides after synthesis (dissolved in ethyl acetate, washed, dried and crystallized by the addition of hexanes). A negative ninhydrin test confirmed that there was no free amino group present indicating the acetylation reaction had indeed taken place. The combined crops of Ac-L-Phe-L-Ala-O-Et (yield 0.65 gm, 45%) had a m p of 153.5-155.0°C. Even though a literature m p could not be found for the product the 1.5°C range and TLC (R_f = 0.88 with 97:3, $CHCl_3$:MeOH) indicated pure product.

Ac-L-Phe-Gly-O-Et. The HCI-L-Phe-Gly-O-Et residue from hydrogenolysis (approximately 32 mmoles) was dissolved in 200 ml DMF and NMM (3.5 ml, 32 mmoles) added with vigorous stirring. N-acetoxysuccinimide (4.5 gm, 32 mmoles) was then added directly to the solution and the reaction allowed to proceed for 6 hours at room temperature with

stirring. After rotary evaporation with a vacuum pump and trap, the material was treated in the same manner as the previous reaction. A negative ninhydrin test on this material also confirmed that the reaction had taken place. Two crops were obtained (yield 3.26 gm, 31%) which had different m p s (crop 1 - 140-142°C and crop 2 - 115-125°C). Despite the discrepancy between the obtained m p for crop 1 and the literature value of 133-135°C (117) this material appeared pure from the TLC values. Crop 1 (2.5 gm) was therefore, used for the final reaction.

Ac-L-Phe-L-Ala-NH_2. Ac-L-Phe-L-Ala-O-Et (0.65 gm, 1.9 mmoles) was dissolved in 50 ml of anhydrous methanol (MeOH) in a three neck 200 ml round bottom flask fitted with a bubbler, a thermometer and a vent. The solution was brought to 0°C with an ice bath and stirred vigorously as ammonia gas was bubbled through for 3.5 hours. The vessel was then equilibrated to room temperature and left overnight with stirring. The volume of MeOH was reduced to dryness by rotary evaporation two days later. The dry material was dissolved in a small volume of MeOH with heating and upon equilibration overnight at 4°C crystals formed. The three crops collected (yield 0.46 gm, 87%) had a m p of 246-248°C (literature m p 242-244°C (118)). TLC also revealed a homogeneous product (R_f = 0.85, 4:1, $CHCl_3$:MeOH). This product was used for peptide hydrolysis assays without further recrystallization.

Ac-L-Phe-Gly-NH_2. Ac-L-Phe-Gly-O-Et (2.5 gm, 7.6 mmoles) was dissolved in 50 ml MeOH in a three neck 200 ml flask fitted with a bubbler, a thermometer and a vent. The solution was brought to 0°C with an ice bath and stirred vigorously as ammonia gas was bubbled through

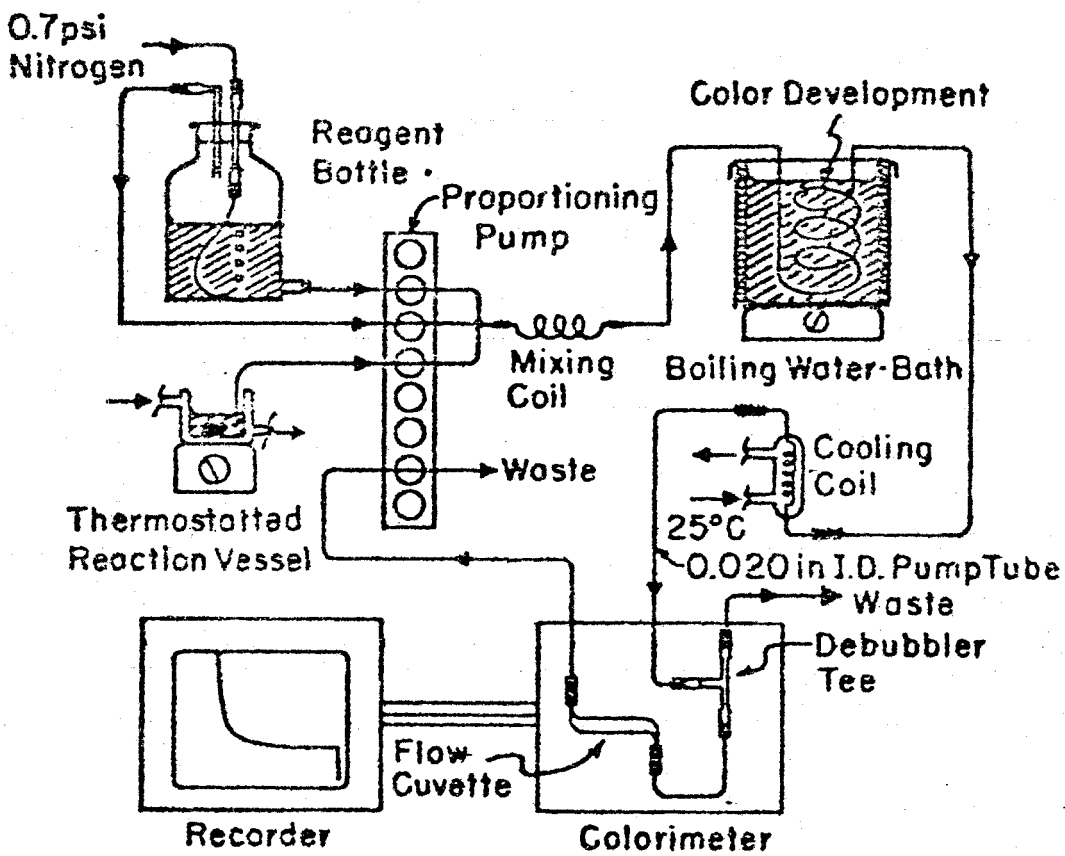
for 7 hours. The vessel was stoppered, equilibrated to room temperature and left for four days. The product was rotary evaporated to dryness and crystallized from ethyl methyl ketone-ethanol. The combined crops collected (yield 1.93 gm, 97%) had a m p of 182-184°C (literature m p 183-184°C (117)). TLC also indicated a pure product (R_f = 0.70, 4:1, $CHCl_3$:MeOH). This product was also used for peptide hydrolysis assays without further purification.

Hydrolyses of Peptide Substrates

The hydrolyses of the two peptide substrates by δ-Cht and monosulfoxide-Met192-δ-Cht (MS-δ-Cht) were performed on a continuous automatic ninhydrin development system devised by Lenard and coworkers (119). It is composed of a Technicon amino acid analyzer and a Technicon auto analyzer. A diagram of the system is shown in Figure 14. The color intensity of an enzyme reaction solution (due to the ninhydrin reaction with free amino groups) is continuously measured by the colorimeter and registered on a recorder connected to it. In this way, a trace of the enzymic hydrolysis of a peptide substrate can be obtained. Color intensity from the reaction of ninhydrin with the free amino groups of the enzyme can be blanked out by inserting the proper aperture (usually #4) in the sample beam of the colorimeter at the beginning of the assay (in contrast to the aperture arrangement used by Lenard, a #1 aperture was used in the sample beam while a #2 was used in the reference beam).

Ninhydrin reagent solution (i.e. 1 L) was prepared by mixing 4 gm ninhydrin, 0.3 gm hydrindantin, 0.53 L methyl cellosolve, 0.40 L distilled deionized water, 0.07 L 4 N, pH 5.5 acetate buffer, 4 ml Brij 35 solution and bubbling N_2 through for at least 60 minutes prior to use.

Figure 14 - Flow diagram of peptide hydrolysis apparatus of Lenard and coworkers (119).



The system was standardized with ammonium chloride solutions ranging from 0.25 to 2.00 x 10^{-4} M. The extinction coefficient thus obtained was 6.23 x 10^{3} M^{-1} cm^{-1}. The ninhydrin solution was checked at the beginning and end of each set of assays by recording the absorbance for a 2.0 x 10^{-4} M ammonium chloride solution.

The hydrolysis of N-acetyl-L-tryptophan amide was run successfully in a test of the apparatus. The chart speed was set at a constant value of 5 min/inch for the recorder used.

Peptide hydrolyses of Ac-L-Phe-Gly-NH_2 and Ac-L-Phe-L-Ala-NH_2 were performed at 25°C in pH 7.8, 0.1 M carbonate buffer, 0.1 N in sodium chloride. For the hydrolysis of Ac-L-Phe-L-Ala-NH_2, 6.3 ml volumes of substrate (3-25 mM) were mixed with 0.7 ml of enzyme solution (2 x 10^{-7} M for δ-Cht and 6.7 x 10^{-7} M for MS-δ-Cht). The reaction solution was immediately passed through the appropriate line in the system leading to color development and recording of the reaction. It was necessary to heat the Ac-L-Phe-L-Ala-NH_2-H_2O solutions (3.15 ml) to 90-100°C to dissolve the substrate completely. Subsequent addition of distilled deionized water and 3.15 ml of 0.2 M carbonate, 0.2 N sodium chloride pH 7.8 buffer resulted in 6.3 ml of the appropriate solution for the reaction. The hydrolyses of Ac-L-Phe-Gly-NH_2 were done in a similar manner with 9 ml volumes of substrate (4-28 mM) combined with 1 ml of enzyme (2 x 10^{-6} M for δ-Cht and MS-δ-Cht). The hydrolyses of Ac-L-Phe-NH_2 were done in exactly the same manner as those for the previous substrate with the exception of the substrate concentration range (4-50 mM). Vigorous stirring was maintained during the course of all the reactions. Absorbance versus time plots were obtained for each enzyme-substrate pair

and reciprocal ($1/v$ vs $1/[S]$) Lineweaver-Burke plots were made for the δ-Cht and MS-δ-Cht hydrolysis of each substrate.

Hydrolysis of α-Casein

The peptide hydrolyses of α-casein by δ-Cht and MS-δ-Cht were done by the method of Kunitz (120) with modifications for the Cht assay suggested by Laskowski (121, 122). One exception to this procedure was made, that being the use of half as much enzyme in each sample as was used originally. This facilitated a more accurate determination of the slope of the curve obtained.

A stock solution(α-casein from bovine milk)was prepared by mixing 0.5 gm of α-casein in 50 ml of 0.1 M, pH 7.8 borate buffer, 0.005 M in $CaCl_2$ and boiling the solution for 20-25 minutes. The resulting suspension was centrifuged at 2500 rpms for 30 minutes and the supernatant solution retained for the enzyme assays (stored at 5°C). The appropriate volume of a 0.1 mg/ml stock enzyme solution (δ-Cht or MS-δ-Cht) was added to each of nine 15 ml Pyrex centrifuge tubes with λ pipettes. The volume of each tube was adjusted to 1 ml and mixed thoroughly before incubation at 35°C for several minutes prior to the assays. At one minute intervals 1 ml of α-casein solution (preincubated for 5 minutes at 35°C) was added to each tube and mixed to start each reaction. The final enzyme concentration range was 1.25 to 20 μg/ml for each set of reactions. After 20 minutes incubation at 35°C in a shaker bath 3 ml of a 5% trichloroacetic acid solution was added (with vigorous mixing) to each tube to stop the reaction and precipitate out the remaining casein in solution. The tubes were left at room temperature for 1 hour before

centrifugation at 2500 rpms for 30 minutes. The absorbance of each supernatant solution was read at 280 nm and plotted versus enzyme concentration. From the slope of the hyperbolic curve obtained an estimate of the k_{cat} was determined for each enzyme assayed.

Measurement of Rates of Methionine Alkylation with α-Bromo-4-Nitroacetophenone

The alkylation reactions were calculated as pseudo-first-order reactions with first-order rate constants (k_{obsd}) equal to k[M] (k - the second-order rate constant of the reaction; [M] - the concentration of BrNAP). The equations in Figure 15 show that k_{obsd} is actually equal to k_2/K_s x [M] for the modification reaction. Because the concentration of BrNAP does not change appreciably during the reaction it can be treated as a constant (123).

$$v = k[M][E]; \; v = k_{obsd}[E]$$

In most reactions first-order rate constants were calculated by the classical kinetic method. The absorbance of E-M (Cht-NAP) complex at 350 nm at time t(A_t) was subtracted from the absorbance at infinite time (A_∞) and plotted versus time on a semi-logarithmic scale. The slope of the line obtained times 2.303 equaled the first-order rate constant k_{obsd}. In some cases, notably anhydro-α-Cht, biphasic plots were obtained. The first-order rate constants for these reactions were found by subtracting the slope for the slower reacting species from the slope for the faster reacting species and multiplying the resulting slope by 2.303.

Figure 15 - Kinetic equations for alkylation of chymotrypsin with BrNAP.

$$E + M \underset{}{\overset{K_s}{\rightleftharpoons}} E{:}M \xrightarrow{k_2} E\text{-}M + Br^-$$

$$K_s = \frac{[E][M]}{[E{:}M]}$$

$$1)\ \frac{d[E\text{-}M]}{dt} = \frac{k_2[E][M]}{K_s + [M]}$$

If $[M] << K_s$ and $[M] >> [E]$

$$2)\ \frac{d[E\text{-}M]}{dt} = \frac{k_2}{K_s}[E][M]$$

$$k_{obsd} = \frac{k_2}{K_s}[M]$$

Initial rates were used to calculate the first-order rate constants for reactions which proceeded slowly and precipitated before an endpoint could be obtained (123). The rate of change in these reactions d[E-M]/dt was divided by the concentration of enzyme present to get the first-order rate constant, k_{obsd}.

III. RESULTS

Preparation and Characterization of Acetylated(Ile16)-Chymotrypsin

Activity Assays of Modified Chymotrypsin

The acetylated(Ile16), inactive form of Cht was chosen as a model for Chtgen based on previous studies of this modified form (21, 60, 61). In these investigations Chtgen was first acetylated with acetic anhydride at pH 6.7, followed by activation to A-δ-Cht. Acetylation of the newly created N-terminal group of Ile16 was then accomplished under the same conditions. The decrease in ATEE activity observed upon acetylation of Ile16 was proportional to the loss of the N-terminal group. When ^{14}C labeled acetic anhydride was utilized in the Ile16 acetylation step the incorporation of radioactive label was also proportional to the loss of activity. The loss of 90% enzymic activity was attributed to a 90% acetylation of the α-amino group of Ile16. Failure to acetylate free amino groups to a greater extent is not uncommon (124). The investigators reasoned that acetylation of the Ile16 residue in active Cht prevents the acquisition of a positive charge which is a prerequisite for enzymic activity (see Section I Parts B and C).

A more complete acetylation of Ile16 may lead to a more inactive sample of Cht. One of the objectives of this work was, therefore, to maximize the yield of acetylated(Ile16)-Cht and minimize any denatura-

tion in the preparations. In order to do this several different procedures were employed.

In the first acetylation procedure the same conditions were used as described above, with the exception of a second acetylation step. Hopefully, this would increase the yield of A_{16}-A-δ-Cht to more than 90%. The results for the ATEE assays of these acetylated forms of Cht are presented on Table 4. The right hand column in that table indicates the percent values for N-*trans*-cinnamoylimidazole active site titrations of α- and δ-Cht. The other values in that column are the results of the ATEE activity assays corrected for the active site titrations. The ATEE activity of A-δ-Cht(kobsd - 42.4 sec^{-1}) was found to be comparable to the value for native δ-Cht(46.4 sec^{-1}). After one acetylation of this material the activity dropped approximately 35% (kobsd - 22.8 sec^{-1}). Another acetylation resulted in a value (kobsd - 4.2 sec^{-1}, 12% activity) comparable to the results obtained by Hess and coworkers (11% activity) (20) and Ghelis and coworkers (9% activity) (61).

The second method of acetylation of Ile16 was chosen primarily to decrease the activity further without increasing the number of acetylations or the concentration of acetylating reagent. Two direct acetylations of δ-Cht and one of α-Cht were carried out at pH 7.9 (125), and an enzyme concentration of 3-4 mg/ml. Hydroxylamine treatments following acetylation reactions were also included to deacetylate any residues other than amino groups (61). All other conditions remained the same. A primary acetylation of δ-Cht decreased ATEE activity 81.1% and reacetylation decreased the activity 98.2% compared to native δ-Cht. A single acetylation of α-Cht was sufficient to decrease ATEE activity

TABLE 4

N-ACETYL-L-TYROSINE ETHYL ESTER ACTIVITY ASSAYS

Cht	k_{obsd} sec^{-1} [a]		%
α-Cht	45.4 ± 2.2	(4)	79.5[b]
δ-Cht	46.4 ± 7.6	(12)	76.1[b]
A-δ-Cht	42.4 ± 0.7	(4)	-
A_{16}A-δ-Cht	22.8 ± 1.2	(2)	64.5[c]
ReA_{16}A-δ-Cht	4.12 ± 0.08	(2)	12.0[c]
A_{16}-δ-Cht	6.19 ± 0.13	(4)	18.9[c]
ReA_{16}-δ-Cht	0.67 ± 0.08	(6)	1.8[c]
A_{16}-α-Cht	2.07 ± 0.02	(2)	6.0[c]
A_{16}-δ-Cht(F)	1.34 ± 0.07	(5)	3.8[c]
Anhydro-α-Cht	-	-	<1.6
3-Methyl-His57-α-Cht	-	-	<2.0
MS-δ-Cht	19.4 ± 0.7	(3)	54.6[c]

[a] under conditions of [ATEE] > K_m

[b] actual N-*trans*-cinnamoylimidazole active site titration values

[c] modified Cht/native Cht percent ATEE activities corrected for N-*trans*-cinnamoylimidazole active site titrations of native Cht

( ) denotes number of determinations

94.0%. These results were below the values obtained for acetylation at pH 6.7 and reported by others (20, 61).

The third procedure was by far the most specific for Ile16 acetylation and yielded a form of the enzyme in which peripheral lysine residues were not acetylated. This procedure was employed to alleviate precipitation problems encountered in the BrNAP alkylation reactions of acetylated Chts. Acetylation of all lysine residues of Cht drastically decreases the number of positive charges on the molecule. This would lower the pI of the enzyme and consequently cause precipitation problems in the acid pH regions in which the BrNAP alkylation experiment is studied. Use of the reversible modification reagent, dimethylmaleic anhydride (DMMA) for ε-amino groups of lysine made it possible to block these residues in Chtgen. Activation of this form of the enzyme to δ-Cht created the Ile16 N-terminal group which was then selectively acetylated without affecting the lysine residues. Subsequent removal of the reversible modification reagent at pH 5.0 generated A_{16}-δ-Cht(F). Affinity chromatography over a lima bean trypsin inhibitor-agarose column made it possible to separate most of the unreacted active Cht from the A_{16}-δ-Cht. The decrease in ATEE activity observed was 96.2% which was comparable to the other decreases in activity already reported.

Control experiments were done to determine whether the exposure of the enzyme to the acetic acid caused any loss of enzymic activity (denaturation). The conditions for these experiments were similar in concentrations of acetic acid that result from the reactions (1% v/v), pH (7.9), time (120 minutes), enzyme concentration (3-4 mg/ml) and solution (0.01 M $CaCl_2$). A 4.4% decrease in ATEE activity was observed after one acetic acid treatment and a 6.9% decrease observed after the

second treatment. These data indicate that the substantial decreases in enzymic activity were due to acetylation and not denaturation due to exposure to acetic acid. However, some denaturation may occur during the acetylation procedure.

Active Site Titrations of Acetylated Forms of Chymotrypsin

p-Nitrophenyl acetate (*p*NPA), non-specific, active site titrations were performed on most of the acetylated samples previously mentioned. This substrate was used to test for active sites because hydrolysis is non-specific (the aromatic moiety is not directed into the specificity cavity of Cht). The productive orientation of the molecule in the nucleophilic site of the enzyme results in cleavage of the *p*-nitrophenyl moiety from the substrate. The *p*-nitrophenol product is a good chromophore and its appearance, upon hydrolysis, makes it possible to follow the enzymic reaction spectrophotometrically. It also allows one to calculate the normality of active sites from the burst of *p*-nitrophenol upon acylation of the enzyme (113). These titrations were used on the acetylated samples of Cht because it has been implied from a previous study that acetylation of the Ile16 causes distortion of the binding site and does not affect the nucleophilic site in catalysis considerably (61). Since *p*NPA active site determinations involve only the nucleophilic site and not the substrate binding site they were chosen to determine the functional nature of the nucleophilic region of the active site. The results of the titrations can be found on Table 5 for native and acetylated forms of Cht. The ATEE activity values (percent values) are included in the right hand column of Table 5 for comparison. There is relatively good correlation between the *p*NPA active site titrations and

TABLE 5

p-NITROPHENYL ACETATE ACTIVE SITE TITRATIONS

	% Active Sites[a]		% ATEE Activity[b]
α-Cht	74.8 ± 1.0	(3)	-
δ-Cht	76.5 ± 0.5	(5)	-
$A_{1\,6}$-δ-Cht	13.3 ± 0.5	(3)	18.9
$ReA_{1\,6}$-δ-Cht	4.7	(1)	1.8
$A_{1\,6}$-α-Cht	3.0 ± 0.3	(4)	6.0
$A_{1\,6}$-δ-Cht(F)	4.1	(1)	3.8
MS-δ-Cht	61.4 ± 1.3	(3)	54.6

[a] modified Cht/native Cht percent values corrected for *p*NPA active site titrations of native Cht

[b] values from Table 4, corrected for C-I active site titrations of native Cht

( ) denotes number of determinations

the ATEE assays. This was not expected based on the results obtained by Ghélis and coworkers in 1970 (61). They observed approximately 50% active sites in samples of $A_{16}A$-δ-Cht which possessed 9% ATEE activity.

p-Nitrophenyl acetate active site titrations were also performed on MS-δ-Cht. This form of the enzyme displayed 61.4% active sites, whereas, native δ-Cht had 76.5%. The 15% decrease in active sites for MS-δ-Cht is probably due to a limited denaturation during the extensive oxidation procedure (22 hours).

Circular Dichroism Patterns of Acetylated Chymotrypsins

The circular dichroism (CD) experiments revealed that three modified enzymes, ReA_{16}-δ-Cht, A_{16}-α-Cht and A_{16}-δ-Cht, had CD patterns almost identical to that of Chtgen (Figures 16 and 17). The sample of δ-Cht acetylated as Chtgen and then activated had a CD pattern similar to that of native δ-Cht. These experiments have been previously reported and interpreted as a conformational change that accompanies deprotonation or acetylation of the α-amino group of Ile16 (62, 114). The CD results reported here correlate well with the original results and coupled with the dramatic decreases in enzymic activity observed lead to the conclusion that the α-amino group of Ile16 had indeed been acetylated.

Figure 16 - Circular dichroism patterns of various forms of chymotrypsin in the 220-240 nm range: [E] = 2×10^{-5} M, 0.17 M phosphate buffer, pH 6.7, 0.05 M Na_2SO_4, ionic strength 0.2, 1 mm path length, 0.002 scale.

a Chtgen, b ReA_{16}-δ-Cht, c A_{16}-α-Cht
d A-δ-Cht, e δ-Cht, f α-Cht

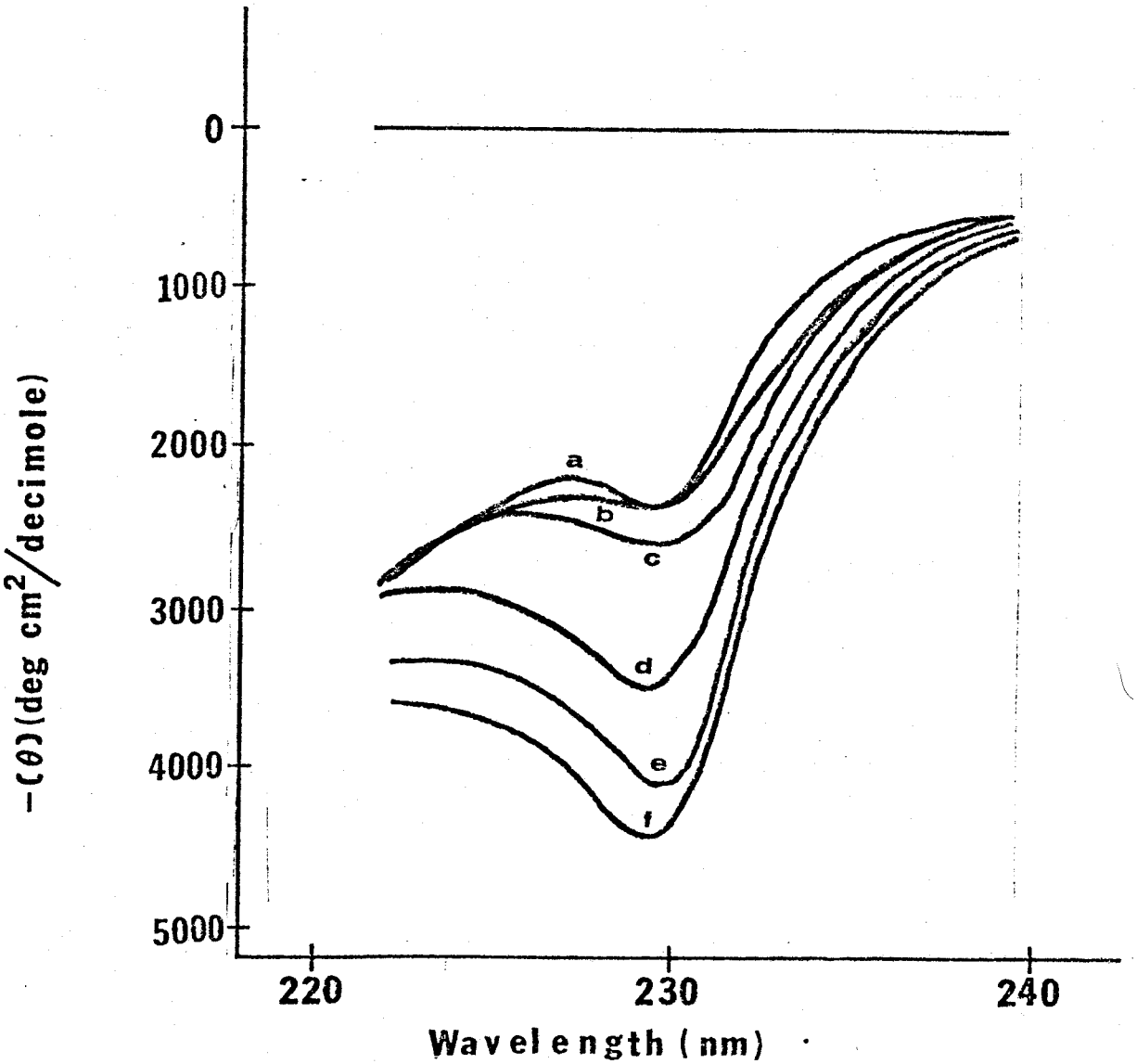
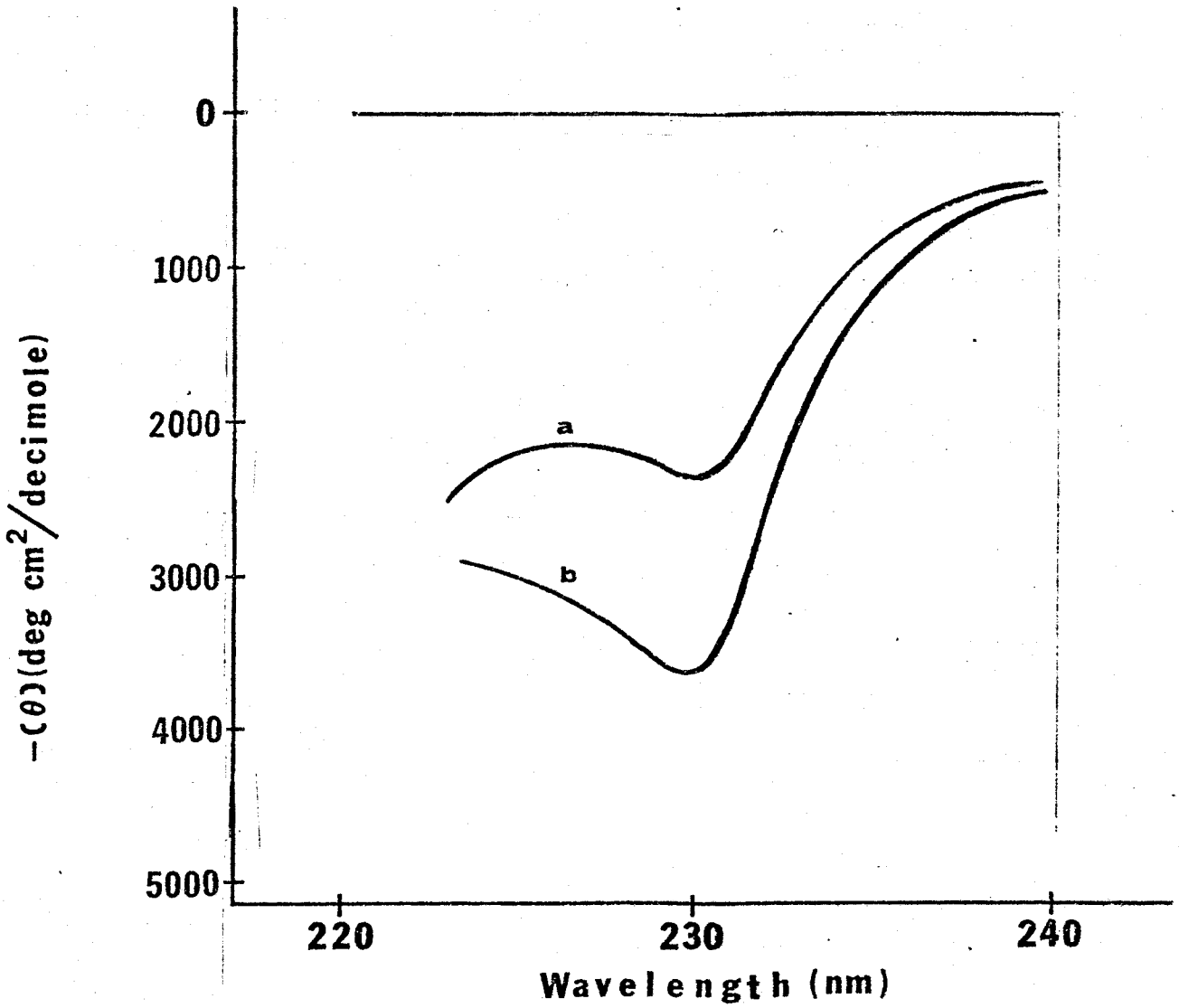


Figure 17 - Circular dichroism patterns of δ-chymotrypsin and A_{16}-δ-chymotrypsin(F) in the 220-240 nm range: conditions same as those stated in preceeding figure.

a Chtgen and A_{16}-δ-Cht, b δ-Cht



Alkylation of Acetyl-L-Methionine Amide and Various Forms of Chymotrypsin with α-Bromo-4-Nitroacetophenone

BrNAP and other phenacyl bromides react with Cht in an active-site-directed manner by virtue of their similarity to natural substrates of Cht (95, 105, 126, 127). A new long wavelength absorption band at 350 nm is observed upon the alkylation of Cht with BrNAP under conditions stated in Section II Part E 5. The ease of following an alkylation reaction of Cht with this reagent at 350 nm with time makes it a convenient way to probe the active site of Cht in respect to the structural position of the Met192 residue.

In this study, the attempted BrNAP alkylation of Met192 of acetylated(Ile16)-Cht was designed to investigate the role of Met192 in the activation mechanism of Cht. As mentioned in the preceding part, acetylated(Ile16)-Cht was chosen as a model for a Chtgen-like molecule. The attempted BrNAP alkylation would reveal the relative accessibility of the Met192 residue in this form of the enzyme. Differences between this and other conformations of the enzyme can be discerned from the Met192 accessibility to BrNAP. In essence, this constituted an indirect test of Freer's "single unified event" hypothesis. In Cht at high pH or acetylated(Ile16)-Cht, the Ile16 possesses no positive charge and therefore, cannot ion-pair with Asp194. According to the theory, the residues involved in the formation of the active site should rearrange into a *zymogen-like* structure with Asp194 reassociating with His40 and Met192 reburying into the hydrophobic interior of the molecule. Experimental

results which support this theory include:

1. The loss of activity by acetylated(Ile16)-Cht (20, 60, 61).

2. Similarity of the CD spectra of acetylated(Ile16)-Cht, Cht at high pH and Chtgen (62, 114).

3. The observation of no alkylation of Met192 in Chtgen with phenacyl bromides (126, 127).

Since the ideal pH for alkylation of Met192 with BrNAP is 5.6, the reaction could not be run at high pH on Cht. For this reason, the acetylated(Ile16)-Cht was best suited to test the "single unified event" theory of activation. Failure to alkylate Met192 of this enzyme form would support Freer's hypothesis while alkylation would indicate an accessibility of Met192 and tend to disprove the theory.

The study of the structural position of Met192 in anhydro-α-Cht and 3-methyl-His57-α-Cht by the BrNAP alkylation reaction was also undertaken to investigate the role of Met192 in the catalytic mechanism of Cht. The two inactive forms of Cht referred to above involve modifications of two essential "charge transfer complex" residues, Ser195 and His57 respectively. Due to the importance of Ser195 and His57 in catalysis (see Section I Part B 2) the effect of modification of these residues on the reactivity of Met192 to BrNAP would be of interest. As a consequence, relationships between the "charge transfer" residues and Met192 heretofore undetected, could be uncovered. The results for the BrNAP alkylation of various forms of Cht follows.

<u>Ac-Met-NH_2, Chtgen, DIP-α-Cht, α-Cht and δ-Cht</u>

Data for the alkylation of Ac-Met-NH_2, Chtgen, DIP-α-Cht, α-Cht and δ-Cht were necessary for comparative purposes. The results obtained for these reactions are presented on Table 6. The rate of BrNAP alkyla-

TABLE 6

ALKYLATION WITH BrNAP

	$k_{obsd} \times 10^4$ sec^{-1}	
Ac-Met-NH_2	0.004 ± 0.002[a]	(3)
Chtgen	N.R.[b]	(3)
DIP-α-Cht	N.R.[b]	(2)
α-Cht	7.42 ± 0.51	(4)
δ-Cht	7.12 ± 0.54	(3)
MS-δ-Cht	N.R.[b]	(2)

[BrNAP] = 2.0×10^{-4} M, [E] = 2.0×10^{-5} M, 0.05 M acetate buffer pH 5.6, 5% acetonitrile, 25°C

[a] [BrNAP] = 1.0×10^{-3} M, [Ac-Met-NH_2] = 2.0×10^{-4} M

[b] no observable reaction

( ) denotes number of determinations

tion of Ac-Met-NH_2 was calculated from the initial increase in absorbance at 350 nm as described in Section II Part E 17, using 2.0×10^{3} $\underline{M}^{-1}$ cm^{-1} as the extinction coefficient for Ac-Met-NH_2-NAP. This extinction coefficient was determined from the 350 nm absorbance (0.04) of a 2×10^{-5} $\underline{M}$ solution of Ac-Met-NH_2-NAP. The calculated first order alkylation rate constant for Ac-Met-NH_2 was 0.004×10^{-4} sec^{-1}.

Alkylation rate constants for α- and δ-Cht were obtained graphically (see Section II Part E 17) from A_∞-A_t readings. The results were 7.42 and 7.12×10^{-4} sec^{-1} respectively, for α- and δ-Cht ($t_{\frac{1}{2}}$ = 15.5 and 16.2 minutes). When these values for the alkylation of Cht are compared to the value for the alkylation of Ac-Met-NH_2, they show that the BrNAP reaction takes place approximately 1800 times faster with Cht than with Ac-Met-NH_2. These data attest to the active-site-directed nature of the reaction of BrNAP with Cht. Chtgen and DIP-α-Cht did not react with BrNAP over a 24-48 hour period. These results agree with those of Schramm and Lawson who were unable to alkylate Chtgen and DIP-α-Cht with similar phenacyl bromides (126).

Acetylated-Chymotrypsins

The results of the BrNAP alkylation of acetylated forms of Cht are summarized in Table 7. δ-Cht which had been acetylated as Chtgen at pH 6.7 and activated with trypsin (A-δ-Cht) displayed an alkylation rate constant approximately 57% that of native δ-Cht. The alkylation reaction of the preparation of ReA_{16}-δ-Cht occurred very slowly. This reaction appeared to be complete after 34 hours as evidenced by a leveling off of the 350 nm absorbance reading (0.078). Alkylation rate constants were calculated using the two different methods outlined in Section II

TABLE 7

ALKYLATION OF ACETYLATED CHT WITH BrNAP

	$k_{obsd} \times 10^4$ sec^{-1}		$t_{1/2}$ hours	
δ-Cht		7.12 ± 0.54	0.27	
A-δ-Cht	3.38	4.02 ± 0.64	0.48	
	4.65			
ReA_{16}A-δ-Cht	0.21		9.2	
	0.38[a]			
ReA_{16}-δ-Cht	0.19[a]		10.1	
	0.27[a]	0.19 ± 0.08	7.1	10.1
	0.12[a]		16.0	
A_{16}-α-Cht	0.08[a]		24.0	
A_{16}-δ-Cht(F)		N.R.	-	

[BrNAP] = 2.0×10^{-4} M, [E] = 2.0×10^{-5} M, 0.05 M acetate buffer pH 5.6, 5% acetonitrile, 25°C

[a] calculated from initial increase in optical density at 350 nm versus time

Part E 17. The rate constant determined by plotting the log of the absorbance readings at time infinity (A_∞, 34 hours) from the log of the absorbance readings at various times (A_t) was 0.21×10^{-4} sec^{-1}. Based on this rate constant the half-time ($t_{\frac{1}{2}}$) of reaction was 9.2 hours. A different rate constant was calculated from the initial increase in absorbance with time (0.37×10^{-4} sec^{-1}, $t_{\frac{1}{2}}$ = 5.1 hours) assuming that the extinction coefficient for $ReA_{16}A$-δ-Cht-NAP is the same as that of δ-Cht-NAP. Only one alkylation reaction was performed on this preparation due to the limited amount of the material available. Attempts to reproduce the alkylation results stated above with subsequent preparations of $ReA_{16}A$-δ-Cht failed due to precipitation phenomenon which precluded monitoring any alkylation reaction.

The procedure for the direct acetylation of δ-Cht was employed in part to alleviate the solubility problems encountered. Millipore filters (0.22 μm pore size) were also used to filter any material from the ReA_{16}-δ-Cht and A_{16}-α-Cht solutions prior to the attempted alkylation reactions. The absorbance versus time trace seemed to be first order for these reactions up to 10-20 hours reaction time. Thereafter, precipitation was obvious, as evidenced by a disproportionate increase in absorbance with time. Eventually, the absorbance reading far exceeded the theoretical value for total alkylation of the enzyme (0.115). Alkylation rate constants were calculated based on the initial increases in absorbance with time (designated a in Table 7). The average value for this rate constant (0.19×10^{-4} sec^{-1}, $t_{\frac{1}{2}}$ = 10.1 hours) was almost identical to one of the two rate constants calculated for $ReA_{16}A$-δ-Cht (0.21×10^{-4} sec^{-1}). The rate constant for A_{16}-α-Cht determined by this method

was 0.08×10^{-4} sec^{-1} ($t_{\frac{1}{2}}$ = 24 hours).

The question remained, however, whether the increase in absorbance in the first 10-20 hours of reaction was due to precipitation of the modified enzyme or alkylation of the Met192 residue. A simple experiment previously developed by Sigman and Blout resolved whether Met192 was actually alkylated or not (96). According to the procedure, performic acid oxidation was carried out to convert all unalkylated methionine (Met180 or Met192) to methionine sulfone prior to acid hydrolysis. This was done because Met192-NAP is not stable to acid hydrolysis wile methionine sulfone is. Therefore, measurement of the methionine sulfone content of hydrolyzed samples allows one to calculate the extent of alkylation of Met192 by BrNAP. Also, Met192-NAP is stable to performic acid oxidation. A BrNAP alkylation reaction solution of ReA_{16}-δ-Cht was chromatographed over a G-25 (fine) Sephadex column to separate the protein from the other reaction components. This was done 7.5 hours after the reaction was begun. Theoretically 0.4 residue of Met192 should be alkylated if the alkylation reaction were occurring. Approximately, 1.6 residue of methionine sulfone would be expected from amino acid analysis of the test sample. Performic acid oxidation and acid hydrolysis at 110°C followed by amino acid analysis revealed the presence of 1.7 residue of methionine sulfone in the test sample (Table 8). The necessary controls were treated in the same manner as the test sample and are included in Table 8 for comparison. It was concluded from the data that alkylation of the Met192 was actually occurring and that precipitation was not the major cause of the increase in absorbance at 350 nm in the first 7.5 hours of reaction.

TABLE 8

AMINO ACID ANALYSIS FOR METHIONINE SULFONE

Sample	mole %	Methionine Sulfone Residues
Performic Acid Oxidized		
δ-Cht	0.90	2.0
δ-Cht-NAP	0.48	1.0
$ReA_{1\ 6}$-δ-Cht[a]	0.77	1.7
Chtgen-NAP[b]	0.77	1.7

[a] treated with [BrNAP] = 2×10^{-4} M, 5% acetonitrile, pH 5.6 for 8 hours

[b] treated with [BrNAP] = 2×10^{-4} M, 5% acetonitrile, pH 5.1, 4.8 M guanidine hydrochloride for 5 hours

The results for the BrNAP alkylation reaction with A_{16}-δ-Cht (Fersht procedure) were negative and are denoted N.R. on Table 7. The initial absorbance readings of these reaction solutions, immediately after mixing the enzyme solution with the acetonitrile BrNAP solution, were 0.036, 0.075 and 0.110. No significant increase in the 350 nm absorbance occurred thereafter (13, 67 and 24 hours respectively). The variation in the initial readings at 350 nm is indicative of some non-specific phenomenon such as precipitation, rather than the specific alkylation of Met192. It is possible that upon mixing the components of the reaction, a limited precipitation could take place at the acetonitrile-aqueous interface. Close observation of the reaction solution upon mixing substantiated this. The difference between the three initial 350 nm readings could be due to a different degree of mixing in each case. Wide variation in the initial increases also points to some non-specific phenomenon. The absence of any increase in absorbance at 350 nm with time shows that no alkylation of the Met192 occurred in the test samples. The above results will be discussed in the next section.

GuCl Treated Chymotrypsinogen

No increase in absorbance at 350 nm was recorded for any of the guanidine hydrochloride denatured Chtgen-BrNAP reactions after five hours. However, when the proteins were isolated (by dialysis and lyophilization) and redissolved in pH 5.6 buffer, their absorbancies at 350 nm were 40-45%of that expected for Cht-NAP. The data indicate that alkylation

of the Met192 had occurred. The amino acid analysis of one of the Chtgen-NAP samples, denatured with 4.8 N guanidine hydrochloride, revealed the presence of 1.7 residue of methionine sulfone (Table 8). Therefore, 0.3 residue of methionine in Chtgen was alkylated during the denaturation-BrNAP treatment. It is not possible from the data to decide whether or not the Met180, Met192 or both residues were alkylated.

Anhydro-α-Chymotrypsin

The anhydro-α-Cht-BrNAP reaction was biphasic in nature displaying a fast reacting component with a rate constant (7.0×10^{-4} sec^{-1}) similar to that of α-Cht (7.42×10^{-4} sec^{-1}) and a slow reacting component with a rate constant (0.5×10^{-4} sec^{-1}) about 7% that of α-Cht (Table 9). A characteristic semilogarithmic plot of the alkylation of α-Cht is shown in Figure 18 followed by a plot for the alkylation of anhydro-α-Cht in Figure 19. The absence of any slow reacting component in the α-Cht alkylation plot is noteworthy, whereas, the definite presence of one in the anhydro-α-Cht plot is obvious. The rate constants for the two components of the reaction with binding and non-binding (LBTI-affinity column) anhydro-α-Cht varied somewhat. However, the standard deviations for these reactions place the average values within the range of the rate constant for α-Cht. There was a greater variation in the values for the percent of each component in the reaction (13-68% and 32-87%). The following four substates of anhydro-α-Cht appear to exist: a fast reacting binding form, a fast reacting non-binding form, a slow reacting binding form and a slow reacting non-binding form. The implications of the alkylation reaction with anhydro-α-Cht (binding and non-binding) will be discussed in the next section.

TABLE 9

ALKYLATION OF ANHYDRO-α-CHT WITH BrNAP

	$k_{obsd} \times 10^4$ sec^{-1}			
	Primary	%	Secondary	%
α-Cht	7.42 ± 0.51	>85	-	<15
Anhydro-α-Cht (binding)	4.44	20	0.22	80
"	6.76	13	0.55	87
"	8.20	50	1.00	50
"	4.17	47	0.31	53
Anhydro-α-Cht (non-binding)	7.86	59	0.43	41
"	9.14	68	0.54	32
Average	7.0 ± 2.8	13-68	0.5 ± 0.2	32-87

[BrNAP] = 2.0×10^{-4} M, [E] = 2.0×10^{-5} M, 0.05 M acetate buffer pH 5.6, 5% acetonitrile, 25°C

Figure 18 - Semilogarithmic plot of the change in absorbance at 350 nm due to reaction of native α-chymotrypsin with BrNAP: [E] = 2×10^{-5} M, [BrNAP] = 2×10^{-4} M, pH 5.6, 0.05 M acetate buffer, 5% acetonitrile, 25°C; k_{obsd} = 7.4×10^{-4} sec^{-1}.

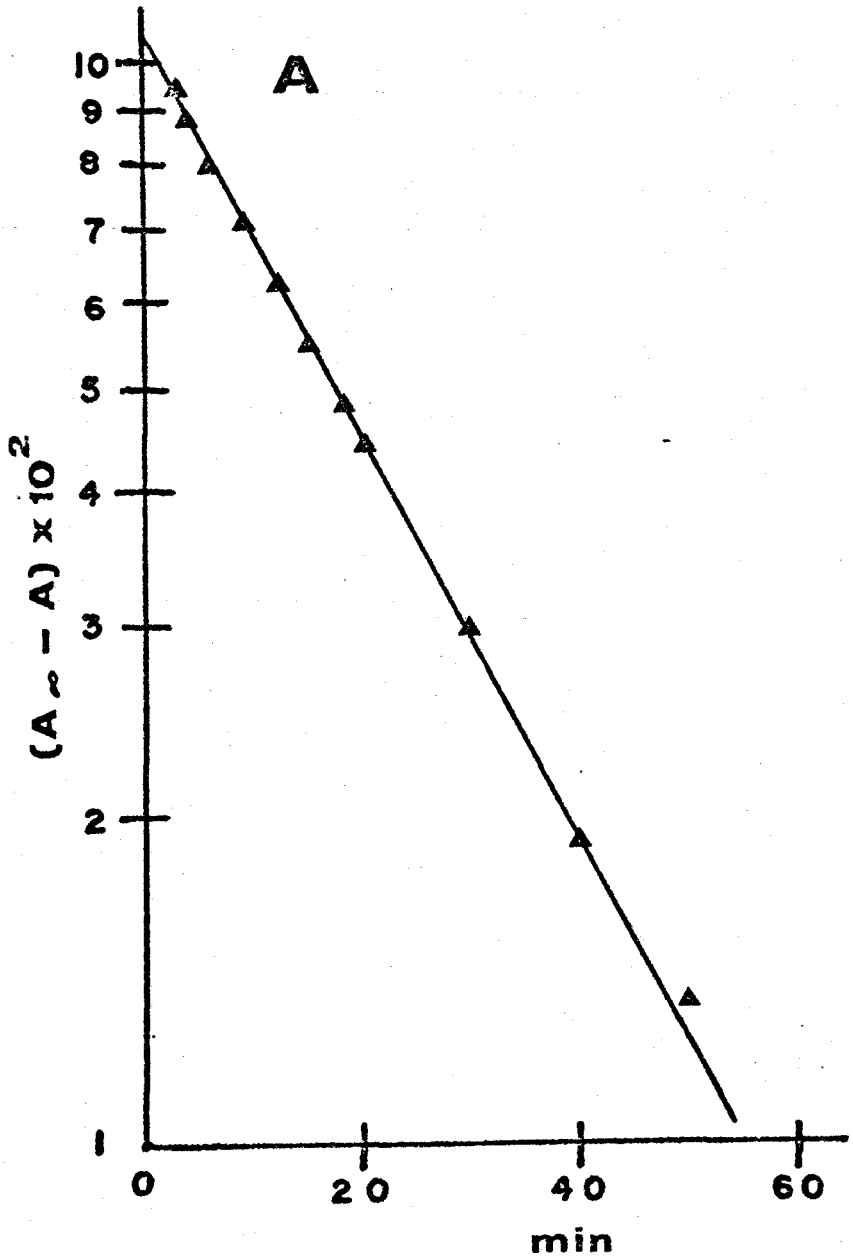
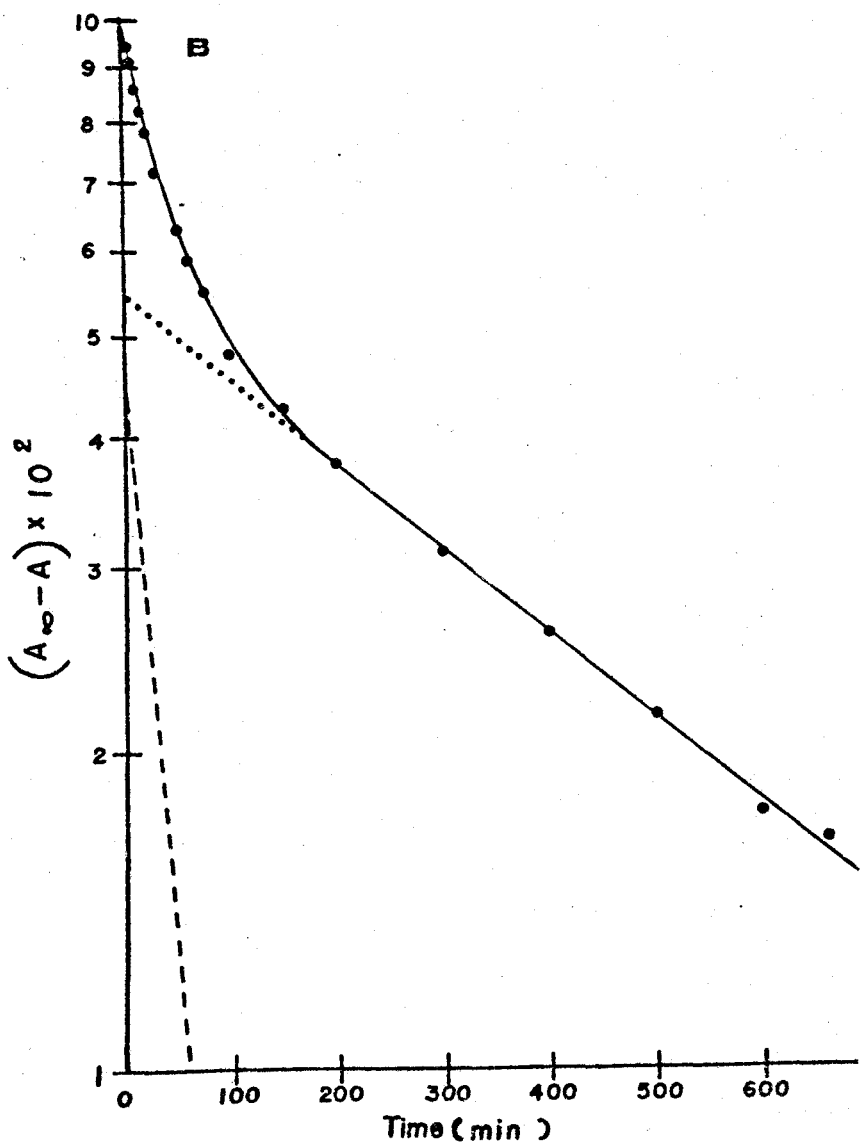


Figure 19 - Semilogarithmic plot of the change in absorbance at 350 nm due to reaction of anhydro-α-chymotrypsin with BrNAP: [E] = 1.6×10^{-5} M, [BrNAP] = 2×10^{-4} M, same solution conditions as in reaction with α-chymotrypsin; -------- fast reacting component ($k_{obsd} = 7.0 \times 10^{-4}$ sec^{-1}) (calculated by subtraction of slow reaction from observed reaction), ····· slow reacting component ($k_{obsd} = 0.5 \times 10^{-4}$ sec^{-1}, ——— observed reaction.



3-Methyl-His57-α-Chymotrypsin

Surprisingly, 3-methyl-His57-α-Cht reacted with BrNAP approximately 6 times faster than α-Cht (41.6×10^{-4} sec^{-1}). The rate constants calculated for several reactions are presented in Table 10 and a characteristic plot of the reaction is shown in Figure 20. The variation between the alkylation reactions with 3-methyl-His57-α-Cht is considerably less than that for anhydro-α-Cht. A slow reacting component (1.38×10^{-4} sec^{-1}) was also observed in the 3-methyl-His57-α-Cht-BrNAP reactions. However, it consistently accounted for less than 20% of the total reaction. These results will also be discussed in the following section.

Spectra of Alkylated Chymotrypsins

When spectra (240-600 nm) of Chtgen-NAP, 3-methyl-His57-α-Cht-NAP and anhydro-α-Cht-NAP were run two basic patterns emerged. These patterns as well as the spectrum of Cht-NAP are shown in Figure 21. Chtgen-NAP had a plateau in the region between 300 and 350 nm whereas, 3-methyl-His57-α-Cht-NAP and anhydro-α-Cht-NAP displayed distinct peaks at 350 nm as seen in the spectrum of Cht-NAP. No significant changes were noted between the spectra for Cht-NAP, 3-methyl-His57-α-Cht-NAP and anhydro-α-Cht-NAP.

CD Spectra of Alkylated Chymotrypsins

CD patterns in the 300-400 nm range are presented in Figure 22 for α-Cht-NAP and 3-methyl-His57-α-Cht-NAP. It was not possible to obtain data for the anhydro-α-Cht-NAP derivative of Cht due to a limited amount

TABLE 10

ALKYLATION OF 3-METHYL-HIS57-α-CHT WITH BrNAP

	$k_{obsd} \times 10^4$ sec^{-1}	
	Primary	Secondary
α-Cht	7.42 ± 0.51	-
3-Methyl-His57-α-Cht	44.8	1.48
"	45.0	0.95
"	35.2	1.70
Average	41.6 ± 4.6	1.38 ± 0.28

[BrNAP] = 2.0×10^{-4} M, [E] = 2.0×10^{-5} M, 0.05 M acetate buffer pH 5.6, 5% acetonitrile, 25°C

Figure 20 - Semilogarithmic plot of the change in absorbance at 350 nm due to reaction of 3-methyl-His57-α-chymotrypsin with BrNAP: [E] = 2×10^{-5} M, [BrNAP] = 2×10^{-4} M, same solution conditions as in reaction with α-Cht; ------- fast reacting component ($k_{obsd} = 41.6 \times 10^{-4}$ sec^{-1}), ——— slow reacting component ($k_{obsd} = 1.38 \times 10^{-4}$ sec^{-1}), -·-·-·- observed reaction.

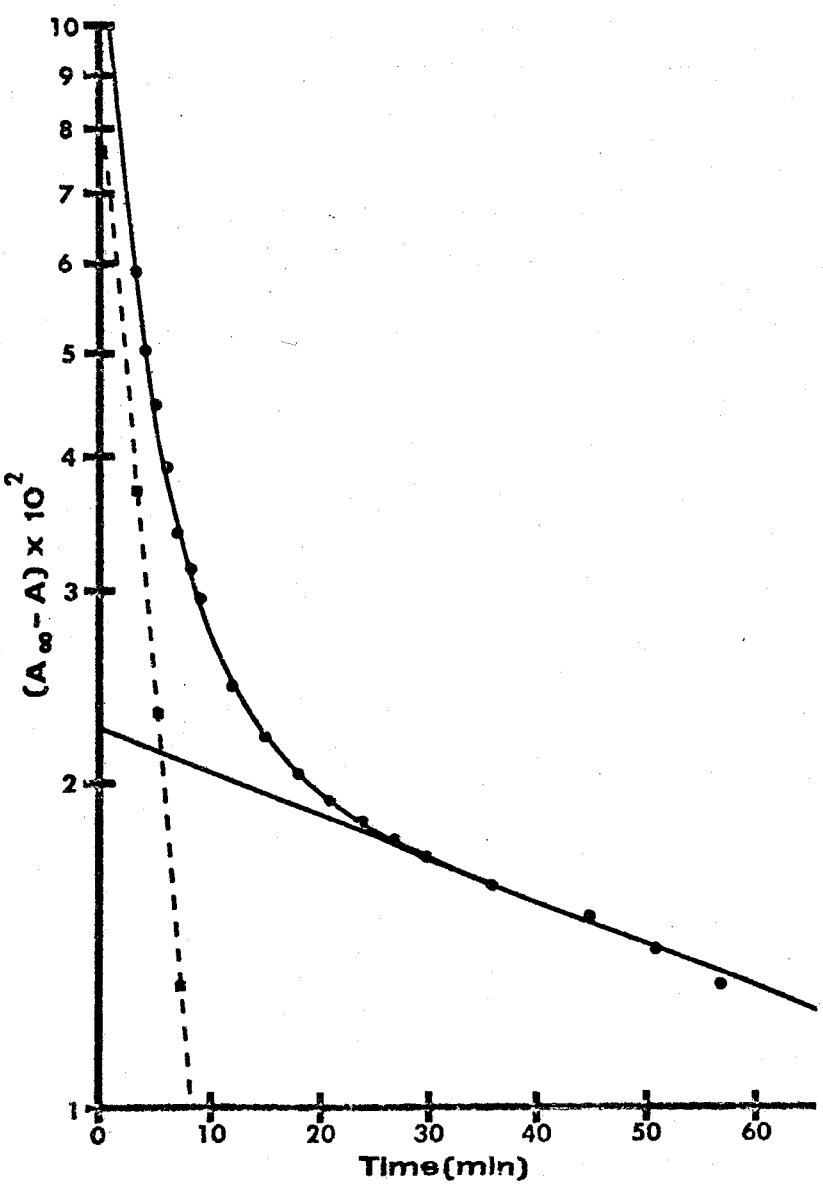


Figure 21 - Absorption spectra characteristic of various forms of chymotrypsin: ——— chymotrypsin-NAP, anhydro-α-chymotrypsin-NAP, 3-methyl-His57-α-chymotrypsin-NAP; ····· chymotrypsinogen-NAP; ------- native-chymotrypsin: [E] = 2×10^{-5} M, pH 5.6, 0.05 M acetate buffer

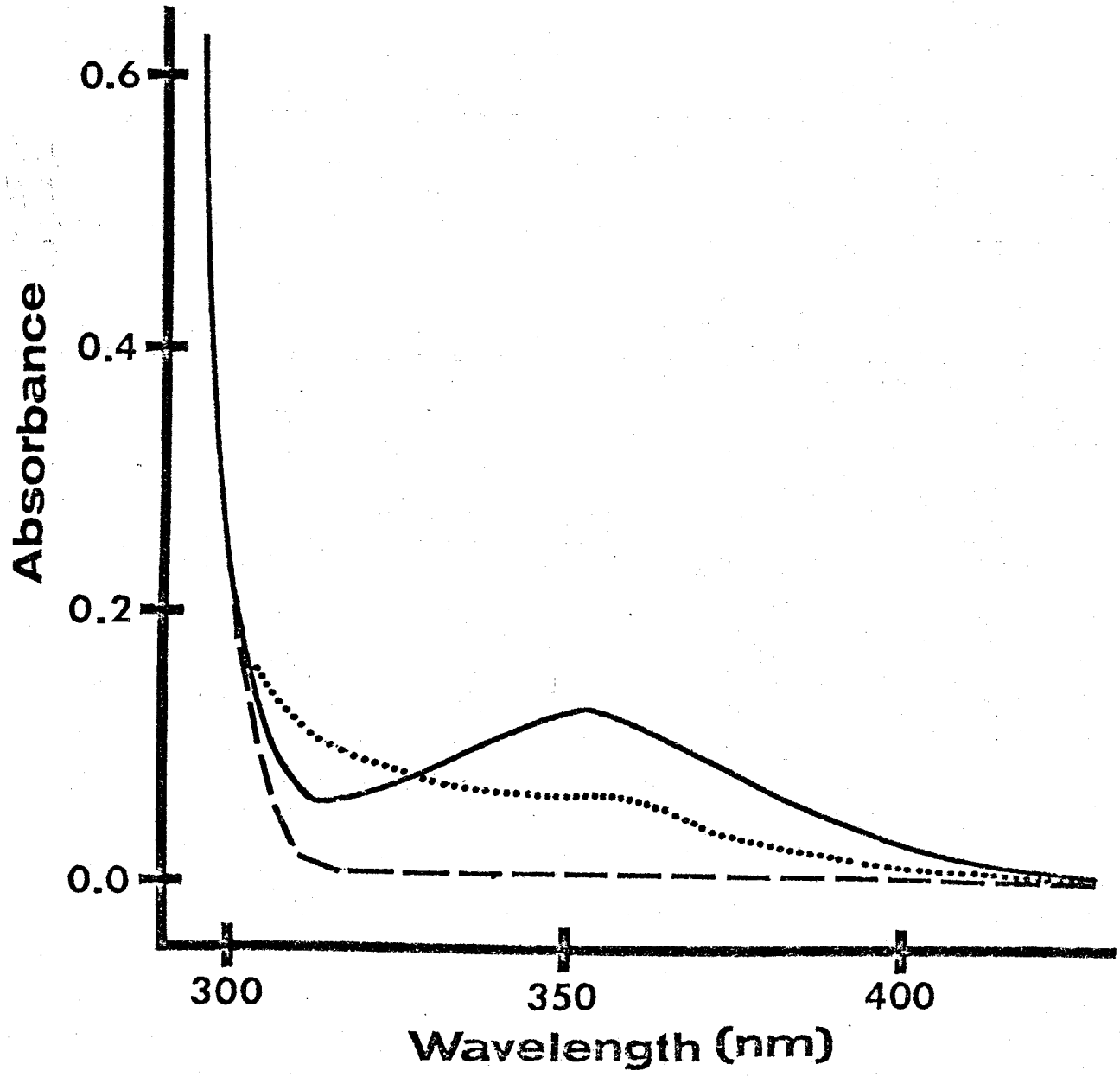
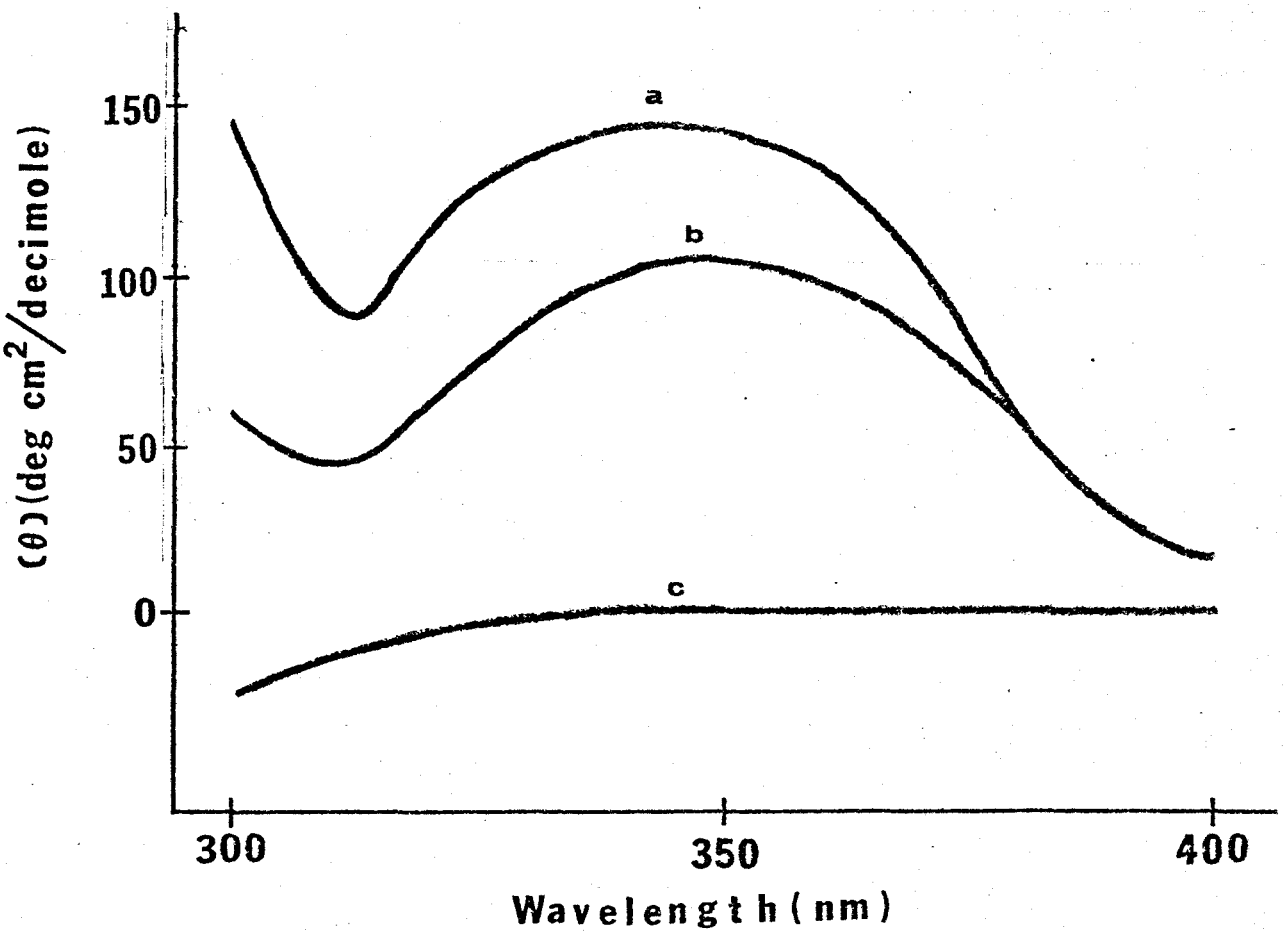


Figure 22 - Circular dichroism patterns of various forms of chymotrypsin in the 300-400 nm range: [E] = 4 x 10^{-5} M, 10^{-3} M HCl, 1 cm path length, 0.002 scale.



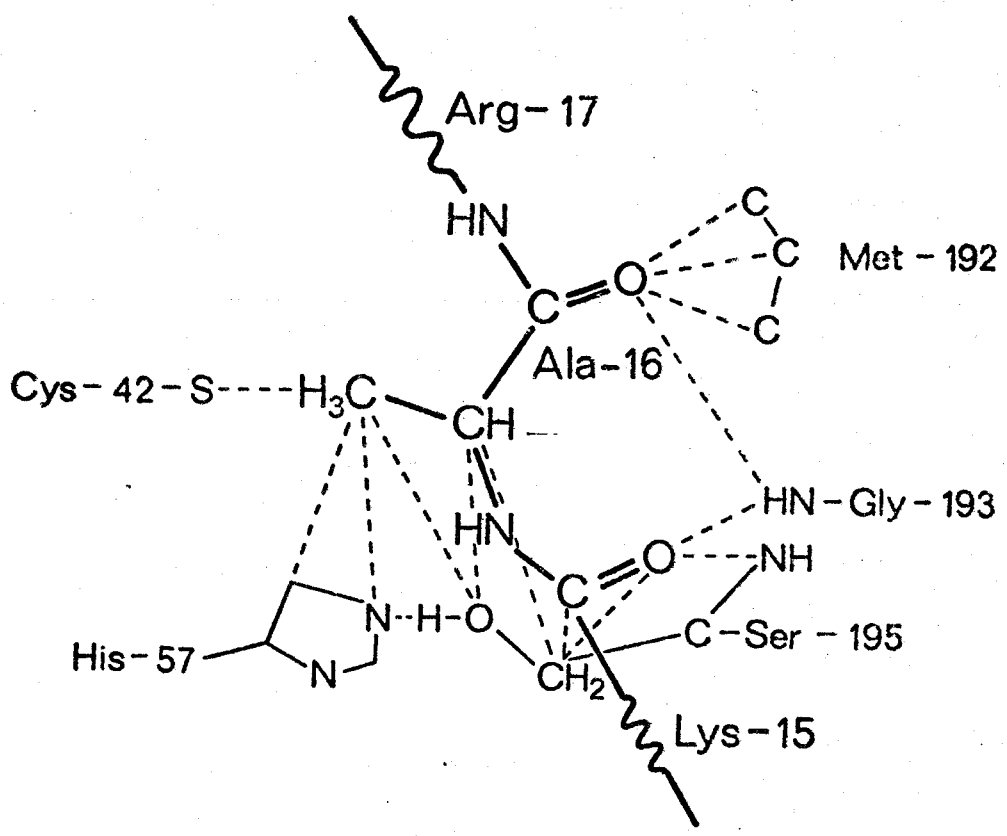
of the material. No significant changes were noticed in the spectra of α-Cht-NAP and 3-methyl-His57-α-Cht-NAP.

Kinetic Parameters of the Peptide Hydrolysis of Several Substrates by δ-Chymotrypsin and Monosulfoxide (Met192)-δ-Chymotrypsin

The literature reviewed in Section I points out the extensive work done on Met192 modified Chts concerning monoamino acid ester or amide substrates. There has been little work done on the effect of Met192 modification on peptide substrates. One of the three theories on the participation of Met192 in catalysis presented in Section I (Fersht's theory) suggests that the Met192 residue maintains repulsive contacts with the leaving group of peptide substrates and in this way helps form a secondary substrate binding site for the leaving group. These contacts are thought to distort bound peptide substrates toward the transition state of the reaction, thus lowering the energy of activation and promoting the rate of catalysis. The theory is built basically upon an x-ray model of Cht and pancreatic trypsin inhibitor (shown in Figure 23) and is supported by some experiments on the regeneration of peptide substrates from acyl-enzyme intermediates and amino acid amides (118).

Weiner and coworkers looked at the relative activity of mono- and dimethionine sulfoxide Cht toward several peptides (110). These experiments measured relative rates of hydrolysis and did not distinguish changes in kinetic parameters, K_m and k_{cat}. The general results concerning MS-Cht showed a decrease in the relative rate of hydrolysis (2-6 fold) of three CBZ peptides (CBZ-Phe-Leu, CBZ-Trp-Ser and CBZ-Trp-Gly). Without more specific knowledge about the decreases in activity (k_{cat} or K_m changes) this data is only of marginal value.

Figure 23 - Interactions of the reactive peptide bond (Lys15I-Ala16I) of pancreatic trypsin inhibitor and chymotrypsin. The inhibitor residues are in heavier type (104).



Since the small substrates used to study Met192-modified Chts in the past lack the leaving group normally present in peptide substrates, and since the studies available on peptide substrates are of limited value, the role of Met192 in peptide hydrolysis remains to be elucidated. For this reason, hydrolysis of two diamino acid peptides (Ac-Phe-Ala-NH_2 and Ac-Phe-Gly-NH_2) and one natural substrate (α-casein) were picked for study in this investigation. Hydrolysis of Ac-Phe-NH_2 was also studied for comparison. For these hydrolyses MS-δ-Cht was chosen as the Met192 modified form of Cht to compare with δ-Cht. Theoretically, the bulky hydrophilic sulfoxide side chain of MS-δ-Cht would prevent the Met192 from carrying out its normal function in catalysis.

Fersht's theory predicts that modification of Met192 would cause a decrease in the catalytic rate constant (k_{cat}) of polypeptide hydrolysis. This is in marked contrast to monoamino acid ester and amide hydrolysis where the k_{cat} either does not change significantly or even increases. One would expect an increase in K_m for peptide hydrolysis based on the results observed for monoamino acid ester and amide hydrolysis. It was with these ideas in mind that the peptide hydrolysis experiments were undertaken.

The results for the hydrolysis of Ac-Phe-Ala-NH_2, Ac-Phe-Gly-NH_2 and Ac-Phe-NH_2 with δ-Cht and MS-δ-Cht are displayed in tabular and graphical form on Table 11 and Figures 24-26. The initial velocities of the peptide hydrolysis reactions at different substrate concentrations are reported and displayed as Lineweaver-Burke plots. A least square analysis computer program was used to obtain intercepts, slope and standard error of estimate for each plot.

TABLE 11

INITIAL RATES OF HYDROLYSIS OF PEPTIDE SUBSTRATES

AT 25°C, pH 7.8, 0.1 M $CaCl_2$, 0.1 M CARBONATE

Substrate	[S] 10^3 M	δ-Cht $10^8 v$ M sec^{-1}		MS-δ-Cht	
Ac-Phe-Ala-NH_2	0.23	2.41	(1)	-	
	0.30	-		0.51[a]	(1)
	0.40	-		0.70[a]	(1)
	0.68	5.57	(1)	0.97[a]	(1)
	1.00	-		0.94[a]	(1)
	1.35	10.39	(1)	-	
	1.50	-		1.71[a]	(1)
	2.50	12.74	(1)	2.52[a]	(1)
Ac-Phe-Gly-NH_2	0.30	2.07	(2)	0.44	(4)
	0.40	2.68	(2)	0.69	(4)
	0.60	3.14	(2)	0.82	(2)
	1.00	4.41	(2)	0.96	(2)
	1.40	4.75	(2)	1.15	(2)
	2.80	7.89	(2)	1.63	(2)
Ac-Phe-NH_2	0.40	-		0.50	(1)
	0.50	1.41	(1)	0.54	(1)
	0.67	1.90	(1)	0.79	(1)
	1.00	2.43	(1)	1.07	(1)
	2.00	5.24	(1)	1.79	(1)
	3.00	3.61	(1)	2.43	(1)
	5.00	5.35	(1)	-	

[a] values normalized to [E] of 2.0×10^{-7} M, for [E] of other reactions see Table 13

( ) denotes number of determinations

Figure 24 - Lineweaver-Burke plots for the hydrolysis of Ac-Phe-Ala-NH_2 by a. δ-Cht and b. MS-δ-Cht.

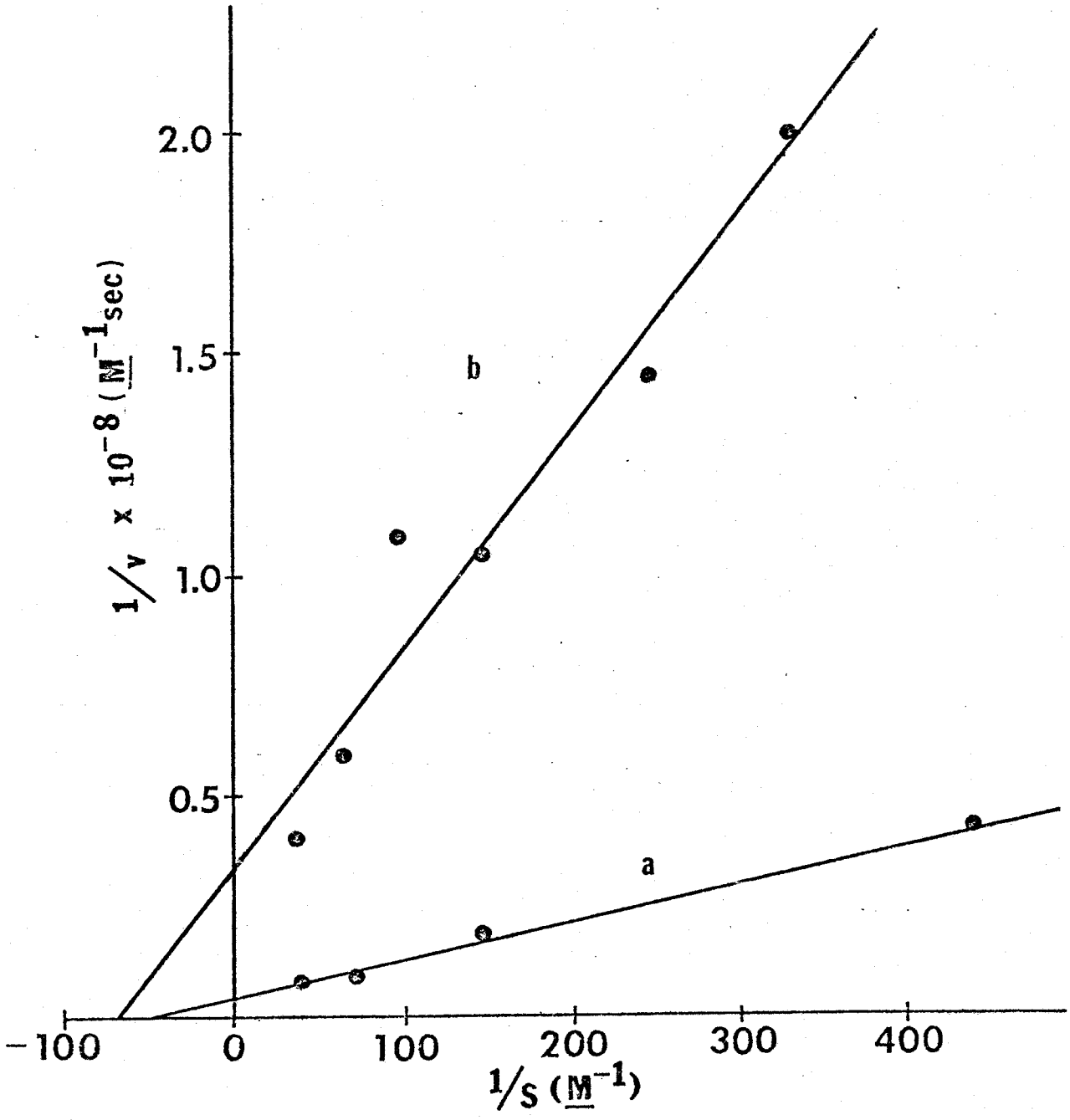


Figure 25 - Lineweaver-Burke plots for the hydrolysis of Ac-Phe-Gly-NH_2 by a. δ-Cht and b. MS-δ-Cht.

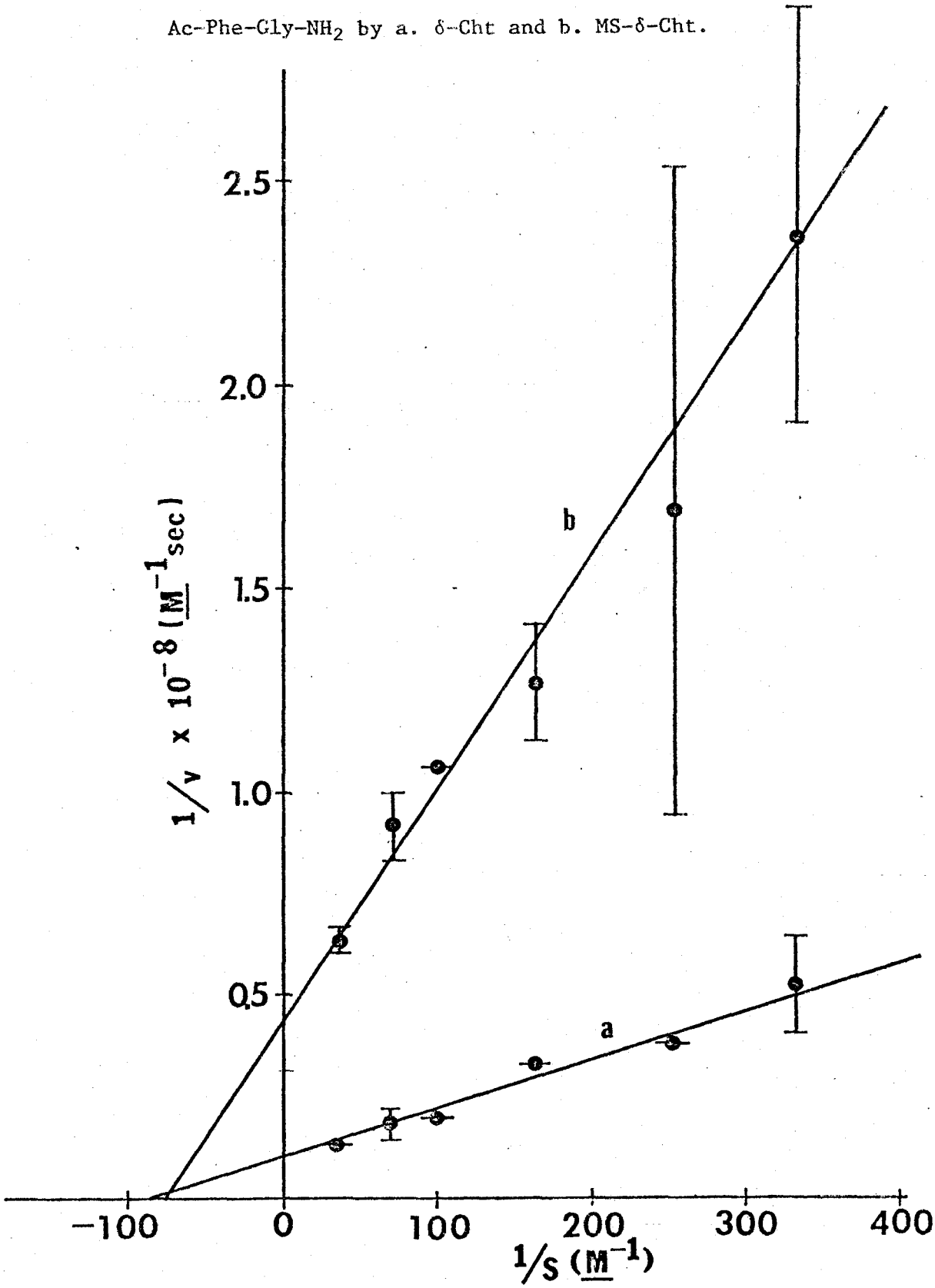
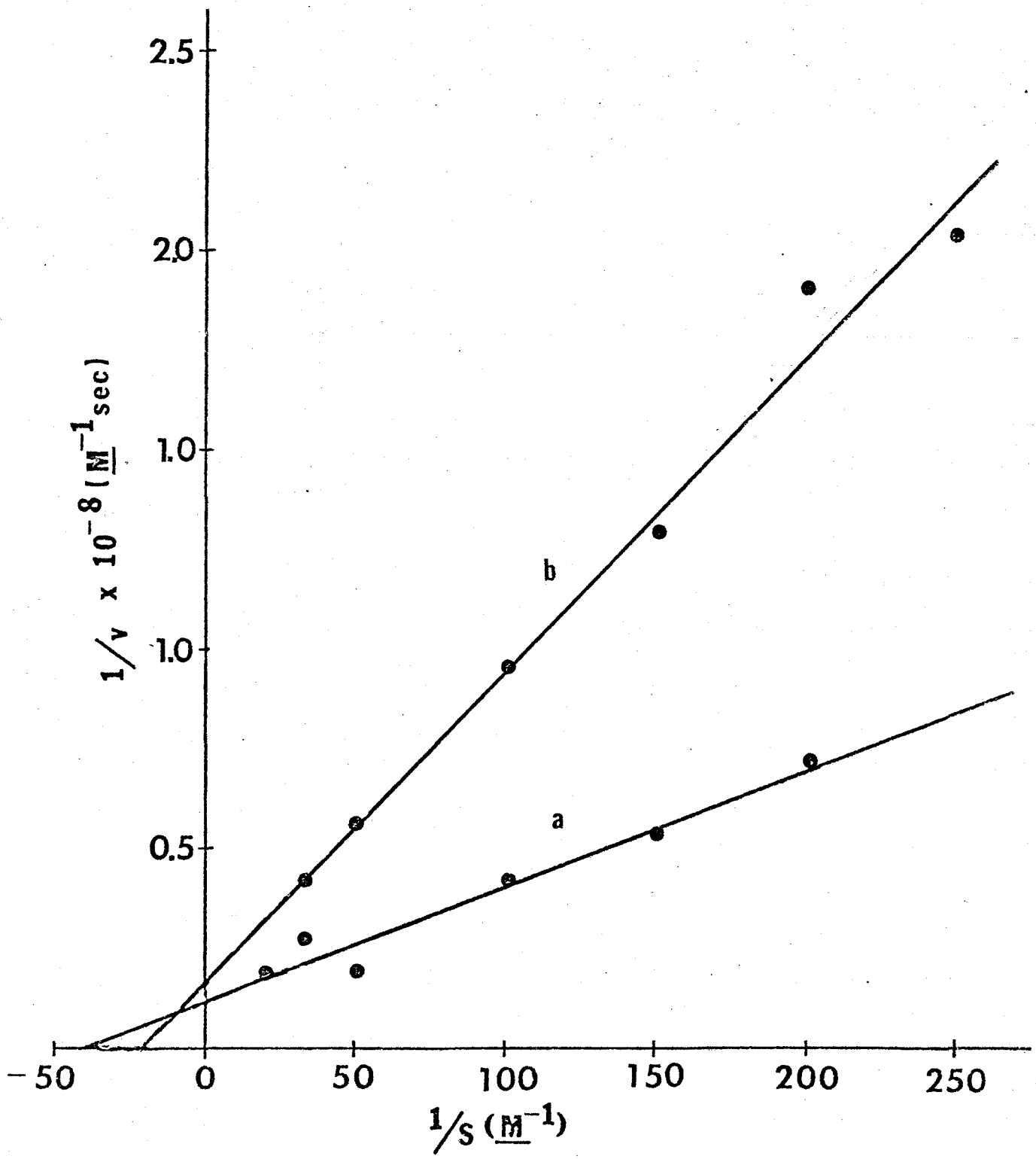


Figure 26 - Lineweaver-Burke plots for the hydrolysis of Ac-Phe-NH_2 by a. δ-Cht and b. MS-δ-Cht.



The data for the hydrolysis of α-casein follows on Table 12 and Figure 27. In this case the absorbance readings at 280 nm reflect the concentration of peptides released upon the partial hydrolysis of α-casein. These values were obtained under steady state conditions (0.5 mg/ml α-casein concentration) at low concentrations of enzyme for the 20 minute hydrolysis at 35°C. When graphed the slope of the kinetic plot can be used as an estimate of k_{cat} expressed as O.D./μg/ml/20 minutes.

Tables 13-15 summarize the kinetic parameters measured for the various peptide and amide hydrolyses (Table 13) and compare the results with literature values where they are available (Tables 14 and 15). The kinetic values for the δ-Cht catalyzed hydrolysis of Ac-Phe-NH_2 agree with values reported by two other groups of investigators (Table 14). The relationship between the α-Cht and MS-α-Cht catalyzed hydrolysis of Ac-Phe-NH_2 and that reported by Knowles for hydrolysis of Ac-Trp-NH_2 were also very close (72, 119, 128, 129). In the latter case the k_{cat} for the hydrolysis of the amide substrates was not affected significantly while the K_m increased by a factor of 2. Table 15 compares the experimental kinetic parameters obtained for peptide hydrolysis using a ninhydrin development system with values obtained by a different method using a pH Stat. The pH Stat method was indirect as it was necessary to compensate for the buffering capacity of the product by calculation (117). The K_ms for both methods were in good agreement but the k_{cat}s arrived at by the pH Stat method were always significantly greater than the k_{cat}s from the ninhydrin system (by a factor of 2).

The data obtained in this study for the MS-δ-Cht catalyzed hydrolysis of two peptide substrates differs from that available on the MS-Cht catalyzed hydrolysis of monoamino acid esters and amides. This is

TABLE 12

α-CASEIN HYDROLYSIS AT 35°C, pH 7.8,

0.005 M $CaCl_2$, 0.1 M BORATE

[E] 10^7 mM	δ-Cht	MS-δ-Cht
	O.D. 280 nm	
0.5	0.124	0.039
1.0	0.325	0.075
1.5	0.457	0.132
2.0	0.604	0.163
3.0	0.681	0.272
4.0	0.723	0.341
5.0	0.750	0.415
6.0	0.760	0.474
8.0	0.798	0.552
8.0	-	0.015[a]
-	0.014[b]	

[a] enzyme blank

[b] buffer blank

Figure 27 - Standard activity curves for the hydrolysis of α-casein by a. δ-Cht and b. MS-δ-Cht.

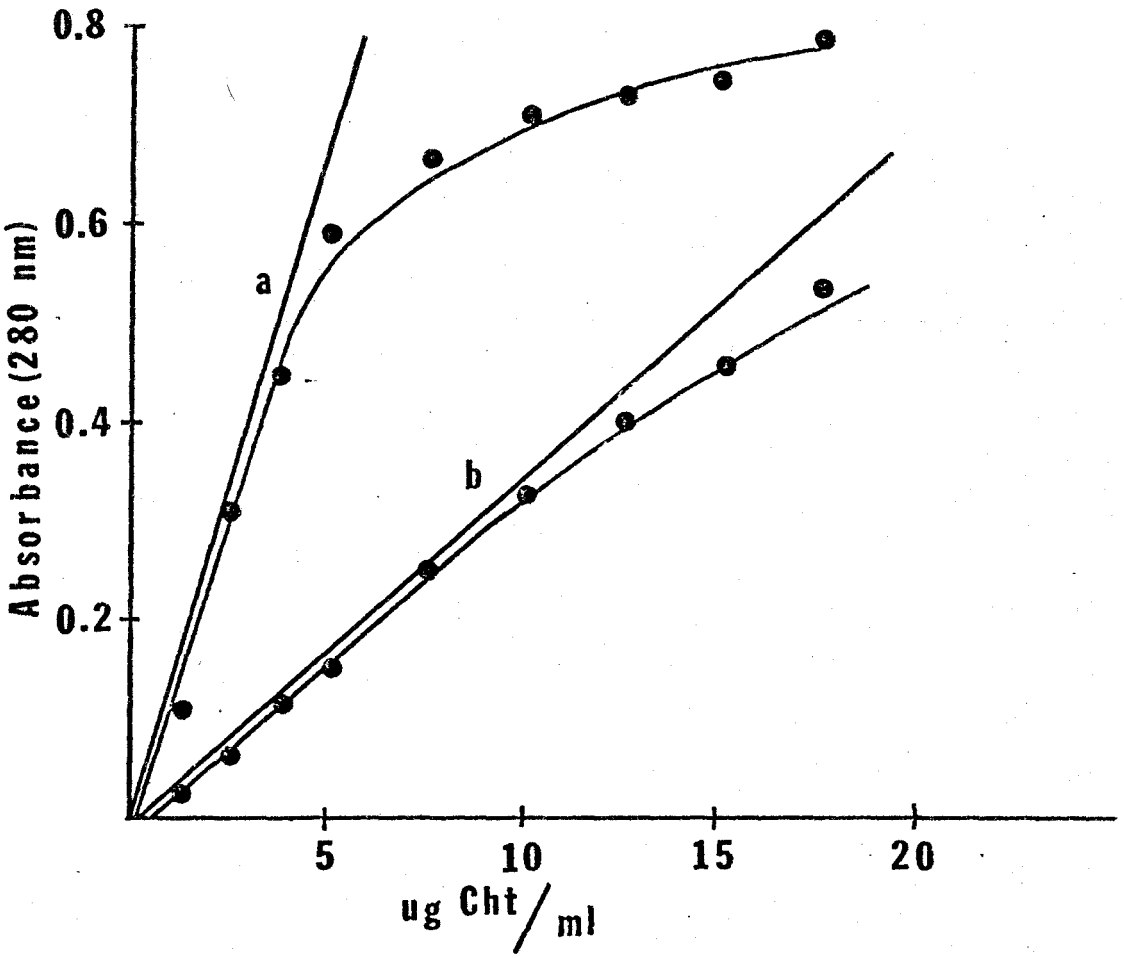


TABLE 13

KINETIC PARAMETERS FOR THE HYDROLYSIS OF PEPTIDE SUBSTRATES

Substrate	Enzyme	[E] 10^{-6} M	[S] 10^{3} M	k_{cat} sec^{-1}	K_m mM
Ac-Phe-Ala-NH_2	δ-Cht	0.20 (0.15)	2-25	1.491 ± 0.239	19.6 ± 3.2
	MS-δ-Cht	0.65 (0.40)	3-25	0.259 ± 0.101	14.3 ± 6.3
Ac-Phe-Gly-NH_2	δ-Cht	1.92 (1.47)	3-28	0.063 ± 0.011	11.7 ± 2.1
	MS-δ-Cht	2.05 (1.26)	3-28	0.018 ± 0.003	12.1 ± 2.3
Ac-Phe-NH_2	δ-Cht	2.01 (1.54)	5-50	0.053 ± 0.017	26.8 ± 10.1
	MS-δ-Cht	2.12 (1.30)	4-30	0.045 ± 0.021	51.8 ± 27.4
α-Casein	δ-Cht	-	-	0.160[a]	-
	MS-δ-Cht	-	-	0.038[a]	-

[a] k_{cat} estimates, O.D./μg/ml/20 min
( ) concentration of active sites based on *p*NPA titrations

TABLE 14

COMPARISON OF THE KINETIC PARAMETERS OF AMIDE HYDROLYSIS

Substrate	k_{cat} sec^{-1}	K_m mM	Enzyme	pH	Reference
Ac-Phe-NH_2	0.055	31 ± 3	α-Cht	7.9	(128)
	0.046	30.00	α-Cht	7.9	(129)
	0.053 ± 0.017	26.8 ± 10.1	δ-Cht	7.8	-
	0.045 ± 0.021	51.8 ± 27.4	MS-δ-Cht	7.8	-
Ac-Tryp-NH_2	0.0468 ± 0.0001	4.8 ± 0.2	α-Cht	7.9	(72)
	0.0476 ± 0.0001	10.6 ± 0.4	MS-α-Cht	7.9	(72)
	0.039 ± 0.001	4.1 ± 0.3	α-Cht	8.3	(119)
	0.036	5.00	α-Cht	7.9	(128)
	0.026	4.00	α-Cht	7.0	(129)

TABLE 15

COMPARISON OF THE KINETIC PARAMETERS OF PEPTIDE HYDROLYSIS

Substrate	k_{cat} sec^{-1}	K_m mM	Enzyme	pH	Reference
Ac-Phe-Ala-NH_2	2.80 ± 0.2	25.0 ± 1.2	α-Cht	7.9	(109)
	1.491 ± 0.239	19.6 ± 3.2	δ-Cht	7.8	-
	0.259 ± 0.101	14.3 ± 6.3	MS-δ-Cht	7.8	-
Ac-Phe-Gly-NH_2	0.140 ± 0.006	14.6 ± 0.3	α-Cht	7.9	(109)
	0.063 ± 0.011	11.7 ± 2.1	δ-Cht	7.8	-
	0.018 ± 0.003	12.1 ± 2.3	MS-δ-Cht	7.8	-

evident when δ-Cht/MS-δ-Cht ratios of the kinetic parameters of the hydrolysis data are calculated (Table 16). The k_{cat} ratio of 1.2 for Ac-Phe-NH_2 agreed with the data for monamino acid esters and amides where k_{cat} remained relatively the same. However, the k_{cat} ratios for the hydrolysis of the two peptide substrates and α-casein increased to 5.8, 3.5, and 4.0. These values are significant changes and indicate that this modification of Met192 (oxidation to the sulfoxide form) has an effect on the catalytic steps of peptide hydrolysis. As expected, there was a decrease in the ratio for the K_m of Ac-Phe-NH_2 hydrolysis (0.5). Surprisingly, there was little or no change in the ratio for the K_m of hydrolysis of the two peptide substrates. This was in direct contrast to what had been found for the monoamino acid ester and amide substrates where the K_m increases δ-Cht/MS-δ-Cht ratio decreases). The results will be discussed in the next section.

TABLE 16

RATIOS OF KINETIC PARAMETERS

FOR THE HYDROLYSIS OF PEPTIDE SUBSTRATES

Substrate	δ-Cht/MS-δ-Cht k_{cat} sec^{-1}	δ-Cht/MS-δ-Cht K_m mM
Ac-Phe-Ala-NH_2	5.8	1.4
Ac-Phe-Gly-NH_2	3.5	1.0
Ac-Phe-NH_2	1.2	0.5
α-Casein	4.0[a]	-

[a] k_{cat} estimate

IV. DISCUSSION

Relationship Between Methionine-192 and Isoleucine-16

The x-ray studies of α-Cht definitely show that the Met192 and Ile16 residues reside in the vicinity of the active center (4, 5). The common link between them is the Asp194 residue which is two residues away from Met192 and forms an ion-pair with Ile16 in active forms of Cht. When the ion-pair between Ile16 and Asp194 is broken (by deprotonation or chemical modification of the α-amino group of Ile16) the enzyme becomes inactive and the side chain of Asp194 is free to reassociate with other residues (20, 60, 61). His40 is the most probable candidate because it is with this residue that Asp194 is ion-paired within Chtgen (6). The resulting movement of the side chain of Asp194 to the surface of the enzyme occurs simultaneously with the movement of Met192 and also with a rotation of the main chain that connects them. This statement is based primarily on the x-ray structures of Cht and Chtgen showing Met192 in a buried position in the zymogen in the absence of the Ile16-Asp194 ion-pair as opposed to an exposed position in active Cht where the ion-pair exists (Freer's "single unified event" hypothesis). The maintainence of the ion-pair is, therefore, critical with respect to the position of Met192.

An experimental model system was chosen to test the "single unified event" hypothesis on a chemical modification basis. Acetylated(Ile16)-Cht was selected to represent an inactive *zymogen-like* molecule. The evidence for the chemical and conformational similarity of this form of

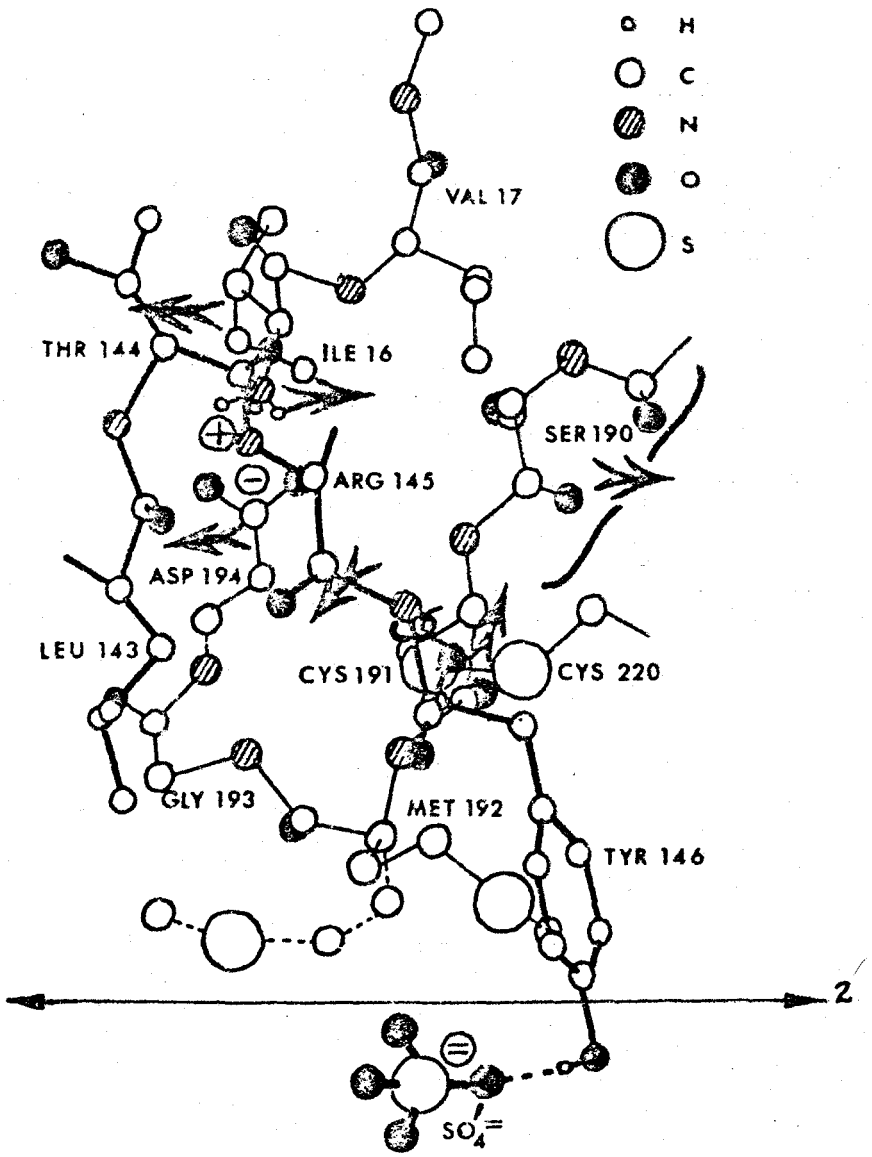
Cht to Chtgen has been stated previously in Section I Part B 3 and Section II Part E1. BrNAP was picked as a structural and functional probe of the position of the Met192 residue in Cht. Its specificity for Met192 has been shown in past studies (96, 105).

Samples of δ- and α-Cht which were totally acetylated (on Ile16 and peripheral lysines) displayed a slow rate of alkylation with BrNAP. The rate of alkylation for A_{16}-δ-Cht was 19-37 times slower than that of δ-Cht depending on the method used to calculate the rate constant. For a comparable sample of α-Cht the rate of alkylation was 93 times slower than α-Cht alkylation with BrNAP. Positive verification that alkylation of Met192 was responsible for the increase in absorbance at 350 nm (wavelength used to monitor the BrNAP reactions) was shown by amino acid analysis (see preceding section Part B 2). The BrNAP alkylation of these samples demonstrates that Met192 is slightly accessible to reagent.

Ghélis and coworkers have previously suggested, from chemical modification experiments, that acetylation of Ile16 disrupts the substrate binding site of Cht (60, 61). According to the x-ray structure based hypothesis of Freer and coworkers, this is what should happen when the electrostatic interaction between Ile16 and Asp194 is blocked (6). However, a slow rate of alkylation of Met192 is observed indicating that binding affinity is only substantially decreased and not completely disrupted in this form of the enzyme. A decreased ability to bind substrate-like molecules such as BrNAP would decrease the BrNAP alkylation reaction due to the active-site-directed nature of the reaction. This is one possible explanation for the observed slow rate of BrNAP alkylation of the totally acetylated samples of δ- and α-Cht.

Alternatively, the slow reactivity of the Met192 may not be due to poorer binding, but to a reduced accessibility of the Met192 to active-site bound BrNAP. BrNAP does not react with Met192 in Chtgen because the thio-ether side chain is buried in the hydrophobic interior of the molecule. The x-ray structure of Chtgen clearly shows that this is the case. The Freer hypothesis predicts not only disruption of the substrate binding site when the ion-pair is blocked by acetylation of Ile16, but also the reburying of the side chain of Met192. Accordingly, one would expect the side chain of Met192 to be inaccessible to BrNAP. The observation of alkylation of the Met192 residue in totally acetylated samples of Cht contradicts this hypothesis and may indicate the Met192 is not buried. A repositioning, but not a reburying, of the Met192 side chain to an intermediate position in totally acetylated-Cht may take place. Supportive evidence for this explanation of the alkylation results for A_{16}-Cht comes from the x-ray structure elucidated for α-Cht at pH 8.3 (90). Tulinsky and coworkers found (from the x-ray structure of α-Cht at pH 8.3) that the Met192 side chain had rotated 180° in respect to its position in the structure of α-Cht at pH 3.6 (Figure 28). However, it remained in a surface position in the active center rather than reburying into the interior of the molecule. As previously stated, the acetylated(Ile16)-δ-Cht form of Cht has been equated to the high pH form of the enzyme. If the Met192 side chain occupies the same structural position in the solution conformation of A_{16}A-Cht as it does in the crystalline structure of α-Cht at pH 8.3 this would explain the observed accessible nature of the Met192 to BrNAP (Table 7).

Figure 28 - Drawing depicting the changes of the structure of α-chymotrypsin at pH 3.6 with change in pH to 8.3 in the vicinity of the active site (90). Broken lines denote position of Met192 at pH 8.3.



Another possible explanation of the data would be the inability of A_{16}-Cht to reform into the zymogen structure due to the absence of constraint imposed on the Chtgen molecule by the Leu13-Ser14-Arg15-Ile16 peptide segment. This loss of constraint may prevent the acetylated-Ile16 from assuming the zymogen structure. An intermediate structure somewhere between the active and zymogen structures would exist for A_{16}-Cht. The slow rate of Met192 alkylation in A_{16}-Cht supports this view (130).

It is also very possible that extensive chemical modification is responsible for the unexpected alkylation results obtained in the samples mentioned above. Modification of 14 peripheral lysine resudes during the acetylation of the N-terminal Ile16 could affect the tertiary structure of the molecule in such a way that Met192 becomes slightly accessible to reagent, since charged functional groups may be involved in maintaining the tertiary structure of the protein. Loss of 14 positively charged groups at neutral or acidic pH could adversely affect the integrity of the molecule.

The procedure in which the zymogen was modified with dimethylmaleic anhydride (DMMA), activated with trypsin, acetylated with acetic anhydride and treated at pH 5.0 resulted in an inactive enzyme in which only the Ile16 was modified (A_{16}-Cht(F)). All lysine residues were in their natural chemical form. The inability to alkylate the A_{16}-δ-Cht(F) preparation with BrNAP (see Table 7) suggests that the modified lysine residues cause a structural change ultimately leading to a slightly accessible Met192 residue. When the lysine groups are not modified Met192 is inaccessible as predicted by the Freer hypothesis. Therefore, the experi-

mental results for the BrNAP alkylation of A_{16}-Cht(F) support the "single unified event" theory for activation of Chtgen proposed by Freer and coworkers.

The anomalous BrNAP results with fully acetylated-Cht may account for the controversial diversity of results that have appeared in the literature regarding this form of the enzyme (21, 60, 61, 125).

Juxtaposition of Methionine-192, Serine-195 and Histidine-57 in the Active Site

It is suggested, from x-ray structural studies that the Met192 not only participates in zymogen activation, but in addition, may have an important role in the catalysis of substrates (see Section I Part D 2). It is difficult to imagine the exact function of Met192 in catalysis from these studies alone. Chemical modification of active site residues in Cht has, in the past, led to some of the important breakthroughs concerning the mechanism of catalysis as we now know it. In light of this fact, chemical modification was used to study the juxtaposition of Met192, Ser195 and His57 in the active site of Cht. Specifically, Cht was inactivated by modification of Ser195 or His57 and the effect on the rate of BrNAP alkylation of Met192 observed.

Chemical modification of Ser195 and His57 in Cht leads to inactivation of the enzyme (16, 17, 18, 19, 48, 49, 101). Therefore, these residues are generally referred to as essential catalytic residues. X-ray studies show that these residues along with Asp102 make up the catalytically important "charge transfer complex" in Cht (4, 5). The proper orientation of the Ser195, His57 and Asp102 in this complex is crucial in respect to the catalytic activity of the enzyme. This fact becomes evident when the x-ray structures of Chtgen and Cht are compared. The "charge transfer complex" appears to be preformed in the zymogen (6). Only a slight repositioning of the imidazole ring of His57 is obvious in the reorientation of these residues upon activation. Other residues in the vicinity of the active site rearrange to a greater degree than the

"charge transfer complex" residues. This general concept emphasizes the sensitivity of essential catalytic residues in the active site of Cht.

The Ser195 was chemically converted to dehydroalanine by first sulfonating the residue with PMSF and then carrying out a Hofmann elimination reaction on the sulfonated derivative. This modification removes the hydroxyl portion of the side chain and renders the molecule inactive (see Table 4). The modification disrupts the "charge transfer complex" due to the removal of the hydroxyl group which normally hydrogen bonds the N3 atom of the imidazole side chain of His57. Lack of this hydrogen bond may allow residue-195 and His57 to change their positions relative to native unmodified Cht. Such changes in the active site conformation may be detected from changes in the rate of Met192 modification between anhydro-Cht and native Cht.

The observed BrNAP alkylation reaction rates revealed the presence of a fast and slow reacting enzyme species in anhydro-α-Cht which bound to a LBTI affinity column and anhydro-α-Cht which did not bind to a LBTI affinity column. Spectra of anhydro-α-Cht preparations showed a variance in extinction coefficient at 240 nm (131). Anhydro-α-Cht with a higher extinction coefficient at 240 nm (4.9×10^{4} $\underline{M}^{-1}$ cm^{-1}) was found to possess predominantly the slow BrNAP reacting enzyme species whereas anhydro-α-Cht with a lower extinction coefficient at 240 nm (4.0×10^{4} $\underline{M}^{-1}$ cm^{-1}) was found to possess the fast reacting species. These data support the existence of four conformational states for anhydro-α-Cht at room temperature (131). One pair of conformational states is separated by their different affinities toward lima bean trypsin inhibitor. Another pair of conformational states are apparent from the variation in absorption at 240 nm and the observation of two distinct rates of BrNAP alkylation.

Changes in the environment appear to facilitate interconversions among this second pair of substrates.

The decreased rate of BrNAP modification for the slower reacting anhydro-α-Cht conformation may be due either to a poorer binding of BrNAP by the active site or a decreased accessibility of the Met192 to bound BrNAP. If one assumes the decreased rate is due to a poorer binding constant, then a similar decrease in binding strength of 14-fold may be predicted for the binding of other small substrate analogs to this conformational substate of anhydro-α-Cht. The factor of increase for any one anhydro-α-Cht sample will be dependent on the mixture of conformational substates in that particular sample and, accordingly, should be between 1- and 14-fold. In agreement with this prediction, Weiner et al. (132) found the binding constants for substrate analogues to anhydro-α-Cht to be increased between 3- and 8-fold.

These data infer that the serine-195 hydroxymethyl moiety may have a structural role, in addition to its well known catalytic role, in giving stability to the productive active site conformation of native α-Cht. Its elimination in anhydro-α-Cht appears to result in four different conformational forms, some of which bind substrates less well than the native conformation of α-chymotrypsin (131).

The nature of the chemical modification of His57 was different from that of Ser195. The N3 atom of the His57 imidazole side chain was methylated using methyl-*p*-nitrobenzene sulfonate (48) and the preparation shown to be relatively inactive (Table 4, <1.6%). This modification is similar to the Ser195 modification, in that both disrupt the hydrogen bond between the His57 and Ser195. The modification of His57, however,

adds a methyl group to the active site and leaves the hydroxyl group of Ser195 intact in contrast to the Ser195 modification which eliminates the hydroxyl group of the Ser195 and leaves the His57 intact.

The x-ray crystalline structure of 3-methyl-His57-α-Cht has been reported (133). The different electron density maps between native and 3-methyl-His57-α-Cht reveal that a reorientation of His57, Ser195 and Met192 occurs on methylation of His57. The His57 moves approximately 0.3 Å from a position on the enzyme surface to a position in solution. It also expels two of the three water molecules in the area of the active site. There is a movement of Ser195 characterized by rotation of the C_α-C_β bond by 50° and 100°. This movement places the hydroxyl group either in a position where it can interact with a water molecule and hydrogen bond to Ser214 or in a position which it occupies in the indoleacryloyl-Cht, acyl-enzyme intermediate (134). The His57-Ser195 hydrogen bond is broken in 3-methyl-His57-α-Cht with the distance between atoms increasing from 3.0 Å in Cht to 4.6 Å in the modified enzyme. A slight rearrangement in the Gly197-Ser190 peptide segment (including the Met192 side chain) also occurs. In the x-ray structure of native α-Cht the side chain of Met192 occupies two alternative positions. In the x-ray structure of 3-methyl-His57-α-Cht a redistribution of the Met192 side chain occurs such that one of the two positions is favored 65% of the time.

In contrast to the BrNAP results found for anhydro-α-Cht (either the same rate as native α-Cht or a slower rate), 3-methyl-α-His57 was found to react with BrNAP 6 times faster than with native α-Cht. Several explanations can account for the increased reactivity of methylated-Cht

with BrNAP. The available x-ray data for this form of the enzyme facilitates choosing the most plausible interpretation of the results.

A. Alteration of the position of Met192 due to a movement of the Ser195 upon methylation of His57. The observed rotation of the C_α-C_β bond of Ser195 would be expected to affect residues adjacent to it (i.e. Asp194, Gly193, Met192, etc.). The change in position of Met192, obvious from its redistribution in the x-ray structure of 3-methyl-His57-α-Cht, supports this view. The favored position for the side chain of Met192 either brings the side chain into better juxtaposition with the bound BrNAP molecule, enhancing the productive orientation for the reaction or brings it closer to the BrNAP molecule permitting more frequent contact according to the "flexible lid" hypothesis of Blow and Steitz (75).

B. Steric interaction of the methyl group of His57 with the reactive portion of the bound BrNAP molecule leading to a better orientation of BrNAP with respect to the Met192 in its normal conformation. This interpretation is also supported by the x-ray study of 3-methyl-His57-α-Cht because the proper alignment for acylation of amino acid substrates is obstructed by the methyl group on the His57 residue (133). The relatively large size of the reactive moiety of BrNAP (due in part to a bromine atom) suggests that the BrNAP molecule occupies the site where acylation takes place. This may lead to a close proximity between BrNAP and the side chain of Met192 causing an increased rate of alkylation as seen experimentally.

C. The concerted action of a movement of the Met192 side chain and steric interaction of the methyl group of His57 with BrNAP. The significant increase in reactivity of BrNAP with 3-methyl-His57-α-Cht

(see Table 10) may be due to the concerted action of the two effects discussed above.

The following points are of interest in the study of Met192 in respect to Ser195 and His57:

1. Inactive forms of Cht modified on the Ser195 and His57 residues of the "charge transfer complex" display major differences in their ability to react with the Met192 specific alkylating reagent, BrNAP. Anhydro-α-Cht reacts with BrNAP either at the same rate or a slower rate while 3-methyl-His57-α-Cht reacts approximately 6 times faster.

2. The results presented here for the anhydro-α-Cht-BrNAP reactions show that "charge transfer complex" not only has a role in catalysis but also a role in stability of Cht. Other studies on the His57 residue of Cht have come to a similar conclusion (135).

3. The increased reactivity of 3-methyl-His57-α-Cht is surprising when compared to the results for anhydro-α-Cht. The enhanced reactivity of this form of the enzyme is apparently not due to a better binding of the alkylating reagent because small substrate molecules do not bind to 3-methyl-His57-α-Cht any better than to native Cht (132). Furthermore, 3-methyl-His57-α-Cht does not bind to a LBTI affinity column (136).

4. The alkylation studies of the modified-Chts referred to above demonstrate the usefulness of BrNAP as a structural and functional probe of the active site. The results also demonstrate that BrNAP can be used to titrate the active site concentration of inert forms of the enzyme.

The Role of Met192 in the Catalytic Mechanism of Peptide Hydrolysis

As stated in sections I and II several groups of investigators have modified the Met192 residue of Cht and measured the kinetic parameters for the hydrolysis of various monoamino acid ester and amide substrates (22, 46, 47, 72, 73, 74, 94, 95). In these studies, the K_m of hydrolysis increased significantly while the k_{cat} usually changed very little. From these data, it is inferred that Met192 has a function in substrate binding. A similar increase in K_m may be expected in the hydrolysis of large peptide substrates. However, the kinetic parameters found in this dissertation for the MS-δ-Cht catalyzed hydrolysis of two diamino acid peptides and α-casein show that it would be incorrect to make this inference (Table 13). It appears that the role of Met192 is different in peptide hydrolysis than it is in monoamino acid ester or amide hydrolysis.

The finding of a significant decrease in k_{cat} and little or no change in K_m for the MS-δ-Cht hydrolysis of peptides implies the participation of the Met192 residue in the catalytic steps of peptide hydrolysis. These results can be considered supportive evidence for two hypotheses introduced in Section I Part D 2 on the participation of Met192 in chymotrypsin catalysis.

The suggestion that the side chain of Met192 acts as a "flexible hydrophobic lid" during catalysis was proposed by Blow and Steitz (75). It predicts a movement of the hydrophobic thio-ether side chain of Met192

based on x-ray studies of Cht and Cht with small bound substrate-like molecules. Apparently, the Met192 side chain is observed to act as a lid on the specificity pocket holding bound substrate molecules in the proper orientation for catalysis. Changing the Met192 side chain to the sulfoxide form (MS-Cht) would make it difficult for the side chain to properly function in this role. Without the thio-ether side chain to maintain the substrate in the proper orientation, catalysis would take place at a slower rate. The decreased k_{cat} for the MS-δ-Cht hydrolysis of peptide substrates supports this view.

One could envision the possibility where by the Met192 side chain cannot act as a "flexible lid" in the hydrolysis of large peptide substrates, but can do so in small ester and amide hydrolysis by Cht. Contacts made with the large leaving group of peptide substrates would prevent the thio-ether side chain from covering the specificity cavity of the enzyme. The thio-ether side chain of Met192 could be flexible in the hydrolysis of monoamino acid esters and amides because the large leaving group present in peptide substrates would not be present to hinder its movement. This would conveniently explain the increase in K_m observed in the MS-Cht hydrolysis of small esters and amides.

Fersht and Blow have proposed that Met192 participates in the Cht catalyzed hydrolysis of peptide substrates. Based on a model for the association of bovine pancreatic trypsin inhibitor with Cht, they assert that the α, β and γ carbons of Met192 make important repulsive van der Waals contacts with the carbonyl oxygen of the Ala16I leaving group of the inhibitor (104, 137). The hypothesis fits into the scheme presented above very well. Small amino acid ester and amide substrates lack the

leaving group necessary to make the proper hydrophobic contacts with Met192 while peptides possess such a leaving group.

From the model for bovine pancreatic trypsin inhibitor and Cht Fersht and Blow suggest that "The hydrophobic binding energy is used to lower the activation energy of the chemical steps." (104) They conclude that "Specificity is expressed in k_{cat} and not K_m." (104) The decrease in k_{cat} observed for the hydrolysis of peptides by MS-δ-Cht supports their conclusion very well. Modification of the thio-ether side chain of Met192 to the sulfoxide form would disrupt the hydrophobic contacts due to the size and hydrophilic nature of the sulfoxide group. This would cause a decrease in the k_{cat} as seen in the experimental results (see Table 14).

More specifically, it is proposed that the flexible nature of the sulfoxide side chain increases k_{-1} (see Figure 2) by preventing the Met192 from holding small ester and amide substrates in the substrate binding site. This would explain the increased K_m ($K_m = \frac{k_{-1}k_2}{k_1}$) observed in small ester and amide hydrolysis by MS-Cht. In peptide hydrolysis however, the sulfoxide group is prohibited from its holding role in substrate binding. Therefore, the K_m remains the same in MS-δ-Cht catalyzed hydrolysis of peptides. This proposal explains all three theories presented in Section I by integrating them into one hypothesis.

The experimental results obtained for peptide hydrolysis by MS-δ-Cht quantitate the contribution of Met192 in Cht catalysis of peptides under the conditions stated. The decrease in activity observed for MS-δ-Cht catalyzed hydrolysis of several peptides (92% for Ac-Phe-Ala-NH_2 and 72% for Ac-Phe-Gly-NH_2 as calculated from Table 17) is small

TABLE 17

RATIOS OF KINETIC PARAMETERS

FOR THE HYDROLYSIS OF PEPTIDE SUBSTRATES

Substrate	Enzyme	$k_{cat}/K_m \times 10^3$ sec^{-1} mM^{-1}
Ac-Phe-Ala-NH_2	δ-Cht	76.1
	MS-δ-Cht	6.3
Ac-Phe-Gly-NH_2	δ-Cht	5.4
	MS-δ-Cht	1.5
Ac-Phe-NH_2	δ-Cht	2.0
	MS-δ-Cht	0.9

when compared to the acceleration factor for enzyme catalyzed peptide hydrolysis (10^8) (138). Therefore, the contribution of Met192 to the overall hydrolysis of peptides is small but significant.

Earlier, Koshland stated

> It is clear therefore, that the methionine modification (oxidation to the sulfoxide form) results in a complex effect in which several parts of the substrate and several parts of the enzyme change their relationships to produce the observed changed specificity. (110)

The proposed explanation presented for the results obtained in this thesis supports the above statement.

Conclusions

The conclusions reached in this dissertation are the following:

1. The Met192 thio-ether side chain is not accessible to solvent in the acetylated (Ile16)-δ-Cht model for the zymogen, Chtgen.

2. The Ser195 hydroxymethyl moiety has a structural as well as functional catalytic role in stabilizing the active conformation of Cht.

3. The alkylating reagent, BrNAP is a useful active site probe in forms of Cht devoid of catalytic activity. It can be used as an active site titrant for inactive forms of Cht.

4. The role of the Met192 residue in Cht catalyzed hydrolysis of peptide substrates is different from that in the Cht catalyzed hydrolysis of monoamino acid ester and amide substrates.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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